DEPARTMENT OF MEDICAL CHEMISTRY
FACULTY OF MEDICINE
UNIVERSITY OF DEBRECEN

LABORATORY PRACTICALS IN MEDICAL CHEMISTRY

Edited by
VIKTOR DOMBRÁDI

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Revised Edition

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DUPress

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Preface

The aim of this book is to deepen and extend the chemical foundations for medical students. While doing an experiment in the laboratory, the student understands the applications of chemical theories and through direct personal experience materializes the concepts mentioned during the lectures. In the recent years, there was a steady increase in the number of students, a decrease in the duration of practical classes, and the Department of Medical Chemistry moved into new premises. The above changes as well as the progress in the field necessitated the reorganization of chemistry teaching, including the practical classes. The present book is updated and *contains all of the essential information* that is required for the understanding of medical chemistry practices.

As the students approach the chemistry laboratories located in the Theoretical Building, the first thing they may notice is a row of cupboards along the corridor. Because of safety and fire regulations the students must store their personal belongings before entering the laboratory room. Besides the writing utensils and a labcoat, you will carry this book and use it as the sole written source of information during the class. The book has been written in such a way that it can guide the student through all the steps required for understanding and performing the experiments.

Each chapter starts with a short, general introduction containing the theoretical background and technical considerations related to the experiments. This is followed by the detailed description of the practical work including step by step experimental protocols. Safety measures and environmental hazards are emphasized at the beginning of the book and in each chapter again when applicable. We extended the new book with sample calculations in order to help the students in the evaluation of their results. Through several examples we explain how to convert units and how to calculate the final results from raw data. In each example we give one possible way of solving the problem, but of course any other alternative approach giving a correct answer will be accepted. In addition, review questions and a few additional exercises are provided for testing the knowledge and preparedness of the students before the class. If you go through all of these pages you can be sure that you are ready for the practical work. For each class there are specially prepared results sheets with empty spaces for the recording of data and conditions. As you proceed with the experiments, please, fill in these pages and evaluate your results by answering the questions. After completing the *Results* go to the *Appendix*, fill in the date and title of the experiment and ask for the signature of the supervising teacher. The signature proves that you performed the experiments with acceptable results.

Although the book was significantly extended by the theoretical treaties, the page number was decreased as we included only those experiments that were performed by the students or demonstrated by the supervisors in the year of publication. While writing the book the authors and the editor relied on teaching experience of the previous years and utilized some of the chapters from the book "Experiments in Medical Chemistry" published in 2001. We would like to thank the useful advises from our students and colleagues that helped us to correct earlier mistakes. We are especially obliged to Dr. Kornélia Szűcs, Dr. Béla Tóth, and Dr. György Vereb who provided the authors with teaching materials from earlier publications. We are grateful to Mrs. Andrea Hoszpodár for her help in typing and electronic editing of the manuscript. We thank Dr. Pál Gergely, the head of the Department, for his continuous support and encouragement.

Although the authors and the editor did their best to avoid typos and spelling mistakes, after the previous edition (2009) some modifications had to be implemented in order to optimize the time and material requirements of some experiments and to correct small mistakes. Thanks are due to Dr. Béla Tóth for his useful suggestions that made these corrections possible. The authors would appreciate any further comments and suggestions concerning both the form and content of the present edition.

Debrecen, March 10, 2016 the Authors and the Editor

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LABORATORY SAFETY INSTRUCTIONS AND FIRE REGULATIONS

The chemical laboratory is a dangerous workplace. During the experimental work one cannot avoid the usage of corrosive, poisonous, flammable and hazardous materials. Experience proves that laboratory accidents and fires could be evaded by the observance of the proper precautions. Therefore everybody must know the sources of danger as well as the tools and modes of prevention. For these reasons everyone has to get acquainted with the most important safety rules of universal application before starting the laboratory work. Please, respect the following rules to save your own and your colleagues' health. The knowledge of safety and fire regulations is a requirement of every laboratory work.

Laboratory safety instructions

1. Meal and alcohol consumption are prohibited. Do not eat anything and drink only water in the laboratory, for drinking use the marked glass.
2. Wear laboratory coat to protect your skin and clothing whenever you are working in the laboratory. Leather shoes are preferred to canvas or open-toped shoes.
3. Fasten up your hair if it is long and do not wear long necklace, bracelets and earrings.
4. Do not store more book or exercise-book than necessary on your desk.
5. Wear safety goggles and rubber gloves when working with concentrated acids or bases.
6. Keep table tops clean. If acids or bases spill neutralize and wipe up them promptly. Neutralize the acid with 2 % $NaHCO_3$ solution, the base with 0.5 % acetic acid.
7. When a chemical spills on your skin, flush the area immediately with plenty of water, **except sulfuric acid which has to be wiped with a piece of dry cloth**.
8. When acids squirt into the eyes, irrigate them with water followed by 2% borax solution, bases should be neutralized by 2% boric acid solution following irrigation with water.
9. Concentrated acids or bases, serum or crude tissue extract must not be pipetted by mouth, but only with safety or automatic pipettes.
10. Infectious material should be washed off with 0.2% Neomagnol solution (obtained by dissolving one tablet in 0.5 L of water) from the skin, then wash the area with soap water finally rinse it with plenty of lukewarm water.
11. When working with toxic gases or volatile toxic substances (e.g. H_2S, NO_2, NO, Cl_2, Br_2), work in the hood.
12. Never taste anything in the laboratory and smell the substances carefully.
13. Do not forget that poisonous materials could get into the human body in three different ways: through the nose, through the mouth or through the skin. For these reason be careful, every time read the description of the experiment and interpret it for yourself. Only after that start to do the actual work. After finishing the experiments do not forget to wash your hands.
14. When heating liquids in test tubes, never point the tube toward yourself or anyone else. Never heat the test tubes directly at the bottom, but tilt the tube and heat it gently between the bottom of the tube and the top of the liquid.
15. Never return chemicals to bottles of their origin it may contaminate the entire batch.
16. Collect the organic solvents in a special waste bottle after use and do not pour in the sink or drain because these chemicals pollute the environment.
17. Mercury from broken thermometer should be sprinkled by powdered sulfur, the resulting mercury(II)sulfide is not poisonous.
18. Do not use broken china- or glass-equipment.

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19. Follow instructions carefully and do not perform unauthorized experiments. Always read the label twice before using a chemical reagent. Be sure that the concentration, as well as the name of the reagent, is correct for the experiment.
20. Before using the centrifuge balance the centrifuge tubes against each other.
21. Report all accidents, no matter how minor, to the instructor.

Fire-regulations

1. Smoking is prohibited in the laboratory, and as a matter of fact in the entire building. Smoking is allowed only at the appointed place.
2. Gas burners are allowed to be used only for the experiments. After finishing the work the gas taps must be turned off at the end of the rubber pipes.
3. Electric heaters should only be employed with heat-insulating pads. After finishing the experiment electric instruments must be switched off and the plugs should be pulled out.
4. Operation with inflammable fluids is allowed only if throughout ventilation in the laboratory is ensured. Do not heat organic solvents on direct flame.
5. Chemicals which may react with each other with the risk of fire and explosion must not be mixed, transferred in the sinks or drains and stored together either.
6. Solutions containing inflammable or explosive agents or substances capable of generating gas must not be mixed, transferred in the sinks or drains and stored together either.
7. Know the proper use of all safety devices available including the fire-extinguishers.
8. In case of a fire shut off the gas, switch off the electric current and try to put out the fire by the available devices. **Electrical fire must not be extinguished with water**; a carbon dioxide extinguisher should be used. Fire must be reported to the laboratory instructor.
9. The telephone-number of the fire brigade is 105. Never call them without a good reason.

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LABORATORY TECHNIQUES

Laboratory equipments

The commonly used laboratory tools are shown in *Fig. 1*. The beakers and flasks are for storing liquids and the conical flask is used for heating liquids. The funnel helps pouring or filtrating liquids. With the separatory funnel one can separate inmiscible organic or inorganic liquids. Watch glass is for measuring powders and solids. Solid materials can be powdered in the mortar. A funnel or flask can be fixed by iron-ring, a burette with burette clamp to the holder stand. Gas burners are for heating. One can heat materials on naked flame, but it is more convenient and safer to put the crucible on clay triangle and the Erlenmeyer flask on wire gauze. Crucible tongs are for holding hot objects in the laboratory.

Volumetric apparatus

The most important volumetric apparatus are depicted in *Fig. 2*. Graduated cylinders, pipettes and burettes are calibrated to deliver, the volumetric flasks to contain the quantities marked on them at the given temperature (e.g. 20 °C). To read off the liquid level on these apparatus, one has to hold them at eye level to avoid the parallaxes error which originates from refraction. The surface of liquids wetting the wall of the glass (e.g. aqueous solutions) is concave. The volume of these solutions is read off from the position of the lowest point of the surface (it is called meniscus) on the volumetric apparatus scale (see *Fig.2*). An exception to the rule is a nontransparent solution, in this case the upper point of the surface is read off.

One has to fill up the volumetric flask, the graduated cylinder and the burette (if it is necessary with aid of a funnel) to the mark corroded into the glass. In pipettes the liquid has to be sucked up over the mark. Put your pointer finger on the end of the pipette to stop the flow (*Fig. 2*). If you want to leave the liquid to flow out, lift carefully your finger. Set the liquid level to the mark by letting out the excess. When the liquid is running down, touch lightly the tip of the pipette to the wall of the container. From a burette one can measure out liquids drop by drop with opening and closing the stopcock. The volumetric pipette is for measuring a defined volume, with the graduated pipette we measure in a defined range of volume. **Don't pipette hazardous or corrosive materials by mouth!** These materials have to be measured by a dispenser or a micropipette. To measure liquids with dispenser first of all set the desired volume at the scale and pull the piston up until bumping than push it down and let the solution flow out (*Fig. 2.*). For accurate measurements of very small volume of liquids use a micropipette. Push the lower end of the micropipette into a disposable plastic tip and push down the top knob of the pipette till the first bumping (*Fig. 2.*). Submerge the plastic tip into the liquid and permit the knob to ascend (the liquid will be sucked up). Dispense this volume into another test tube, touch the tip lightly to the wall of the tube than push down the knob (first bumping - the "measured" volume flows out; second bumping - "wash down" the liquid sticked to the wall of the tip with air). After the measurements collect the filthy plastic tips in a container.

Exercise

Gain practice in the use of the different volumetric apparatus by measuring out different volumes of distilled water.

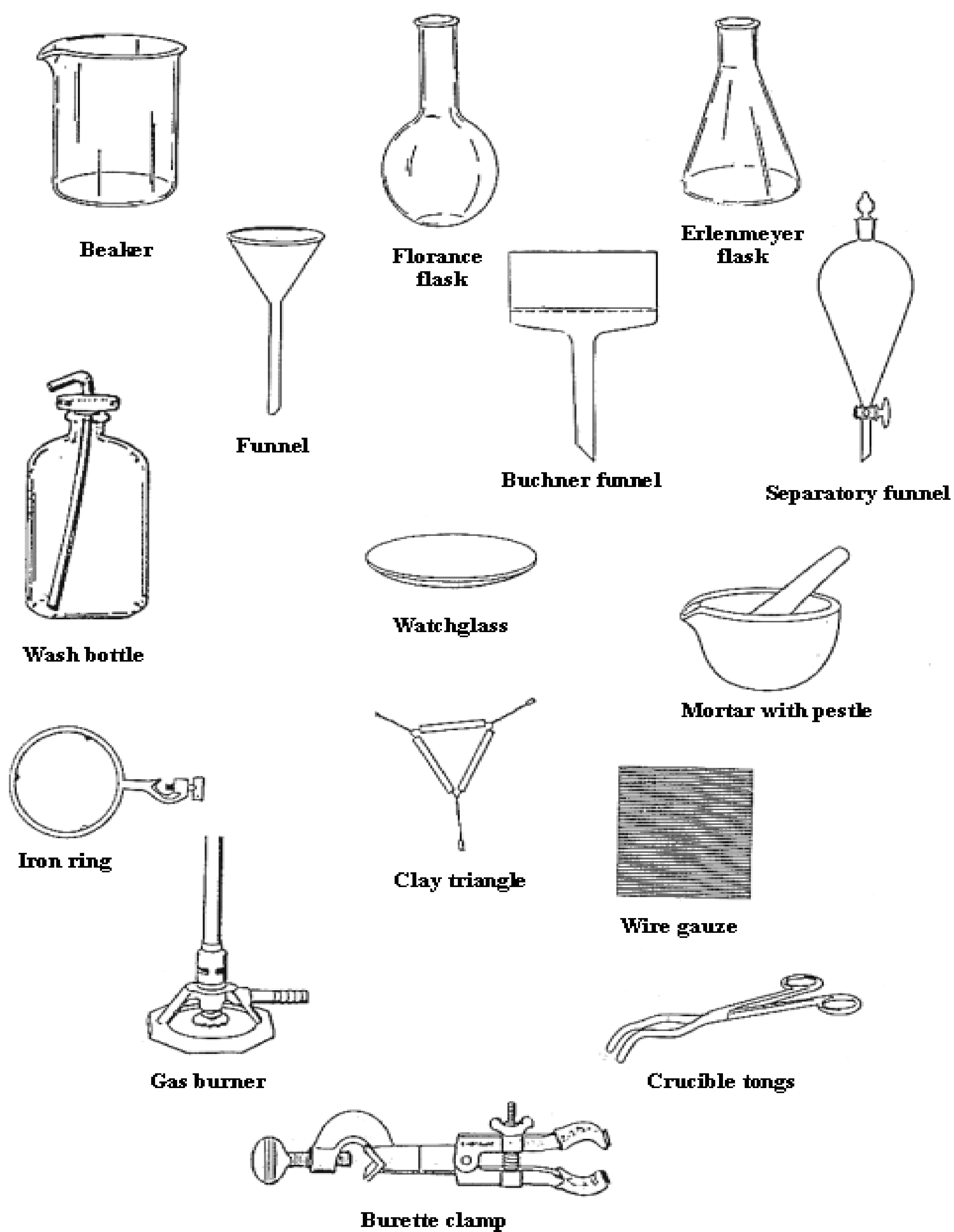


Figure 1. Common laboratory tools

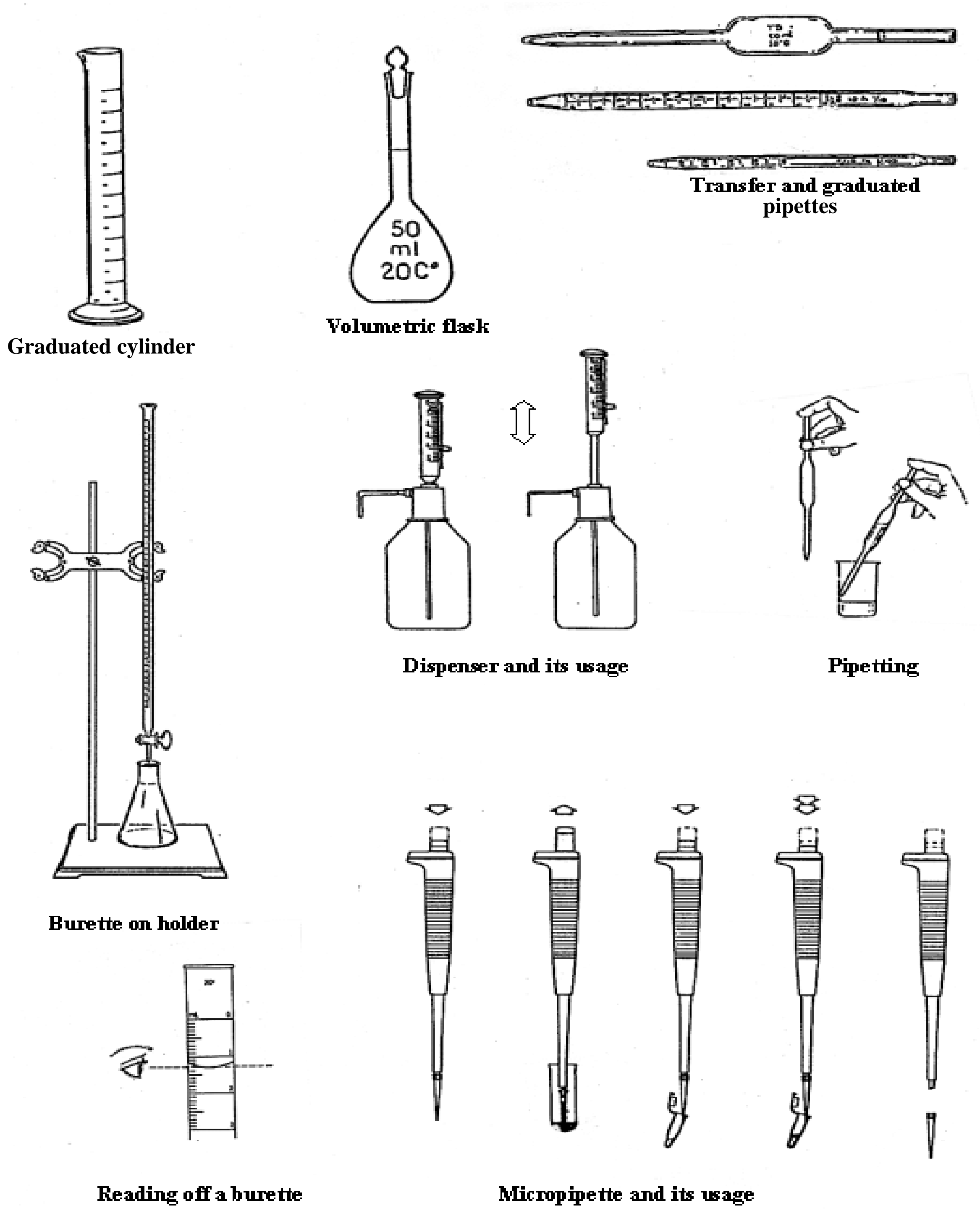


Figure 2. Volumetric apparatus

Filtration

The purpose of filtration is to separate a solid from a liquid in which it is suspended, thus it is suitable for separation of the components of a heterogeneous system. For filtration porous materials with large inner surface (e.g. filter paper, porous glass filter disks and plastic filter plates) are used. The diameter of the pores is smaller than the particles of solid to be retained by the filter while the liquid can flow through.

Experiment 1. *Filtration through filter paper*

Fold a square filter paper in half and then in quarters, after that cut it circularly. Open the paper so that it forms a rectangle with three thicknesses of paper on the one side and a single thickness on the other (*Fig. 3*). Then put the paper into the funnel and moisten it thoroughly with distilled water to fit the funnel. The top of the filter paper should be 0.5-1 cm below the rim of the funnel.

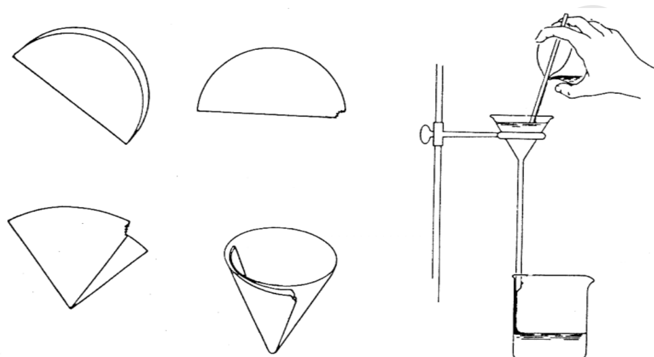
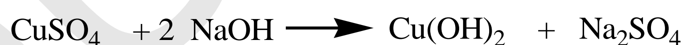


Figure 3. Preparation of the filter paper and filtration

Pour 3-4 cm^3 of 0.5 M $CuSO_4$ solution into a test tube and add 1 cm^3 of 0.5 M NaOH solution to it. Observe the formation of a blue precipitate according to the following chemical equation:



$$CuSO_4 + 2\ NaOH \longrightarrow \underline{Cu(OH)_2} + Na_2SO_4$$

Pour down the liquid to be filtered into the filter using the stirring rod as shown in *Fig. 3*.
Filtrate the precipitate, and after filtration add 0.5 M NaOH solution in excess to the filtrate.

Preparation of solutions

A solution is a liquid, homogeneous system, which contains two or more components (solvent and dissolved material).The concentration of the solution can be expressed as molarity, molality, mole fraction or percent concentration units (*Table 1*).

Table 1. Concentration units

Definition	Unit	Symbol
g of a solute per 100 g of solution	mass percent	m/m %
cm^3 of a solute per 100 cm^3 of solution	volume percent	v/v %
g of a solute per 100 cm^3 of solution	mass/volume percent	m/v %
number of moles of a solute per 1 dm^3 of solution	molarity	M, mol/dm^3
number of moles of a solute per 1 kg of solvent	molality	m, mol/kg
number of moles of a solute per number of moles of solution (solutes and solvent together)	mole fraction	x

A solution can be prepared either by dilution from a more concentrated solution or by dissolving a weighted substance in an appropriate volume of solvent. Weighing is done on an electronic balance (*Fig. 4*).

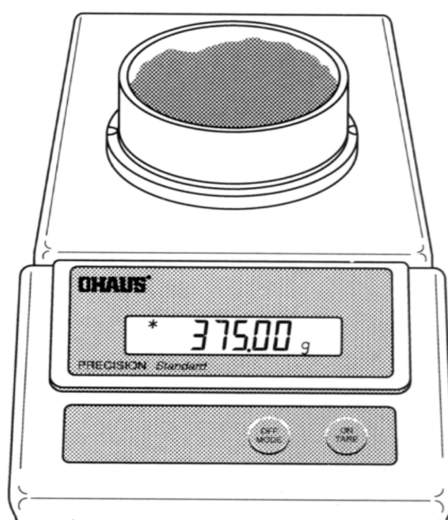


Figure 4. Electronic balance

Instruction manual for the electronic balance

With no load on the pan, turn the balance ON by pressing the ON TARE button. When first switched ON, all segments of the display should be shown. The checklist will be displayed briefly, then the model number of the balance followed by a software revision number. The display will momentarily blank and then indicate zero.

When weighing material or objects that must be held in a container, taring enables you to store the container weight in the balance′s memory.

1. Place an empty container on the pan. Its weight will be displayed.

2. Press ON TARE.
   The display will show zero and the container′s weight will be stored in memory.

3. Add material in to the container. As material is added, its net weight will be displayed.

4. Removing the container and material from the pan will cause the balance to display the container′s weight as a negative number.

Tared weight remains in the balance memory until ON TARE is pressed again.

Experiment 2. *Preparation of a solution*

Prepare, either a sodium chloride, a sodium sulfate or a sucrose solution of the concentration given by the instructor. Volumetric flasks are available in the capacities of 50, 100 and 250 cm^3. Express the concentrations in terms of molarity and mass/volume percent. The molar masses in g/mol are as follows, NaCl=58.5; Na_2SO_4 x 10 H_2O=322.2; sucrose=342.3.

Calculate how much solid substance you need for the preparation of the solution.
Weigh out the substance on a piece of paper and transfer into a beaker. Add distilled water in an amount only just enough to dissolve the solid by shaking. Pour the solutions in a funnel inserted into the neck of a volumetric flask. Wash the beaker and rinse with distilled water into the funnel three times. Wash thoroughly the funnel and then remove it from the flask. The content of the flask is made up to the mark with distilled water. Finally, shake up the solution by turning over the stoppered flask.

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Sample calculations

1. Prepare an 8 m/v % (mass/volume percent) NaCl solution in a 50.0 cm^3 volumetric flask. What mass of solute you need for the preparation of the solution?

8 m/v % concentration means that 100.0 cm^3 of solution contains 8 g of NaCl. The mass of solute in 50 cm^3 can be calculated as follows:

$$Mass\ of\ \ solute = 50.0\ cm^3 x \frac{8.0\ g}{100.0\ cm^3} = 4.0\ g$$

2. What is the (a) molality and the (b) molarity of a solution that was made by dissolving 2.0 g of table sugar (sucrose, $C_{12}H_{22}O_{11}$) in 150.0 cm^3 of water? The density of sucrose solution is 1.006 g/cm^3 and the molar mass of sucrose is 342.3 g/mol.

(a) Molality is the number of moles of solute per kilogram of solvent. First, you have to find out how many moles are in 2.0 g of sucrose?

The number of moles of sucrose is:

$$2g\ x\ \frac{1\,mol}{342.3\ g} = 5.84\,x\,10^{-3}\,mol$$

Next, you calculate the mass of 150.0 cm^3 water in kg unit. Since the density of water is 1.0 g/cm^3, 150.0 cm^3 of water has a mass of 150.0 g, or 0.150 kg.

The molality of the solution is:

$$Molality = \frac{5.84\,x\,10^{-3}\,mol}{0.150\,kg} = 0.039\,mol/kg$$

(b) Molarity is the number of moles of solute per dm^3 of solution.

Since the density of sucrose solution is 1.006 g/cm^3, the mass of the solution is 152.0 g (150.0 g of water and 2.0 g of sucrose). The volume of sucrose solution is:

$$Volume = \frac{152\ g}{1.006\ g/cm^3} = 151.1\,cm^3 = 0.1511\,dm^3$$

The molarity of the solution is:

$$Molarity = \frac{5.84\,x\,10^{-3}\,mol}{0.1511\,dm^3} = 0.0386\ mol/dm^3$$

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Questions

List the name of five volumetric equipments.

Define the molarity of a solution, and give its concentration unit.

Define the molality of a solution, and give its concentration unit.

Define the mass percent of a solution, and give its concentration unit.

Define the mass/volume percent of a solution, and give its concentration unit.

Define the mole fraction of a solution.

Exercises

1. Prepare a 4 m/v % (mass/volume percent) $CaCl_2$ solution in a 150.0 cm^3 volumetric flask. What mass of solute you need for the preparation of the solution?

2. Assuming that seawater is an aqueous solution of NaCl, what is its molarity? The density of seawater is 1.025 g / cm^3 at 20°C, and the NaCl concentration is 3.50 mass %.

3. What is the mass percent concentration of a sodium-hydroxide (NaOH) solution prepared by dissolving 1.5 mol of NaOH in 0.7 dm^3 of water? The molar mass of NaOH is 40.0 g/mol.

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Results

Date:

Experiment 1. *Filtration through filter paper*

What did you observe after mixing the $CuSO_4$ and NaOH solutions?

Write the chemical equation for the reaction.

What was the color of the filtrate?

What happened when you added an excess of NaOH solution, to the filtrate? Explain your observation.

Experiment 2. *Preparation of a solution*

Name of the solid:
Concentration to be prepared:
Volume of the volumetric flask:

Calculation

Mass of the weighed solid:

The concentration of the prepared solution is mol/dm^3;
m/v%.

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QUALITATIVE ANALYSIS

Chemical analysis is concerned with the detection or identification of what substances are present in a material and how much of each is present. Chemical analysis consists of two main parts: *qualitative analysis* and *quantitative analysis*.

Qualitative analysis deals with the identification of an element or compound and the detection of the components in a mixture. Physical properties - density, melting point, boiling point, color, odor, etc. - can be important factor is in the identification of substances. However, a reliable conclusion about the chemical composition can only be drawn from its chemical behavior. Therefore, a series of chemical reactions are carried out between the substance to be analyzed and appropriate reagents. Reagents are selected to induce fast, sensitive and characteristic reactions. Reactions producing a color change or formation of a new phase are preferred.

Qualitative analysis of a substance is carried out with its solution. The solubility of a substance in water, acids or bases is a characteristic property. The strict chemical analysis begins when the substance has been dissolved. To several aliquot parts of the solution various reagent solutions are added and precipitation or any other change is recorded. Most of the reactions are fast and are carried out in test tubes, using small volumes of reactants. Instrumental methods of qualitative analysis are beyond the topic of this textbook.

Chemical analysis of drinking-water

All natural waters contain ions of various salts. The salt concentration of seawater is especially high, Cl^-, Na^+ and Mg^{2+} ions are most abundant. Fresh waters contain lower concentrations of ions accumulated as the water courses over the land, dissolving soluble salts from rocks and minerals. Therefore the salt concentration of fresh waters depends on geological and environmental conditions. Natural waters contain Ca^{2+}, Na^+, Mg^{2+}, K^+ , Fe^{2+}, Fe^{3+} and NH_4^+ cations as well as HCO_3^-, CO_3^{2-}, OH^-, SO_4^{2-}, Cl^-, NO_3^-, F^-, and PO_4^{3-} anions most frequently. Drinking-water must not contain harmful substances.

Natural waters on the surface or in the ground may be polluted by various substances. The presence of ammonia (and ammonium ions), nitrite and nitrate ions is indicative of organic pollutant being decomposed by bacteria. Nitrate ions alone can derive from a pollution that occurred long ago or from a recent contamination with fertilizers. Phosphate ions indicate pollution with fertilizers or detergents. Waters containing high concentrations of iron, sulfate, and chloride ions are not suitable for direct consumption either because of their unpleasant odor and taste.

As long as the quantities of pollutants dissolved in water do not become excessive, the self purification of water occurs naturally. Dissolved gases and other volatile impurities are flushed out by air mixed with the water as it trickles over shallow stream beds. Solids settle out in pools and lakes. Microorganisms in water help to decompose organic materials from plants and animals.

The detection of various ions in aqueous solution is based on specific chemical reactions yielding a color change or precipitation. The concentration of an ion is proportional to the intensity of the color or the mass of precipitate. Thus the concentration can be determined semi-quantitatively by bear eye (see experiments below) or quantitatively, by photometric and gravimetric methods (see quantitative analysis).

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Experiment 1. *Determination of ammonium ions*

The determination is based on the formation of basic mercury(II)-amido-iodide upon addition of Nessler's reagent*, an alkaline solution of potassium tetraiodomercurate(II), to a water sample containing ammonium salts.

At low concentration of ammonium ions, an orange-brown color is produced and at higher concentration, a red-brown precipitate is formed.

$$NH_4Cl + 2\ K_2[HgI_4] + 4\ KOH \rightarrow \underline{HgO{\cdot}Hg(NH_2)I} + KCl + 7\ KI + 3\ H_2O$$

The alkaline reagent would precipitate the dissolved calcium and magnesium salts as hydroxides. Therefore, Seignette salt (potassium sodium tartrate) is added to Nessler's reagent before use. Tartrate in the "mixed" Nessler's reagent** prevents the precipitation of calcium and magnesium ions by forming complexes with them.

Procedure

Place 50 cm^3 of water in a large special test tube (Nessler tube), add 5 cm^3 of "mixed" Nessler's reagent and mix well. From the intensity of the resulting orange-brown color determine the concentration of ammonium ions, using the data in *Table 2*.

Table 2. Determination of ammonium ions in water

Sample color		Ammonium ion content	
from above	from the side	Classification	Concentration range (mg/dm^3)
Colorless	Colorless	Free of ammonium ion	0
Faint yellow	Colorless	Slight traces	0-0.05
Light yellow	Hardly visible yellow	Traces	0.05-0.2
Yellow	Pale yellow	Moderate concentration	0.2-1.0
Orange-brown	Yellow	High concentration	1.0-3.0
Dark orange-brown	Orange-brown	Extremely high concentration	Above 3.0

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**Preparation of Nessler's reagent*: Grind 10 g of HgI_2 in a porcelain mortar containing a small amount (about 40 cm^3) of distilled water. Dissolve 5 g of KI in about 10 cm^3 of distilled water, add to the suspension of HgI_2 and stir well. Transfer the resulting mixture into a 100 cm^3 volumetric flask, rinse the mortar with a few cm^3 of distilled water and pour the rinse in the flask, too. Dissolve 20 g of NaOH in about 40 cm^3 of distilled water, cool it down and add to the mixture in the flask. Fill up the flask to 100 cm^3 and set aside for a few days to clarify

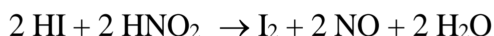
***Preparation of mixed Nessler's reagent*: Mix the clear Nessler's reagent with a 50% solution of Seignette salt in a 1:1 ratio just before use. Than dilute the mixture twofold with distilled water.

Comments and classification

Ammonia is formed during the decay of organic matter from plants and animals. The presence of ammonium ions may indicate that the water sample has been polluted with excrement of humans or animals (and presumably with pathogenic bacteria) recently. Therefore tap water must not contain ammonium ions (upper limit is 0.1 mg/dm^3). Spring waters may contain traces (0.05-0.2 mg/dm^3) of ammonium ions since they can derive from ammonia in the air.

Experiment 2. *Determination of nitrite ions*

The determination is based on the oxidation of iodide ions with nitrite ions in acidic solution, producing iodine:



$$2\ HI + 2\ HNO_2 \rightarrow I_2 + 2\ NO + 2\ H_2O$$

Iodine gives a blue color with starch solution. The intensity of the color is directly proportional to the nitrite content of water.

Procedure

Transfer 50 cm^3 of water to be analyzed, 1 cm^3 of 25% phosphoric acid, 4 cm^3 of 0.5% starch solution and 1 cm^3 of 10% potassium iodide solution into a Nessler tube. Mix and set aside for 30 minutes in the dark. Estimate the concentration of nitrite ions using the data in *Table 3*.

Table 3. Determination of nitrite ions in water

Sample color		Nitrite ion content	
from above	from the side	Classification	Concentration range (mg/dm^3)
Colorless	Colorless	Free of nitrite ion	0
Pale blue	Colorless	Slight traces	0-0.03
Light blue, transparent	Pale blue	Traces	0.03-0.1
Dark blue, opaque	Light blue	Moderate concentration	0.1-0.3
Black, opaque	Dark blue	High concentration	0.3-0.5
Black, opaque	Bluish-green	Extremely high concentration	Above 0.5

Comments and classification

While ammonium ions (see experiment 1.) suggest a recent pollution of the water the presence of nitrite ions indicates a contamination that occurred long ago. Tap water and spring water must not contain more than 0.03 mg/dm^3 and 0.1 mg/dm^3 of nitrite ions, respectively.

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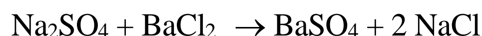
Experiment 3. *Determination of sulfate ions*

Sulfate ions react with $BaCl_2$ to give the white precipitate of $BaSO_4$ in an acidic solution:



$$Na_2SO_4 + BaCl_2 \rightarrow \underline{BaSO_4} + 2\ NaCl$$

Procedure

Transfer 25 cm^3 of water into a Nessler tube. Add 25 cm^3 of 5% barium chloride that contains hydrochloric acid*. Beware, $BaCl_2$ is poisonous! Mix well and allow to stand for 30 min. Now put the tube on a black surface and watch it from above. Then place the tube in front of a black surface and observe the extent of turbidity from the side. Give the approximate concentration of sulfate ions using the data in *Table 4*.

Table 4. Determination of sulfate ions in water

Sample		Sulfate ion content	
from above	from the side	Classification	Concentration range (mg/dm^3)
Clear	Clear	Free of sulfate ion	0
Slightly turbid	Clear	Traces	0-12
White, turbid	Slightly turbid	Low concentration	12-40
White, turbid	Precipitation observable	Moderate concentration	40-200
White	Fast sedimentation of a white precipitate observable	High concentration	200-1000
White	White powder sediment at the bottom	Extremely high concentration	Above 1000

Comments and classification

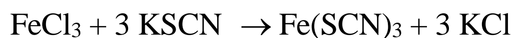
Sulfate ions in water usually originate from minerals. An increase in sulfate ion concentration may indicate pollution with domestic sewage or industrial wastewater. Drinking waters must not contain more than 50 mg/dm^3 of sulfate ions. Waters with higher sulfate ion content are not suitable for human consumption because of their unpleasant odor and taste.

*Preparation of $BaCl_2$-HCl reagent: Dissolve 50 g of $BaCl_2 \cdot 2H_2O$ in a small volume of distilled water, add 500 cm^3 of 10% HCl and then make up to 1 dm^3 with distilled water in a volumetric flask.

DUPress

Experiment 4. *Determination of iron ions*

Iron(III)ions react with potassium thiocyanate in acidic solution to produce a pink to red color due to the formation of iron(III) thiocyanate. The color intensity increases with increasing iron(III)ion concentration.



$$FeCl_3 + 3\ KSCN \rightarrow Fe(SCN)_3 + 3\ KCl$$

Iron(II)ions do not react, thus they should be oxidized to iron(III)ions with a small amount of $KMnO_4$ before carrying out the determination. The pink color of $KMnO_4$ does not interfere since unreacted $KMnO_4$ will be discolored by KSCN used in large excess.

Procedure

Transfer 50 cm^3 of water sample, 2.5 cm^3 of 10% hydrochloric acid, and 3 drops of 0.02 M $KMnO_4$ into a Nessler tube. Mix and allow standing for 10 min. Then add 2.5 cm^3 of 20% KSCN solution and allow standing for another 10 min. From the intensity of the color produced estimate the iron concentration in the water sample in mg/dm^3 using *Table 5*.

Table 5. Determination of iron ions in water

Sample color		Iron ion content ($Fe^{2+} + Fe^{3+}$)	
from above	from the side	Classification	Concentration range (mg/dm^3)
Colorless	Colorless	Iron ion free	0
Faint pink	Colorless	Very slight traces	0-0.1
Pink	Faint pink	Slight traces	0.1-0.3
Pink	Pink	Traces	0.3-0.5
Light red	Pink	Medium concentration	0.5-1.5
Red	Light red	High concentration	1.5-3.0
Dark red	Red	Extremely high concentration	Above 3.0

Comments and classification

The iron content of surface waters comes from wastewaters of metallurgical and tanning plants or surface mining works. Drinking waters must not contain more than 0.1 mg/dm^3 of iron ions. Waters with high iron concentration are not suitable for direct consumption.

Questions

Write the chemical reaction used in the determination of ammonium ions in water.
Write the chemical reaction used in the determination of nitrite ions in water.
Write the chemical reaction used in the determination of sulfate ions in water.
Write the chemical reaction used in the determination of iron ions in water.

DUPress

Results

Date:

Number of unknown water sample:
Give the results of analysis by filling in the table below.

Ion	Color		Classification	Concentration range (mg/dm^3)
	from above	from the side		
NH_4^+				
NO_2^-				
SO_4^{2-}				
Fe^{3+}, Fe^{2+}				

A drinking-water should meet the following criteria:

Color, transparency: colorless, fully transparent, no floating particles present.
Odor: odorless
Temperature: 14-18°C
Ammonium ion concentration: not more than 0.05 mg/dm^3
Nitrite ion concentration: not more than 0.05 mg/dm^3
Sulfate ion concentration: not more than 50 mg/dm^3
Iron ion concentration: not more than 0.1 mg/dm^3

Classify the water sample according to the above requirements by underlining the correct statement.

The No.   water sample is potable/not suitable for human consumption.

Explain your answer.

DUPress

Analysis of inorganic salts and complexes

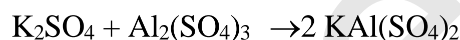
Compounds formed between metal or polyatomic cations and the anions of acids in a reaction of acids with bases are called *salts*. For example, KCl, Na_2SO_4, $Mg_3(PO_4)_2$, and $NaHCO_3$ are all referred to as salts. With a few exceptions, salts are crystalline ionic compounds. Melting or dissolving a salt in water frees the ions from their fixed positions in the solid. As a result, molten salts and aqueous solutions of salts conduct electricity.

Regular salts are formed between one kind of metal ion and anion, respectively [for example, NaCl, K_2,SO_4, $Al(NO_3)_3$ are called regular salts].

Acidic salts contain hydrogen ions in addition to metal ions and the anions of acids. Not all of the hydrogen ion of a polyprotic acid is replaced by a metal ion in the reaction producing acidic salt [for example, $NaHSO_4$, KH_2PO_4].

Basic salts contain hydroxide ions besides metal ions and the anions [for example, $Bi(OH)_2NO_3$].

Double salts are produced by the crystallization of two salts together, for example



$$K_2SO_4 + Al_2(SO_4)_3 \rightarrow 2\ KAl(SO_4)_2$$

Potassium aluminum sulfate is a double salt also known as alum. This substance is one of a general class of compounds, the alums that have a general formula where Na^+, K^+, NH_4^+ and Al^{3+}, Cr^{3+}, and Fe^{3+} are the most common cations.

$$M^+M^{3+}(SO_4)_2 \cdot 12\ H_2O$$

In the solutions of double salts the components - ions - keep their original properties.

Complex ions are formed between a metal ion that accepts one or more electron pairs from ions or neutral molecules (ligands) which donate electron pairs. The number of monovalent ligands bonded to a metal ion in a complex is the coordination number. The charge of a complex ion such as $[Ni(CN)_4]^{2-}$ or $[Co(NH_3)_4]^{3+}$, equals to the sum of the charges of the constituents. Ligands bonded to the central metal ion by coordinate covalent bonds make up the *first coordination sphere*. The tendency for the ligands to dissociate is usually small, depending on the stability of the complex. As a result, most of the properties of the complex differ from those of the free constituents. Complex formation is often accompanied by color change or an increase in solubility. The stability of a complex depends on the strength of the ligand-metal bond. The stronger is the bond the smaller is the tendency for a complex to dissociate. The constituents of a stable complex cannot be detected in the solution of the complex.

Complex salts, such as $K_2[Ni(CN)_4]$ or $[Co(NH_3)_4]Cl_3$, are formed between complex ions and other ions which are oppositely charged. The counterions establish a *second coordination sphere* around the central ion/atom by means of electrostatic interactions. Complex salts completely dissociate into complex ions and counterions in aqueous solution.

BUPress

Experiment 1. *Preparation of complexes*

(a) Into a few cm^3 of copper(II)sulfate solution pour aqueous ammonia dropwise until the resulting precipitate dissolves. The complex between Cu^{2+} and NH_3 has a coordination number of 4.

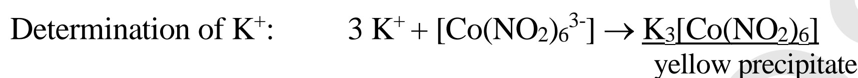
(b) To 1-2 cm^3 of nickel(II)sulfate solution add a small amount of NaOH solution and then excess of aqueous ammonia. The coordination number of Ni^{2+} in the complex with NH_3 is 6.

Experiment 2. *Dissociation of double salts and complex salts*

(a) Grind a small amount of alum $[KAl(SO_4)_2]$ in a mortar and dissolve it in about 10 cm^3 of distilled water. Divide the solution into three portions in test tubes for the identification of K^+, Al^{3+}, and SO_4^{2-} ions.

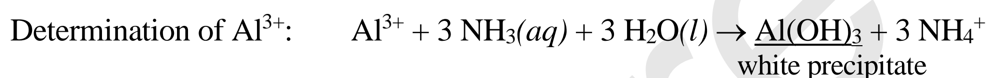
(b) Pour 2 cm^3 of 1.7 m/m% $AgNO_3$ into a test tube and add 30 m/m% KSCN solution dropwise until the precipitate generated dissolves. A complex has been formed with the formula $K[Ag(SCN)_2]$. Divide the solution into three aliquot portions for the detection of K^+, Ag^+, and SCN^-.

The identification of ions is based on the following net ionic reactions:



Determination of K^+: $3\,K^+ + [Co(NO_2)_6^{3-}] \rightarrow \underline{K_3[Co(NO_2)_6]}$

yellow precipitate



Determination of Al^{3+}: $Al^{3+} + 3\,NH_3(aq) + 3\,H_2O(l) \rightarrow \underline{Al(OH)_3} + 3\,NH_4^+$

white precipitate

Determination of SO_4^{2-}: $SO_4^{2-} + Ba^{2+} \rightarrow \underline{BaSO_4}$

white precipitate

Determination of Ag^+: $Ag^+ + Cl^- \rightarrow \underline{AgCl}$

white precipitate

Determination of SCN^-: $3\,SCN^- + Fe^{3+} \rightarrow Fe(SCN)_3$

red compound

Questions

Give an example of the regular salts (name and formula).
Give an example of the acidic salts (name and formula).
Give an example of basic salts (name and formula).
Give an example of double salts (name and formula).
Give an example of complex salts (name and formula).
Write the chemical equation of copper (II)-sulfate with ammonia. Give the name of the product. (The coordination number of Cu^{2+} in the complex with NH_3 is 4.)
Write the chemical equation of nickel (II)-sulfate with sodium-hydroxide and the product with excess amount of ammonia. Give the name of the products. (The coordination number of Ni^{2+} in the complex with NH_3 is 6.)
Explain the most important difference between a double salt and a complex salt.
Write the formula of alum and the equation for its dissociation.
Write the formulas for silver chloride and diamminesilver(I) chloride.

DUPress

Results

Date:

Experiment 1. *Preparation of complexes*

(a) Record your observations upon adding aqueous ammonia to copper(II) sulfate solution.

Color of solution before the experiment:

Color of precipitate generated:

Color of complex formed:

Write chemical equations for the reactions.

(b) What changes did you observe when you added NaOH and then aqueous ammonia to nickel(II) sulfate solution?

Color of solution before the experiment:

Color of precipitate generated:

Color of complex formed:

Write the equations for the reactions.

Experiment 2. *Dissociation of a double salt and a complex salt*

(a) Write the equation for the dissociation of alum. Which ions could you detect in the solution of alum?

(b) Write the equations for the formation of potassium dithiocyanatoargentate(I) and for the dissociation of this complex salt. Which ions can be identified in the complex salt's solution?

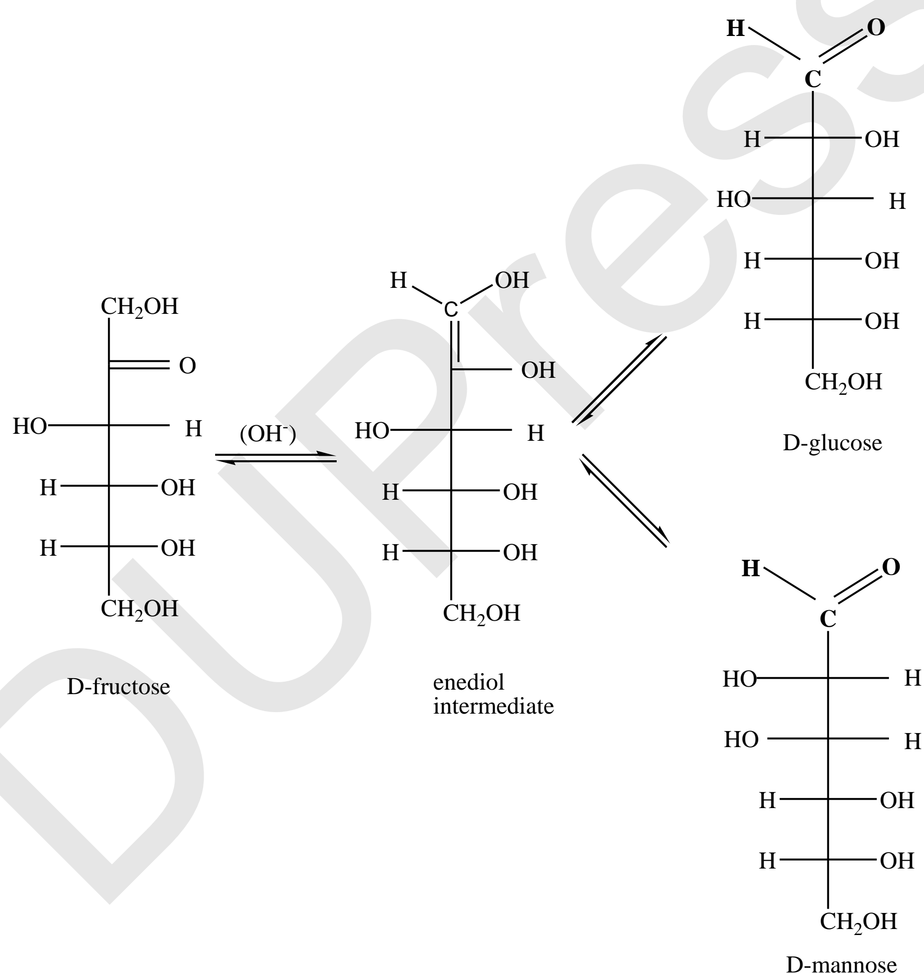
DUPress

Analysis of mono- and disaccharides

Carbohydrates are abundant organic compounds in plants and animals. Mono- and oligosaccharides serve as direct energy fuels for the cells. From a chemical point of view, carbohydrates are polyhydroxyaldehydes or polyhydroxyketones and their derivatives. The detection of carbohydrates is based on reactions of the carbonyl and hydroxyl groups which result in a color change or precipitation.

Reduction tests

Due to their free carbonyl (glycosidic hydroxyl) group, monosaccharides can act as reducing agents. In addition to the aldoses (glucose, galactose, mannose) fructose gives a positive reduction test too, because it is converted to glucose and mannose under alkaline conditions. The conversion can be explained by a keto-enol tautomerisation reaction mechanism:

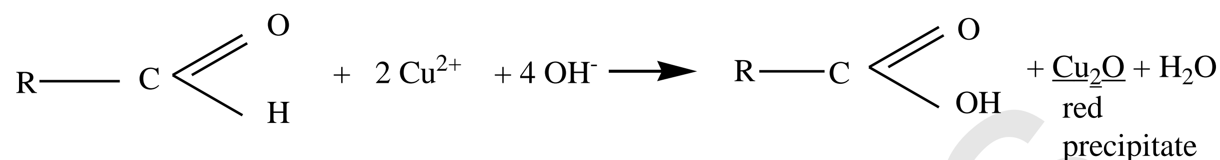


Among oligosaccharides, compounds with free *glycosidic hydroxyl group* have the reducing power.

DUPress

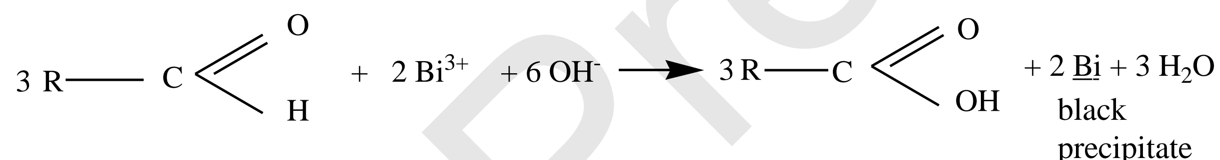
Experiment 1. *Fehling's test*

Prepare Fehling's solution freshly by mixing one volume Fehling I (7% $CuSO_4$) and one volume of Fehling II (3% NaOH + 35% sodium potassium tartrate) solutions. At the beginning, a blue precipitate, $Cu(OH)_2$ is generated which will dissolve due to the formation of a violet complex with sodium potassium tartrate. Then pour about 2 cm^3 of Fehling's reagent (mixture of Fehling I and II) in a test tube and add 2 cm^3 of unknown sugar solution. Heat the mixture for 3 min in a boiling waterbath. A positive reaction (the red precipitate of Cu_2O) indicates the presence of a reducing agent which decreased the oxidation number of copper from +2 to +1.



Experiment 2. *Nylander's test*

Add 2 cm^3 of Nylander's reagent* to 2 cm^3 of the solution to be analyzed. Heat the mixture for 3 min in a boiling waterbath. A positive test (a black precipitate) indicates that bismuth(III) ions have been reduced to bismuth metal.



Reactions based on the dehydration of sugars

In the presence of strong mineral acids, ketohexoses (e.g., fructose) undergo an intramolecular dehydration which yields derivatives of the heterocyclic furfural. In concentrated acid solution, furfural derivatives are converted to lower members of aldehydes which condense with phenol derivatives (e.g., naphthol, anthrone, resorcinol) to give a colored compound. Under well-defined conditions, dehydration reactions are specific to ketohexoses.

Experiment 3. *Seliwanoff's test*

Pour 1-2 cm^3 of the solution to be analyzed in a test tube. Add 1-2 cm^3 of Seliwanoff's reagent (0.5% resorcinol in concentrated hydrochloric acid). Stir and incubate the mixture in a waterbath at 80-90°C for 5 min.

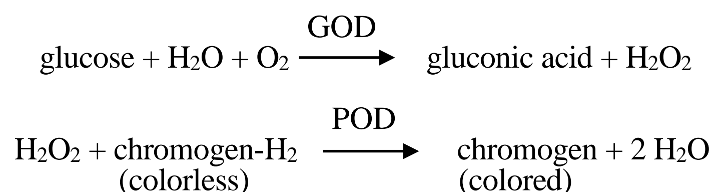
A positive reaction produces a bright red color with or without precipitation. The slightly positive reaction with sucrose when boiled longer than 5 minutes is due to fructose, formed as a hydrolysis product of sucrose in the presence of concentrated HCl (see experiment 5.).

DUPress

* The content of Nylander's reagent: $Bi(OH)_2NO_3$ (3%), NaOH (10%), K-Na tartrate (4%)

Enzymatic detection of glucose

Glucose is oxidized to gluconic acid in the reaction catalyzed by glucose oxidase (GOD). The other product, H_2O_2, can oxidize a chromogen to a colored product in the presence of peroxidase (POD):



Positive reaction occurs only in the presence of glucose since the enzyme glucose oxidase is specific to glucose.

Experiment 4. *Detection of glucose by glucose oxidase and peroxidase*

Various manufacturers produce test papers impregnated with glucose oxidase, peroxidase and a reduced chromogen (for example, o-tolidine). The chromogen can be oxidized to a colored product.

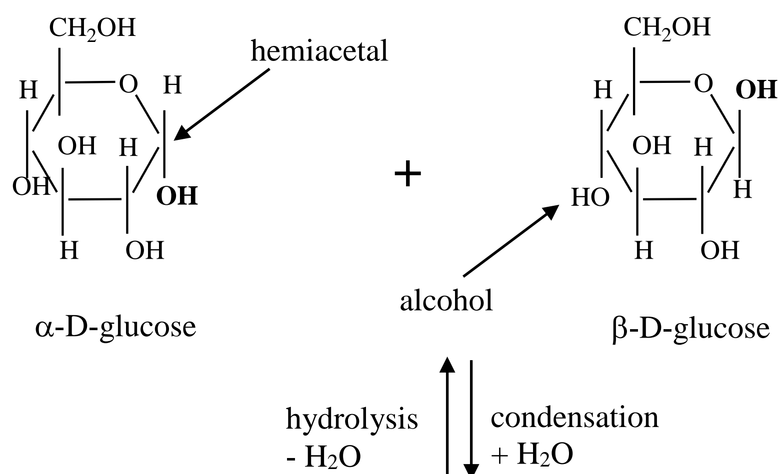
Dip reagent end of a strip in the solution to be examined. Tap the edge of strip against the side of the sample container to remove excess fluid. 30 sec after removing the strip from the sample compare reagent side with the closest matching block in the color chart. Positive reaction is indicated by a color change to blue or violet (depending on the chromogen). Estimate the glucose concentration of the sample.

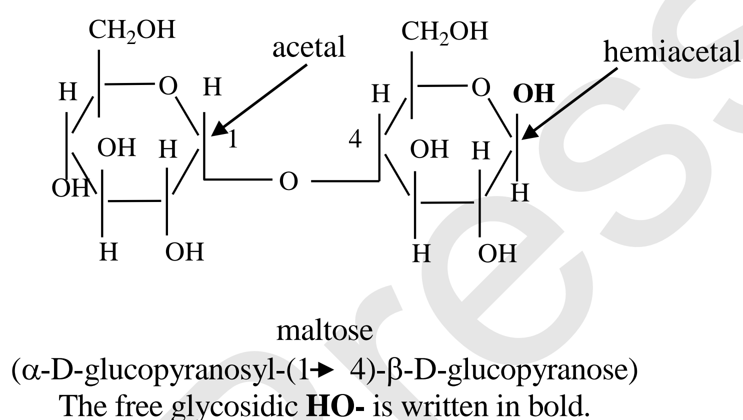
Analysis of disaccharides

Disaccharides consist of two monosaccharides joined by a glycosidic bond. The glycosidic bond breaks upon hydrolysis with acids or specific enzymes. Two sugar molecules can be joined in tandem, when a hydroxyl (alcohol) group of one sugar displaces the hydroxyl group on the other sugar's anomeric carbon. The bond formed is called a glycosidic bond. In forming the glycosidic bond, an acetal is generated on one sugar (at carbon 1) in place of the hemiacetal. Convince yourself that the other carbon (carbon 4) is not an acetal. One mole of water is formed per glycosidic bond generated. Disaccharides are divided into two classes.

Reducing disaccharides, the glycosidic bond is formed between the glycosidic hydroxyl group of one monosaccharide and an alcoholic (not the glycosidic) hydroxyl group of the other. As a result, these molecules, for example the disaccharide formed upon condensation of two glucose molecules called maltose (shown in the next page), contain a free glycosidic hydroxyl group conferring them reducing power.

DUPress





maltose
(α-D-glucopyranosyl-(1→ 4)-β-D-glucopyranose)
The free glycosidic **HO-** is written in bold.

Nonreducing disaccharides contain a glycosidic linkage between the glycosidic hydroxyl groups of both monosaccharides. Due to the absence of the glycosidic hydroxyl group, disaccharides in this class (such as sucrose shown in the next page) do not have reducing power.

When sucrose is hydrolyzed in the presence of sulfuric acid it forms a 1:1 mixture of glucose and fructose. This mixture is the main ingredient in honey. It is called invert sugar because the angle of the specific rotation of the plain polarized light changes from a positive to a negative value due to the fact that the specific rotation of D-fructose (a levorotatory sugar) is larger than that of D-glucose (a dextrorotatory sugar).

Experiment 5. *Hydrolysis of sucrose (inversion)*

Place about 5 cm^3 of sucrose solution in a test tube. Add 4-5 drops of 10% sulfuric acid and incubate in boiling waterbath for 15 min. Then add 2 cm^3 of 10% sodium hydroxyde in order to neutralize the acid. Perform Fehling's test and Seliwanoff's reaction with sucrose before and after hydrolysis.

DUPress

sucrose
(α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside)

$+ H_2O$ $+H_2SO_4$

α-D-glucose + β-D-fructose

Questions

Give the name of the method suitable for the specific detection of glucose.

Draw the structural formula for β-D-fructofuranose. Give the name of the reagent suitable for the specific identification of fructose.

Draw the structural formula of α-D-maltose and mark the position of the O-glycosidic bond. Is maltose a reducing or a non-reducing sugar?

Draw the structural formula for sucrose and mark the position of the O-glycosidic bond. Is sucrose a reducing or a non-reducing sugar?

Explain what happen when the Fehling I and Fehling II solutions are mixed.

Write the chemical equation for the Fehling reaction of an aldehyde.

Write the name of the methods suitable for identifying (a) glucose, (b) fructose in a mixture of these sugars.

Write the basic steps of the enzymatic detection of glucose.

What are the components of inverted sugar and how can you prepare it from sucrose?

DUPress

Results

Date:

Perform **Experiments 1-5** with the mono- and disaccharides as indicated in the table below. Designate positive and negative reactions with + and **-**, respectively.
Perform the tests with an unknown solution, too. Which of the sugars is present in the sample?

Test	Glucose	Fructose	Inverted sugar	Maltose	Sucrose	Sucrose after hydrolysis	Unknown No.:
1. Fehling's							
2. Nylander's							
3. Seliwanoff's							
4. Glucose oxidase and peroxidase							

Conclusion

The number   solution contains the following saccharides:

Explain your conclusion.

DUPress

QUANTITATIVE ANALYSIS

The purpose of quantitative analysis is to determine the relative quantity of one or more constituents of a compound or of a mixture. The determination may utilize chemical or physicochemical methods (see Instrumental Methods of Chemical Analysis). The chemical methods of quantitative analysis are based on a reaction between a specific reagent and the substance to be determined. *Gravimetric analysis* is the process of measuring the mass of one of the products formed in the reaction. *Volumetric analysis* consists of measuring the volume of a reagent solution which is required to react quantitatively with the solution to be analyzed. In the *instrumental analysis*, some physicochemical properties are measured which are related to the amount of the substance.

VOLUMETRIC ANALYSIS

In volumetric analysis, a measured volume of the reagent of precisely known concentration is added to a known volume of the solution to be examined until the reaction is complete. The solution of known concentration is called the *standard solution* and the process of adding it to the unknown solution is referred to as *titration*. At the equivalence point, chemically equivalent amounts of the solutions have reacted. The concentration of the solution can be calculated from the volume of standard solution required to reach the equivalence point.

For use in volumetric (titrimetric) analysis, a reaction must fulfill the following criteria:

1. There must be a simple and fast reaction which can be represented by a single chemical equation. The substance to be determined should react completely with the reagent.

2. There must be a marked change in some physical or chemical properties of the solution at the equivalence point (e.g., color change of the solution or of an indicator dye added to the solution, change in the electric conductivity or electromotive force etc.).

According to the type of reaction, volumetric methods are divided into four classes:

(a) acid-base titrations,

(b) redox titrations,

(c) complexometric titrations, and

(d) precipitation titrations.

Acid-base titrations

Acid-base titrations include the titration of bases with a standard acid (alkalimetry), and the titration of acids with a standard base (acidimetry).

The change in the pH throughout the course of an acid-base titration can be represented by a titration curve, a plot of pH versus volume of acid or base added. The curves have different characteristic shapes depending on whether strong or weak acids or bases are involved.

DUPress

In a *strong acid-strong base titration*, the products are water and a salt that does not react with water. Considering the dissociation of strong electrolytes, the net ionic reaction is the combination of H^+ and OH^- to give a water molecule. The pH of the solution before the equivalence point is determined by the acid which has not yet reacted with base. At the equivalence point the pH is 7.0 (*Fig. 5*) since the concentrations of H^+ and OH^- are equal. In the vicinity of the equivalence point, the pH changes sharply. After the equivalence point, the pH of the solution is determined by the excess of base added.

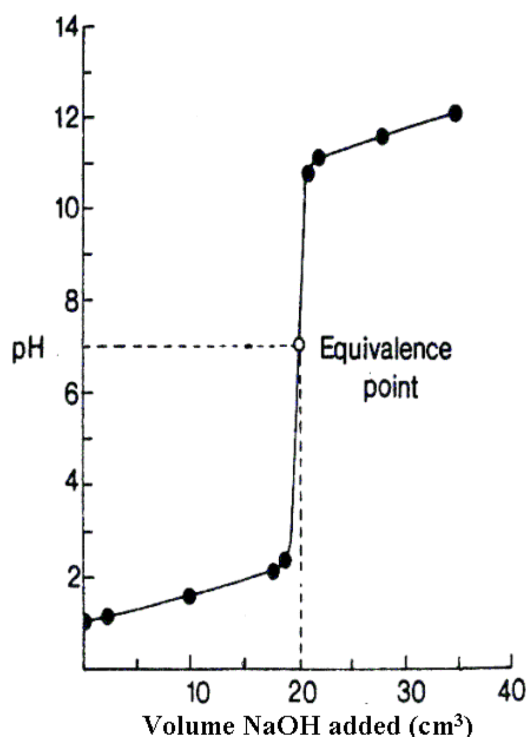


Figure 5. Strong acid-strong base titration
Titration of 20.0 cm^3 of 0.100 M HCl with 0.100 M NaOH.

The comparatively large pH change around the equivalence point in the titration curve allows several methods to be used for end point detection. Any acid-base indicator with a color change interval between pH 3.0 and pH 7.0 is suitable (e.g., methyl orange, pH 3.1 - 4.4, see *Fig. 6*). The end point of the titration can also be established by means of some instrumental (potentiometric, conductometric etc.) methods.

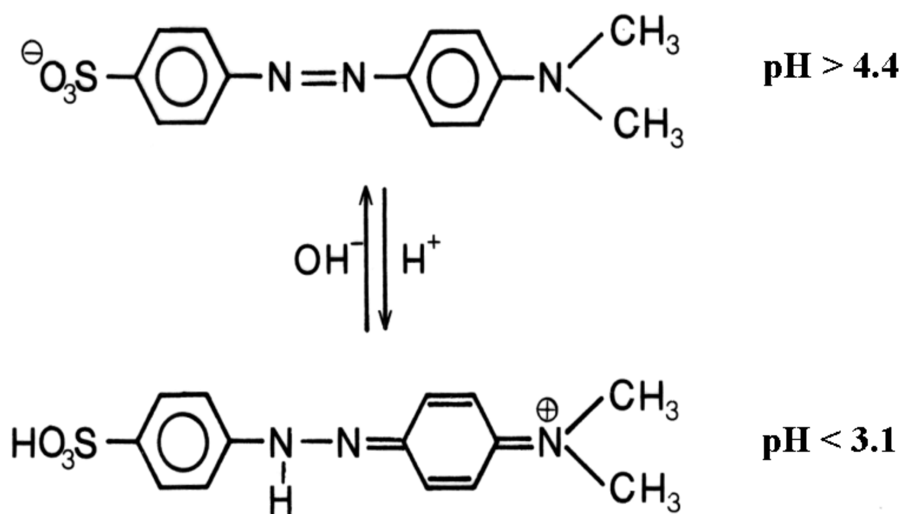


Figure 6. Structure of methyl orange indicator
At pH> 4.4, the indicator is yellow that turns red at pH <3.1.

Figure 7 shows a curve for a *weak acid - strong base titration*. From the addition of the first drops of base and before the equivalence point, the solution contains a buffer system (i.e., acetic acid plus acetate ion).

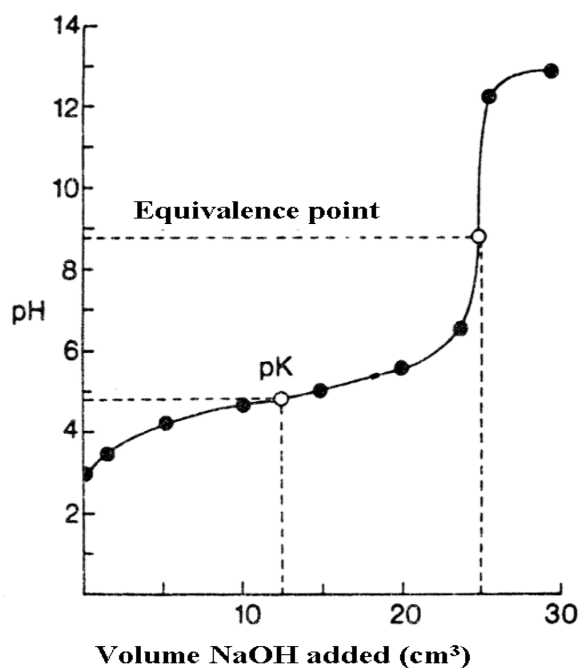


Figure 7. Weak acid - strong base titration
Titration of 25.0 cm^3 of 0.100 M acetic acid with 0.100 M NaOH.

At the equivalence point, the weak acid has been neutralized by the base but the solution is not neutral, because the anions from the salt undergo hydrolysis to give an alkaline pH.

Suitable indicators for weak acid-strong base titrations are those which exhibit a color change in the alkaline region (e.g., phenolphthalein, pH 8.3-10.0, *Fig. 8*).

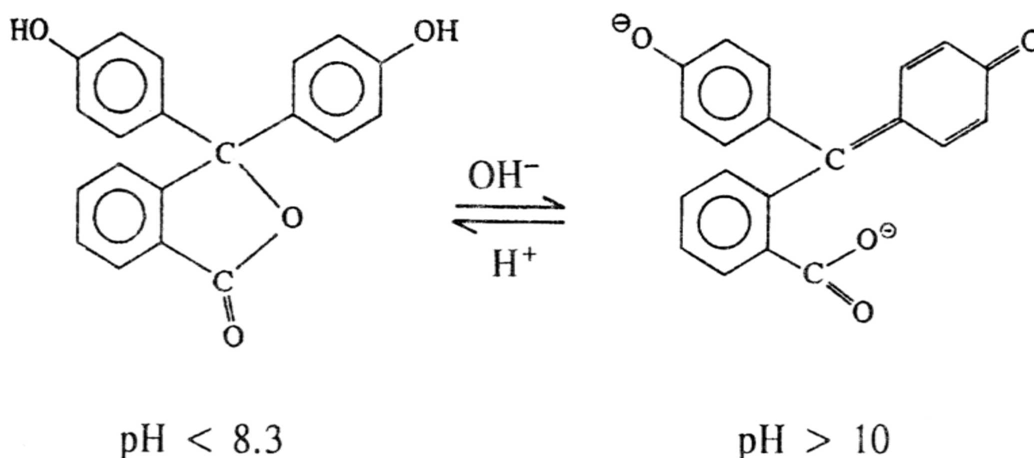


Figure 8. Structure of phenolphthalein indicator
At pH < 8.3, phenolphthalein is colorless, and at pH > 10, it is pink.

General titration procedure

Pour a small volume of the standard solution in the buret through a short funnel. Rinse the buret with the solution and discard the rinse. Then fill the buret to a little above the highest graduation, and remove the funnel. Allow the solution to flow out until the lowest point of the meniscus just touches the top graduation (see *Fig. 2*).

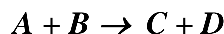
Place a given volume of the solution to be titrated into a conical (Erlenmeyer) flask by means of a volumetric transfer pipette and add a few drops of indicator. Begin to add the standard solution in the buret to the flask slowly. Between additions, swirl the flask to mix the contents. Approaching the equivalence point, the color change remains longer but it disappears when the solution is swirled. Now slow the addition of the standard solution. Swirl your flask after the addition of each drop. At the end point, one drop of standard solution will change the color of the entire contents of the flask and the color change remains stable for a long while. To make the end point easier to see, view the color of the solution against a white background.

Record the buret reading. Repeat the titration with a new portion of the unknown solution until you get reproducible results. Take the final buret readings. The volume of standard solution used is the difference between the final and initial buret readings.

Calculate the concentration of unknown solution from the mean of the volumes of standard solution used in independent titrations.

General calculation method

Consider first a simple chemical reaction in which the mole ratio of the reactants is one to one. The balanced equation for the reaction is



$$\boldsymbol{A + B \rightarrow C + D}$$

where A is the reagent in the standard solution and B is the substance in the unknown solution. Assume that V_A cm^3 of a c_A M standard solution was used to completely react with V_B cm^3 of a c_B M unknown solution.
The number of moles of A in V_A cm^3 of a c_A M solution is

$$(V_A\ cm^3)\left(\frac{1\ dm^3}{1000\ cm^3}\right)\left(\frac{c_A\ mol}{1\ dm^3}\right) = 0.001 c_A x V_A \ \text{mol}$$

which reacts with $0.001 c_A\, x\, V_A$ mol of B. The volume of B in dm^3 is

$$(V_B\ cm^3)\left(\frac{1\ dm^3}{1000\ cm^3}\right) = 0.001 V_B \ \text{dm}^3.$$

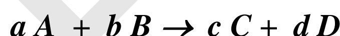
The molarity of B is

$$c_B = \frac{0.001 c_A x V_A}{0.001 V_B} \frac{\text{mol}}{\text{dm}^3} = \frac{c_A x V_A}{V_B} \ \text{mol/dm}^3.$$

Thus, the concentration of the unknown solution can be calculated with the aid of the following formula

$$c_B = \frac{c_A x V_A}{V_B}$$

Next, consider a reaction in which the $A : B$ mole ratio is $a : b$



$$\boldsymbol{aA + bB \rightarrow cC + dD}$$

As before, assume that V_A cm^3 of a c_A M standard solution was used to completely react with V_B cm^3 of a c_B M unknown solution.
The number of moles of A in V_A cm^3 of a c_A M solution is $0.001 c_A$ x V_A (see above).
Since a mol of A reacts completely with b mol of B, $0.001 c_A$ x V_A mol of A reacts with

$$\frac{b\, x\, 0.001 c_A x V_A}{a} \ \text{mol of } B$$

which is present in a volume of $0.001 V_B$ dm^3.
Thus, the molarity of B is

$$c_B = \frac{b\, x\, 0.001 c_A x V_A}{a\, x\, 0.001 V_B} = \frac{b\, x\, c_A x V_A}{a\, x\, V_B} \ \text{mol/dm}^3.$$

The general calculation method in the latter case is

$$\boxed{c_B = \frac{b\, x\, c_A x V_A}{a\, x\, V_B}}$$

DUPress

Strong acid-strong base titrations

Experiment 1. *Standardization of sodium hydroxide*

Solid sodium hydroxide is hygroscopic and absorbs carbon dioxide from air. Therefore, sodium hydroxide always contains a trace amount of sodium carbonate and water. As a consequence, a sodium hydroxide solution with accurate concentration cannot be prepared by dissolving a calculated mass of NaOH in a measured volume of water or solution. In order to find the concentration of an approximately 0.1 M NaOH solution accurately, it is necessary to titrate it against an acid solution with accurate concentration (e.g., against 0.1000 M HCl).

Procedure

Transfer 10.0 cm^3 of 0.1000 M hydrochloric acid to a conical flask. Add 1 or 2 drops of methyl orange and titrate with sodium hydroxide until the color of methyl orange becomes orange. At the equivalence point, an additional drop of the base will change the color of the solution to yellow. At this point, record the volume for NaOH.

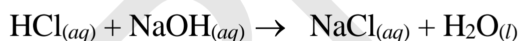
Perform two more titrations of the hydrochloric acid solution in the same way. Calculate the concentration of NaOH solution from the average volume used to neutralize HCl in the three independent titrations.

Calculation

The number of moles of HCl in 10.0 cm^3 of 0.1000 M HCl solution is

$$(10.0\,\text{cm}^3)\left(\frac{1\,\text{dm}^3}{1000\,\text{cm}^3}\right)\left(0.1000\frac{\text{mol}}{\text{dm}^3}\right) = 0.001 \text{ mol}$$

which reacts with 0.001 mol of NaOH according to the equation



$$HCl_{(aq)} + NaOH_{(aq)} \rightarrow NaCl_{(aq)} + H_2O_{(l)}$$

If V cm^3 of the base is required to neutralize the acid, the molarity of NaOH is

$$c_{NaOH} = \left(\frac{0.001\,\text{mol}}{\text{V cm}^3}\right)\left(\frac{1000\,\text{cm}^3}{1\,\text{dm}^3}\right) = \frac{1}{\text{V}}\ \frac{\text{mol}}{\text{dm}^3}$$

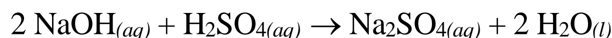
Experiment 2. *Determination of sulfuric acid*

Procedure

Deliver 10.0 cm^3 of unknown H_2SO_4 into an Erlenmeyer flask by a volumetric transfer pipette. Add 1 or 2 drops of methyl orange and titrate with the NaOH standardized in *Experiment 1.* Repeat titration with another 10.0 cm^3 portion of H_2SO_4.

Calculation

According to the equation



$$2\,NaOH_{(aq)} + H_2SO_{4(aq)} \rightarrow Na_2SO_{4(aq)} + 2\,H_2O_{(l)}$$

1 dm^3 of 1.000 M NaOH neutralizes 1/2 mol (49 g) of H_2SO_4. Using the accurate concentration of the NaOH standard solution, calculate the concentration of H_2SO_4 in molarity and mass/volume%.

DUPress

Sample calculations

1. Calculate the molarity of a sodium hydroxide solution if 10.25 cm^3 of this solution is needed to neutralize 10.0 cm^3 of 0.0985 M HCl.

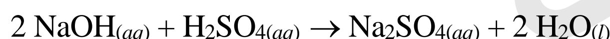
HCl and NaOH react in a 1:1 mole ratio, therefore

$$c_{NaOH} = \frac{c_{HCl} \text{ x } V_{HCl}}{V_{NaOH}}$$

$$c_{NaOH} = \frac{0.0985 \text{ M x } 10.0 \text{ cm}^3}{10.25 \text{ cm}^3} = 0.0961 \text{ M}$$

2. 10.0 cm^3 of a sulfuric acid solution was neutralized with 7.5 cm^3 of 0.0976 M NaOH. Express the concentration of the sulfuric acid solution in (a) molarity, (b) mass/volume %. The molar mass of H_2SO_4 is 98.0 g/mol.

The reaction is represented by the following equation



$$2 \text{ NaOH}_{(aq)} + H_2SO_{4(aq)} \rightarrow Na_2SO_{4(aq)} + 2 \text{ H}_2O_{(l)}$$

(a) 7.5 cm^3 of a 0.0976 M NaOH contains

$$(7.5 \text{ cm}^3)\left(\frac{1 \text{ dm}^3}{1000 \text{ cm}^3}\right)\left(\frac{0.0976 \text{ mol}}{1 \text{ dm}^3}\right) = 7.32 \text{ x } 10^{-4} \text{ mol of NaOH}$$

which reacts with $\frac{7.32 \text{ x } 10^{-4}}{2}$ mol of H_2SO_4 = 3.66 x10^{-4} mol of H_2SO_4. The volume of sulfuric acid solution is 10.0 cm^3 = 0. 01 dm^3.

The molarity of H_2SO_4 is

$$c_{H_2SO_4} = \frac{3.66 \text{ x} 10^{-4} \text{ mol}}{0.01 \text{ dm}^3} = 0.0366 \text{ M}$$

(b) 1000 cm^3 of 0.0366M M H_2SO_4 contains $\left(0.0366 \frac{\text{mol}}{\text{dm}^3}\right)\left(98.0 \frac{\text{g}}{\text{mol}}\right)$ = 3.587 g of H_2SO_4.

Therefore, 100 cm^3 of 0.0366M M H_2SO_4 contains 0.3587 g of H_2SO_4.
Thus, the concentration of H_2SO_4 is 0.3587 m/v%.

DUPress

Questions

Why is it necessary to standardize the NaOH solution prepared for titration of an acid?

How is the accurate concentration of the NaOH solution determined (reaction, indicator)?

How is the concentration of sulfuric acid solution determined by acid-base titration?
Write the equation for the reaction. Would you use methyl orange or phenolphthalein to detect the equivalence point?

Make a sketch of the titration curve expected for the titration of HCl with NaOH. Mark the equivalence point and the pH at the equivalence point.

Exercises

1. Calculate the molarity of a sodium hydroxide solution if 8.75 cm^3 of this solution is needed to neutralize 10.0 cm^3 of 0.1204 M HCl. Give the concentration of NaOH in mass/volume %. (The molar mass of NaOH is 40.0 g/mol.)

2. What is the molarity of a sodium hydroxide solution if 10.0 cm^3 of this solution is neutralized with 5.6 cm^3 of 0.1564 M HCl? How many milligrams of NaOH are present in 0.500 dm^3 of the solution? (The molar mass of NaOH is 40.0 g/mol.)

3. A volume of 12.5 cm^3 of 0.1684 M NaOH is required to completely react with 5.00 cm^3 of a sulfuric acid solution. Calculate the molarity and mass/volume % of sulfuric acid. (The molar mass of H_2SO_4 is 98.0 g/mol.)

4. Calculate the molarity of a sulfuric acid solution if 22.0 cm^3 of this solution is needed to neutralize 10.0 cm^3 of 0.1246 M NaOH. What volume of the solution contains 1.00 g of H_2SO_4? (The molar mass of H_2SO_4 is 98.0 g/mol.)

DUPress

Results

Date:

Experiment 1. *Standardization of sodium hydroxide*

Molarity of HCl: 0.1000 M
Volume of HCl: 10.0 cm^3

	Titration		
	1	*2*	*3*
Initial buret reading (cm^3)			
Final buret reading (cm^3)			
Volume of NaOH used (cm^3)			
Average volume of NaOH used (cm^3)			

Calculation

The accurate concentration of NaOH is   mol/dm^3.

DUPress

Experiment 2. *Determination of sulfuric acid*

Unknown H_2SO_4 No.:
Volume of H_2SO_4:
Concentration of standard NaOH solution from *Experiment 1*:

	Titration	
	1.	2.
Initial buret reading (cm^3)		
Final buret reading (cm^3)		
Volume of NaOH used (cm^3)		
Average volume of NaOH used (cm^3)		

Calculation

DUPress

The concentration of the unknown H_2SO_4 solution No. is mol/dm^3,
m/v %.

Weak acid - strong base titrations

Experiment 1. *Determination of acetic acid*

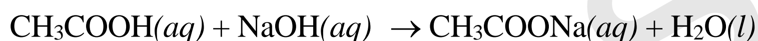
You will use NaOH standard solution to titrate an unknown acetic acid. Due to the hydrolysis of acetate ions from sodium acetate, perform the titration in the presence of phenolphthalein indicator with a color transition interval of pH 8.3-10.0 (changes from colorless to red). Phenolphthalein is weaker acid than carbonic acid therefore it is sensitive to carbon dioxide of air.

Procedure

Transfer 10.0 cm^3 of unknown acetic acid into an Erlenmeyer flask, add 2 or 3 drops of phenolphthalein and titrate with NaOH until the color becomes faint pink. The end point is indicated by a color change that lasts 15 sec after swirling the flask. (Upon standing longer, carbonic acid from atmospheric carbon dioxide makes phenolphthalein colorless again.) Repeat the titration with a second and a third 10 cm^3 aliquot of the unknown acetic acid.

Calculation

According to the equation



$$CH_3COOH(aq) + NaOH(aq) \rightarrow CH_3COONa(aq) + H_2O(l)$$

1 mol of NaOH reacts with 1 mol (60.0 g) of acetic acid. Calculate the milligrams of acetic acid present in 10.0 cm^3 of the solution from the average volume of NaOH used. Express the concentration of acetic acid in terms of mass/volume% and molarity.

Experiment 2. *Analysis of free HCl and total acidity of gastric juice*

Hydrogen ions from a strong acid decrease the extent of dissociation of a weak acid, as a consequence of Le Chatelier's principle. Therefore, hydroxide ions from NaOH combine with hydrogen ions from the strong acid first. The end point for the neutralization of the strong acid can be detected by methyl orange indicator with a color transition interval of pH 3.1-4.4 (*Fig. 6*). Additional amounts of hydroxide ions will react with hydrogen ions from the weak acid. The end point for the second process can be detected by phenolphthalein indicator with transition interval of pH 8.3-10.0 (*Fig. 8*).

Gastric juice contains hydrochloric acid and weak acids (hydrogen phosphates, and in pathological cases, lactic acid). The acid content of gastric juice was characterized by the free HCl and total acidity. Free HCl content (termed free acidity), given in mmol HCl/dm^3, is identical numerically with the number of cm^3 of 0.100 M NaOH required to cause methyl orange to change color in 100.0 cm^3 of gastric juice. Total acidity, in mmol total acid/dm^3, is identical numerically with the number of cm^3 of 0.100 M NaOH causing the color change of phenolphthalein in 100.0 cm^3 of gastric juice.

It should be noted that in the clinical diagnostics, gastric acid secretion has been characterized recently by the HCl secretion per hour, expressed in mmol/hr. The test begins with the removal of stomach fluid from a fasting patient. To measure the *B*asal *A*cid *O*utput (BAO), stomach contents are withdrawn continuously for an hour (or every 15 min for an hour). The volume of gastric fluid is measured, and aliquot portions (usually 5 cm^3) are titrated. Then HCl secretion is stimulated by a Pentagastrin injection and the *M*aximal *A*cid *O*utput (MAO) is determined.

Then the contents of the stomach are withdrawn continuously for an hour (or every 15 min for an hour). The volume of the secretum is measured and aliquot portions (usually 5 cm^3) are titrated. After the determination of the *B*asal *A*cid *O*utput (BAO), HCl secretion is stimulated by a Pentagastrin injection and the *M*aximal *A*cid *O*utput (MAO) is measured.

DUPress

Procedure

Measure 10.0 cm^3 of a gastric juice model solution into a conical flask, add 1 or 2 drops of methyl orange and 2 or 3 drops of phenolphthalein. Titrate with the standardized 0.1 M NaOH to orange, the transition color of methyl orange. Read the buret and then continue adding NaOH until the faint pink color of phenolphthalein appears. Read the buret again.

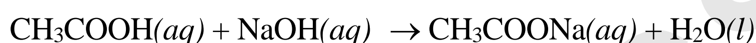
Give the concentration of the strong acid (HCl) and the total acid concentration in terms of mmol/dm^3.

Sample calculations

1. Calculate the molarity and mass/volume % of an acetic acid solution if neutralization of 10.0 cm^3 of this solution required 14.55 cm^3 of 0.1025 M NaOH. How many milligrams of acetic acid are present in 10.0 cm^3 of the solution? (The molar mass of acetic acid is 60.0 g/mol.)

Solution

The chemical equation for the reaction is



$$CH_3COOH(aq) + NaOH(aq) \rightarrow CH_3COONa(aq) + H_2O(l)$$

The number of moles of NaOH used is

$$\left(14.55\ cm^3\right)\left(\frac{1\,dm^3}{1000\,cm^3}\right)\left(\frac{0.1025\,mol}{1\,dm^3}\right) = 1.491\ x10^{-3}\ mol$$

that neutralized 1.491 x10^{-3} mol of acetic acid dissolved in 10.0 cm^3 sample volume.

The molarity of acetic acid is $\dfrac{1.491\,x10^{-3}\ mol}{0.01 dm^3} = 0.1491$ M.

1 dm^3 of 0.1491 M acetic acid contains $\left(\dfrac{0.1491\,mol}{1\,dm^3}\right)\left(\dfrac{60.0g}{1\,mol}\right) = 8.946$ g of acetic acid.

Since 1 dm^3 = 1000 cm^3, 100 cm^3 of 0.1491 M acetic acid contains $\dfrac{8.946\,g}{10}$ = 0.8946 g of acetic acid. Thus, the concentration of acetic acid is 0.8946 m/v%.

10 cm^3 of 0.1491 M acetic acid contains $\dfrac{0.8946\ g}{10}$ = 0.08946 g = 89.46 mg acetic acid.

2. 10.0 cm^3 of gastric juice was titrated with 0.1025 M NaOH in the presence of methyl orange and phenolphthalein. The buret reading was recorded before the addition of the NaOH solution and at the color change of the indicators (see the table below). Calculate the free HCl acidity and the total acidity of the solution in mmol/dm^3.

Initial buret reading	0.15 cm^3
Buret reading at the transition of methyl orange	3.80 cm^3
Buret reading at the transition of phenolphthalein	9.25 cm^3

DUPress

The volume of NaOH used for the neutralization of HCl is 3.80 cm^3 - 0.15 cm^3 = 3.65 cm^3.

The number of moles of NaOH that reacted is

$$(3.65\,cm^3)\left(\frac{1\,dm^3}{1000\,cm^3}\right)\left(\frac{0.1025\,mol}{1\,dm^3}\right) = 3.74 \times 10^{-4}\ mol$$

which neutralized 3.74 x 10^{-4} mol of HCl dissolved in 10.0 cm^3 sample volume.

The free HCl acidity of the solution is

$$\left(\frac{3.74 x 10^{-4}\ mol}{10.0\,cm^3}\right)\left(\frac{1000\,cm^3}{1\,dm^3}\right)\left(\frac{1000\,mmol}{1\,mol}\right) = 37.4\ mmol/dm^3.$$

The volume of NaOH used for the neutralization of the strong and weak acids together (total acidity) is 9.25 cm^3 - 0.15 cm^3 = 9.10 cm^3.

The number of moles of NaOH that neutralized strong and weak acids was

$$(9.10\,cm^3)\left(\frac{1\,dm^3}{1000\,cm^3}\right)\left(\frac{0.1025\,mol}{1\,dm^3}\right) = 9.33 \times 10^{-4}\ mol.$$

The number of moles of HCl plus weak acids was 9.33 x 10^{-4} mol in 10.0 cm^3 of the sample.

The total acidity of the solution was

$$\left(\frac{9.33 x 10^{-4}\ mol}{10.0\,cm^3}\right)\left(\frac{1000\,cm^3}{1\,dm^3}\right)\left(\frac{1000\,mmol}{1\,mol}\right) = 93.3\ mmol/dm^3.$$

Questions

How is the concentration of an aqueous acetic acid solution determined by acid-base titration? (a) Write the equation for the reaction. (b) Suggest an indicator to detect the equivalence point.

Explain how the concentration of a strong acid and a weak acid can be determined in a mixture.

Define the terms free HCl acidity and total acidity of gastric juice.

What do the terms Basal Acid Output and Maximal Acid Output mean?

Make a sketch of the titration curve expected for the titration of acetic acid with NaOH. Mark the characteristic points of the curve.

DUPress

Exercises

1. 22.5 cm^3 of 0.250 M NaOH was required to neutralize 5.0 cm^3 of an acetic acid solution. Calculate the molarity and mass/volume % of acetic acid. How many milligrams of acetic acid are present in 5.0 cm^3 of the solution? (The molar mass of acetic acid is 60.0 g/mol.)

2. 5.0 cm^3 of an acetic acid solution was diluted to 50.0 cm^3 by mixing with 45 cm^3 of water. 10.0 cm^3 of the diluted solution was neutralized with 5.2 cm^3 of 0.145 M NaOH. Give the molarity of the undiluted acetic acid solution. How many milligrams of acetic acid are present in 5.0 cm^3 of the original solution? (The molar mass of acetic acid is 60.0 g/mol.)

3. A 10.0 cm^3 sample of a solution containing hydrochloric acid and acetic acid was titrated with 0.148 M NaOH in the presence of methyl orange and phenolphthalein. Upon addition of 8.5 cm^3 of NaOH, the color of methyl orange changed to orange. Addition of 12.5 cm^3 of NaOH (measured from the start of titration) caused phenolphthalein to change color. Calculate the concentration of hydrochloric acid and acetic acid in (a) mmol/dm^3, and (b) in mass/volume %. (The molar mass of HCl is 36.5 g/mol and the molar mass of CH_3COOH is 60.0 g/mol.)

4. 100.0 cm^3 of an acetic acid and 200.0 cm^3 of a hydrochloric acid solution were mixed to give 300.0 cm^3 of a solution containing CH_3COOH and HCl. A 15.0 cm^3 sample of this mixture was titrated with 0.1520 M NaOH as in *Exercise 3.* Transition of methyl orange required 8.0 cm^3 of NaOH. The red color of phenolphthalein was observed when the total volume of NaOH added was 14.2 cm^3. Calculate the molarity of CH_3COOH and HCl in the mixture and in the original solutions. (Molar masses: HCl, 36.5 g/mol; CH_3COOH, 60.0 g/mol)

DUPress

Results

Date:

Experiment 1. *Determination of acetic acid*

Unknown acetic acid No.:
Volume of acetic acid titrated:
Molarity of standard NaOH:

	Titration		
	1.	2.	3.
Initial buret reading (cm^3)			
Final buret reading (cm^3)			
Volume of NaOH used (cm^3)			
Average volume of NaOH used (cm^3)			

Calculation

10.0 cm^3 of acetic acid solution No contains mg of acetic acid.

The concentration of acetic acid is m/v %,

mol/dm^3.

DUPress

Experiment 2. *Analysis of free HCl and total acidity of gastric juice*

Gastric juice model No.:
Volume of gastric juice model titrated:
Molarity of NaOH standard solution:

	Titration	
	1.	2.
Initial buret reading (cm^3)		
Buret reading at the transition of methyl orange (cm^3)		
Buret reading at the transition of phenolphthalein (cm^3)		
Volume of NaOH neutralizing strong acid (cm^3)		
Volume of NaOH neutralizing strong acid plus weak acid (cm^3)		
Average volume of NaOH neutralizing strong acid (cm^3)		
Average volume of NaOH neutralizing strong acid plus weak acid (cm^3)		

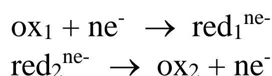
Calculation

Gastric juice model No has free HCl acidity,
and total acidity.

DUPress

Redox titrations

In redox titrations, reducing agents are oxidized with standard solutions of strong oxidizing agents and oxidizing agents are reduced with standard solutions of strong reducing agents. The reaction in a redox titration is an electron interchange between an oxidizing and a reducing agent.



$$ox_1 + ne^- \rightarrow red_1^{ne-}$$
$$red_2^{ne-} \rightarrow ox_2 + ne^-$$

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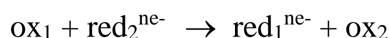


$$ox_1 + red_2^{ne-} \rightarrow red_1^{ne-} + ox_2$$

The greater the difference between the standard reduction potentials of the two redox couples, the larger the equilibrium constant, indicating that the oxidation with the species having more positive standard reduction potential goes essentially to completion.

The potential of the system varies during the course of titration, depending on the concentration ratio of the reduced and oxidized forms. In the vicinity of the equivalence point, the potential changes sharply (*Fig. 9*).

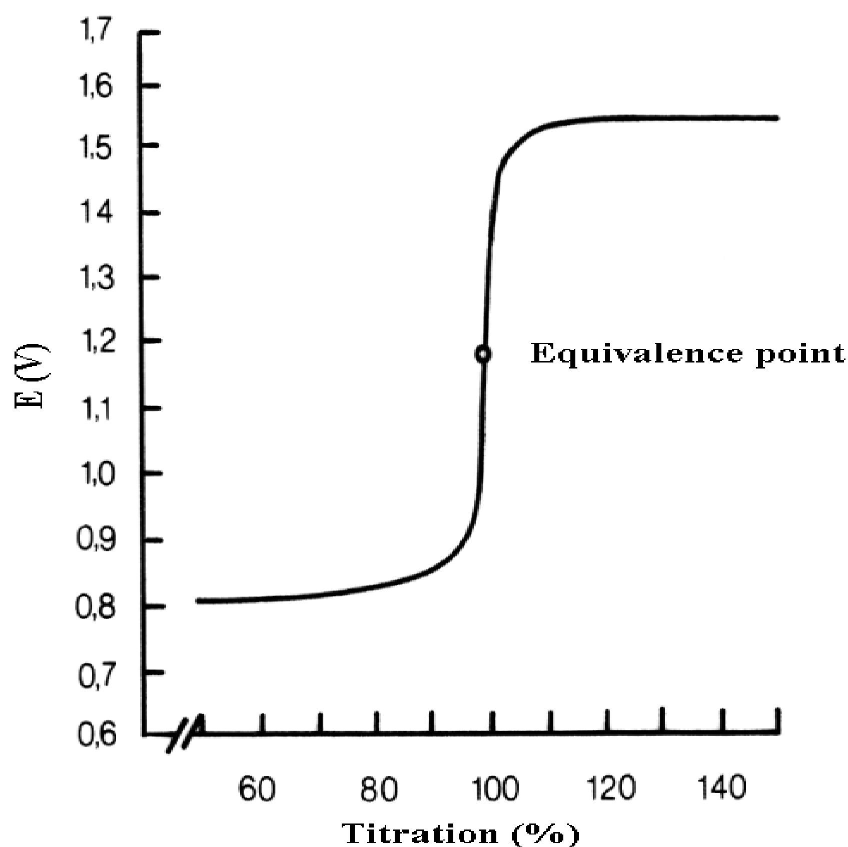


Figure 9. Titration curve for a reducing agent with standard solution of an oxidizing agent

In the titrations with potassium permanganate standard solution, the end point is indicated by the pink color caused by the slight excess of permanganate, therefore no additional indicator is needed. In iodometric titrations, the formation or disappearance of iodine is indicated by its sensitive color reaction with starch. Irreversible redox indicators are organic dyes which lose color when their structure is destroyed by the first excess of oxidizing agent (e.g., methyl orange in bromatometry). Reversible redox indicators can be viewed as reversible redox couples in which the oxidized and reduced forms exhibit different colors. The standard reduction potential of a redox indicator should correspond to the potential at the equivalence point of the system to be titrated. Of the redox titrations, *iodometry* and *bromatometry* will be discussed.

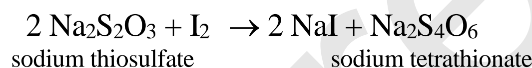
Iodometric titrations

Iodometry can be used for the determination of both oxidizing and reducing agents since the standard reduction potential of the $I_2/2I^-$ couple is of a medium value.

$$I_2 + 2\,e^- \rightarrow 2\,I^- \qquad E° = +0.62\ V$$

Using excess iodine for the oxidation of a redox couple with lower standard reduction potential, the unreacted iodine can be back-titrated. On the other hand, iodide ions reduce redox couples with higher standard reduction potential to yield iodine which can be determined.

In both cases, iodine is titrated with sodium thiosulfate standard solution:

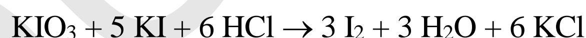


$$\underset{\text{sodium thiosulfate}}{2\,Na_2S_2O_3} + I_2 \rightarrow 2\,NaI + \underset{\text{sodium tetrathionate}}{Na_2S_4O_6}$$

The equivalence point is indicated by starch which gives an intense blue color with traces of free iodine. The color is due to the formation of a molecular complex.

Experiment 1. *Standardization of sodium thiosulfate solution*

Sodium thiosulfate reacts with atmospheric oxygen and carbon dioxide dissolved in the solution. Therefore, the concentration of a sodium thiosulfate solution changes upon storage. Standardization of sodium thiosulfate is carried out against a known amount of iodine which is liberated by a calculated amount of potassium iodate in the following reaction:

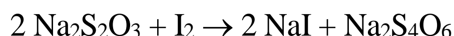


$$KIO_3 + 5\,KI + 6\,HCl \rightarrow 3\,I_2 + 3\,H_2O + 6\,KCl$$

The net ionic equation is

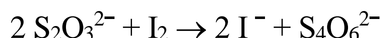
$$IO_3^- + 5\,I^- + 6\,H^+ \rightarrow 3\,I_2 + 3\,H_2O$$

The liberated iodine is titrated with the $Na_2S_2O_3$ solution.



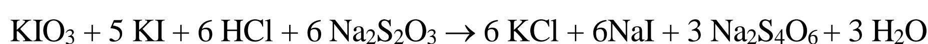
$$2\,Na_2S_2O_3 + I_2 \rightarrow 2\,NaI + Na_2S_4O_6$$

The net ionic equation for the above reaction is



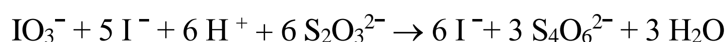
$$2\,S_2O_3^{2-} + I_2 \rightarrow 2\,I^- + S_4O_6^{2-}$$

The overall reaction is



$$KIO_3 + 5\,KI + 6\,HCl + 6\,Na_2S_2O_3 \rightarrow 6\,KCl + 6NaI + 3\,Na_2S_4O_6 + 3\,H_2O$$

The net ionic equation for the overall reaction is



$$IO_3^- + 5\,I^- + 6\,H^+ + 6\,S_2O_3^{2-} \rightarrow 6\,I^- + 3\,S_4O_6^{2-} + 3\,H_2O$$

Thus, 1 mol of IO_3^- reacts with 6 mol of $S_2O_3^{2-}$.

DUPress

Procedure

Pipet 10.0 cm^3 of 1/600 M KIO_3 into a conical flask. Add 5 to 6 cm^3 of 1% HCl (by a graduated cylinder) and 5 cm^3 of 5% KI solution (using another graduated cylinder). KIO_3 is the limiting reactant in the reaction that produces iodine. Iodine forms a brown complex with excess I^- (Lugol solution).

Titrate the solution with an approximately 0.01 M $Na_2S_2O_3$ with constant stirring until the amber iodine color begins to fade. At this point, add a few drops of 1% starch solution and resume titrating. An abrupt color change from dark blue to colorless marks the end point.

Repeat the titration on a second and a third 10.0 cm^3 aliquot of 1/600 M KIO_3.

Calculation

The number of moles of KIO_3 in 10.0 cm^3 of 1/600 M KIO_3 solution is

$$\left(\frac{1\,\text{mol}}{600\,\text{dm}^3}\right)(10.0\,\text{cm}^3)\left(\frac{1\,\text{dm}}{1000\,\text{cm}^3}\right) = \frac{1}{60{,}000}\text{mol}\ .$$

From the overall chemical equation, the mole ratio of KIO_3 to $Na_2S_2O_3$ to is 1 to 6.

The number of moles of $Na_2S_2O_3$ reacted is

$$\left(\frac{1}{60{,}000}\text{mol KIO}_3\right)\left(\frac{6\,\text{mol Na}_2\text{S}_2\text{O}_3}{1\,\text{mol KIO}_3}\right) = 10^{-4}\ \text{mol Na}_2\text{S}_2\text{O}_3.$$

If the average volume of $Na_2S_2O_3$ used is V cm^3, the molarity of $Na_2S_2O_3$ is given by the equation

$$c_{\text{Na2S2O3}} = \left(\frac{10^{-4}\,\text{mol}}{\text{V cm}^3}\right)\left(\frac{10^3\,\text{cm}^3}{\text{dm}^3}\right) = \frac{1}{10\,\text{V}}\ \frac{\text{mol}}{\text{dm}^3}$$

Experiment 2. *Determination of ethanol*

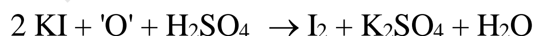
The analysis is based on the oxidation of ethanol with excess potassium dichromate ($K_2Cr_2O_7$). The oxidizing effect of potassium dichromate is attributed to the liberation of oxygen atoms in acid solution in the presence of a reducing agent:

$$K_2Cr_2O_7 + 4\ H_2SO_4 \rightarrow Cr_2(SO_4)_3 + K_2SO_4 + 4\ H_2O + 3\ \text{'O'}$$

Ethanol is oxidized to acetic acid:

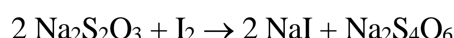
$$CH_3CH_2OH + 2\ \text{'O' (from } K_2Cr_2O_7) \rightarrow CH_3\text{-}COOH + H_2O$$

After the completion of the above reaction, potassium iodide is added to the solution. The excess of potassium dichromate that remained in the solution oxidizes iodide ions to iodine:



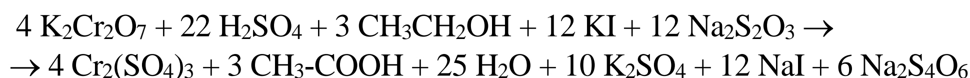
$$2\ KI + \text{'O'} + H_2SO_4 \rightarrow I_2 + K_2SO_4 + H_2O$$

The liberated iodine is then determined by titration with sodium thiosulfate as in *Experiment 1*:



$$2\ Na_2S_2O_3 + I_2 \rightarrow 2\ NaI + Na_2S_4O_6$$

The overall equation for the oxidation of ethanol is



$$4\ K_2Cr_2O_7 + 22\ H_2SO_4 + 3\ CH_3CH_2OH + 12\ KI + 12\ Na_2S_2O_3 \rightarrow$$
$$\rightarrow 4\ Cr_2(SO_4)_3 + 3\ CH_3\text{-}COOH + 25\ H_2O + 10\ K_2SO_4 + 12\ NaI + 6\ Na_2S_4O_6$$

Thus, 1 mol of ethanol is equivalent to 4 mol of $Na_2S_2O_3$ (3:12 mole ratio).

DUPress

A blank reaction is carried out to find the amount of iodine that can be liberated by the entire amount of potassium dichromate. The concentration of ethanol is calculated from the difference between the volumes of $Na_2S_2O_3$ used for the blank and for the sample. The concentration of ethanol is usually expressed in ‰ (same as mg/cm^3). This method was used for the determination of blood ethanol (Widmark's test).

Procedure

Transfer 0.100 cm^3 (100 μl) of a diluted ethanol solution by a micropipette into a special reagent tube. From a dispenser, add 1.0 cm^3 of 0.25% potassium dichromate dissolved in concentrated sulfuric acid. Screw the cap on the tube and mix. Incubate the reaction mixture at 80°C for 30 min. Then cool down the tube in running tapwater.

Place 5 cm^3 of distilled water in an Erlenmeyer flask. Remove the cap of the tube and pour the reaction mixture in the flask. Rinse the tube with a few cm^3 of distilled water three times and transfer each rinse in the flask. Add 2.5 cm^3 of 5% KI (with the help of a transfer pipette). Titrate the sample with standard sodium thiosulfate solution in the presence of starch indicator.

For the blank reaction, place 10 cm^3 of distilled water in an Erlenmeyer flask. Add 1.0 cm^3 of potassium dichromate-sulfuric acid reagent from a dispenser and pipet in 2.5 cm^3 of 5% potassium iodide solution. Titrate the solution with standard sodium thiosulfate in the presence of starch indicator as before.

Subtract the volume of standard solution used for the sample from the volume used for the blank and calculate the concentration of ethanol in mg/cm^3 (same as in ‰).

Calculation

The number of moles of sodium thiosulfate equivalent to ethanol is

$$\left(\frac{\text{c mol } Na_2S_2O_3}{1\,dm^3}\right)(V\,cm^3)\left(\frac{1\,dm^3}{1000\,cm^3}\right) = \frac{cV}{1000}\text{ mol } Na_2S_2O_3$$

where c stands for the molarity of $Na_2S_2O_3$, and $V = V_{blank} - V_{sample}$ is the difference between the volumes used for the blank and for the sample.

From the overall equation, the mole ratio of ethanol to sodium thiosulfate is 1 to 4. The number of moles of ethanol equivalent to $\frac{cV}{1000}$ mol of sodium thiosulfate is given by

$$\left(\frac{1\text{ mol ethanol}}{4\text{ mol } Na_2S_2O_3}\right)\left(\frac{cV}{1000}\right)\text{mol } Na_2S_2O_3 = \frac{cV}{4000}\text{mol ethanol.}$$

The mass of ethanol is

$$\left(\frac{cV}{4000}\text{mol}\right)\left(\frac{46.1\,g}{1\,mol}\right)\left(\frac{1000\,mg}{1\,g}\right) = 11.5\text{ cV mg.}$$

The concentration of ethanol can be expressed as

$$c_{ethanol} = \frac{11.5\text{ cV mg}}{0.100\text{ cm}^3} = 115\text{ cV }\frac{mg}{cm^3}.$$

Sample calculations

1. Calculate the molarity of sodium thiosulfate if 7.1 cm^3 of the solution is required to react completely with iodine liberated from 5.0 cm^3 of 1/600 M KIO_3 in the presence of excess KI and HCl.

The number of moles of KIO_3 in 5.0 cm^3 of 1/600 M KIO_3 solution is

$$\left(\frac{1}{600}\ \frac{mol}{dm^3}\right)\left(5.0\,cm^3\right)\left(\frac{1\,dm}{1000\,cm^3}\right) = \frac{5}{600{,}000}\ mol$$

Since 1 mol of KIO_3 reacts with 6 mol of $Na_2S_2O_3$ (see *Experiment 1*), the number of moles of $Na_2S_2O_3$ reacted is

$$\left(\frac{5}{600{,}000}\,mol\ KIO_3\right)\left(\frac{6\,mol\ Na_2S_2O_3}{1\,mol\ KIO_3}\right) = 5\ x10^{-5}\ mol\ Na_2S_2O_3.$$

It is dissolved in a 7.1 cm^3 sample volume.

The molarity of sodium thiosulfate is

$$c_{Na_2S_2O_3} = \frac{5\ x10^{-5}\ mol}{7.1\,x10^{-3}\ dm^3} = 0.007\ \frac{mol}{dm^3} = 0.007\ M$$

2. In an ethanol test, ethanol in 0.100 cm^3 of the sample was oxidized with 1.0 cm^3 of potassium dichromate-sulfuric acid reagent. The oxidizing agent was present in excess. To determine the oxidizing agent left over, KI was added and the liberated iodine was titrated with 0.010 M $Na_2S_2O_3$. 3.50 cm^3 of 0.010 M $Na_2S_2O_3$ was required to reach the end point. In a separate reaction, iodide ions were oxidized with 1.0 cm^3 of potassium dichromate-sulfuric acid reagent. In the subsequent titration of the liberated iodine, 5.10 cm^3 of 0.010 M thiosulfate solution was used. Calculate the concentration of ethanol in mg/cm^3.

V_{blank} = 5.1 cm^3
V_{sample} = 3.5 cm^3

The volume of 0.010 M $Na_2S_2O_3$ corresponding to the amount of ethanol is

$V = V_{blank} - V_{sample}$
$V = 5.10\ cm^3 – 3.50\ cm^3 = 1.60\ cm^3$

The number of moles of $Na_2S_2O_3$ in 1.6 cm^3 of 0.010 M $Na_2S_2O_3$ is

$$\left(1.6\,cm^3\right)\left(\frac{1\,dm^3}{1000\,cm^3}\right)\left(\frac{0.010\,mol}{1\,dm^3}\right) = 1.6\ x\ 10^{-5}\ mol$$

Since 1 mol of ethanol is equivalent to 4 mol of $Na_2S_2O_3$ (see *Experiment 2*), 1 mol of $Na_2S_2O_3$ corresponds to $\frac{1}{4}$ mol of ethanol.

Thus, the number of moles of ethanol equivalent to 1.6 x 10^{-5} mol of $Na_2S_2O_3$ is

$$\frac{1}{4}\ x\ 1.6\ x\ 10^{-5}\ mol = 4\ x\ 10^{-6}\ mol.$$

The mass of ethanol is

$$\left(4x10^{-6}\ mol\right)\left(\frac{46.1\,g}{1\,mol}\right)\left(\frac{1000\,mg}{1\,g}\right) = 0.184\ mg$$

dissolved in 0.100 cm^3 of the solution.

DUPress

The concentration of ethanol is

$$c_{ethanol} = \frac{0.184\ \text{mg}}{0.100\ \text{cm}^3} = 1.84\ \frac{\text{mg}}{\text{cm}^3}$$

Questions

How can one use the I_2 / $2I^-$ couple in iodometry?

How is the concentration of iodine determined? Write the chemical equation.

How is the equivalence point detected in iodometry?

Briefly describe the determination of ethanol. Write the chemical equations for the reactions.

Exercises

1. The concentration of a sodium thiosulfate solution was determined by titration of iodine which was liberated from 5.0 cm^3 of 0.004 M KIO_3 in a solution containing excess KI and HCl. The liberated iodine required 21.6 cm^3 of $Na_2S_2O_3$ solution to decolorize the blue starch/iodine complex. Calculate the molarity of $Na_2S_2O_3$.

2. Calculate the molarity of a $Na_2S_2O_3$ solution if 11.6 cm^3 of this solution was needed to completely react with iodine liberated from a solution containing 10.0 mg of KIO_3 (molar mass: 214.0 g/mol), excess KI, and HCl.

3. Ethanol present in 0.25 cm^3 of a sample was oxidized with 2.0 cm^3 of potassium dichromate-sulfuric acid as in *Experiment 2*. The remained oxidizing agent liberated iodine that required 2.6 cm^3 of 0.050 M $Na_2S_2O_3$ to react completely. In a blank reaction, 5.1 cm^3 of 0.050 M $Na_2S_2O_3$ was used. Calculate the concentration of ethanol in (a) mg/cm^3, (b) g/dm^3, (c) mol/dm^3. (The molar mass of ethanol is 46.1 g/mol.)

DUPress

Results

Date:

Experiment 1. *Standardization of sodium thiosulfate solution*

Molarity of KIO_3 solution: 1/600 M
Titrated volume of KIO_3: 10.0 cm^3

	Titration		
	1	*2*	*3*
Initial buret reading (cm^3)			
Final buret reading (cm^3)			
Volume of $Na_2S_2O_3$ used (cm^3)			
Average volume of $Na_2S_2O_3$ used (cm^3)			

Calculation

The accurate concentration of $Na_2S_2O_3$ is mol/dm^3.

DUPress

Experiment 2. *Determination of ethanol*

Unknown ethanol solution No.:
Molarity of standard $Na_2S_2O_3$ solution:

	Titration of	
	Blank	Sample
Initial buret reading (cm^3)		
Final buret reading (cm^3)		
Volume of $Na_2S_2O_3$ used (cm^3)		
Blank – sample (cm^3)		

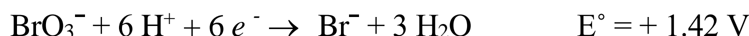
Calculation

The concentration of ethanol in sample No. is mg/cm^3 (‰).

DUPress

Titrations with potassium bromate

Bromatometry is based on the strong oxidizing effect of potassium bromate in acidic medium:



$$BrO_3^- + 6\,H^+ + 6\,e^- \rightarrow Br^- + 3\,H_2O \qquad E^\circ = +\,1.42\ V$$

Some substances are directly oxidized with potassium bromate in the presence of hydrochloric acid. Some other substances do not react with bromate itself but with the bromine. In this case, a standard bromate solution is added to the acidic solution containing bromide in excess.

Bromate generates bromine according to the following equation

$$BrO_3^- + 5\,Br^- + 6H^+ \rightarrow 3\,Br_2 + 3\,H_2O$$

The bromine released quantitatively reacts with the substance. The end point can be detected by the color change of a suitable redox indicator or by the irreversible bleaching of an azo dye.

The determination of certain organic compounds is based on addition, substitution or oxidation with excess bromine. Bromine left over is determined by the addition of potassium iodide and titration of the liberated iodine with sodium thiosulfate.

Experiment 1. *Determination of iodine value of fats and oils*

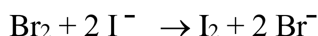
Fats and oils contain various amounts of unsaturated fatty acid building blocks. Unsaturated fatty acids have the ability to add halogens. The equation for bromine addition of oleic acid is given below as an example:

$$CH_3\text{-}(CH_2)_7\text{-}CH{=}CH\text{-}(CH_2)_7\text{-}COOH + Br_2 \rightarrow CH_3\text{-}(CH_2)_7\text{-}CHBr\text{-}CHBr\text{-}(CH_2)_7\text{-}COOH$$

By definition, the *iodine value* is the number of grams of iodine taken up by 100.0 g of a lipid sample. Since iodine is not very reactive, excess bromine is liberated in the lipid solution as described above

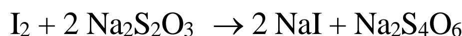
$$BrO_3^- + 5\,Br^- + 6H^+ \rightarrow 3\,Br_2 + 3\,H_2O$$

and is directly used in the addition reaction. To avoid a substitution, the reaction is carried out at room temperature in the dark for a definite time. Then a KI solution is added in excess. The remained bromine oxidizes iodide ions to iodine.



$$Br_2 + 2\,I^- \rightarrow I_2 + 2\,Br^-$$

Iodine is measured by iodometric titration in the presence of starch indicator.



$$I_2 + 2\,Na_2S_2O_3 \rightarrow 2\,NaI + Na_2S_4O_6$$

A blank determination must be carried out to find the volume of $Na_2S_2O_3$ equivalent to the initial amount of bromine.

The difference between the volumes of sodium thiosulfate used for the blank and for the sample is equivalent to the amount of halogen added to unsaturated fatty acids in the lipid.

DUPress

Procedure

The sample solutions are natural oils or fats diluted with CCl_4 or $CHCl_3$. The extent of dilution is marked on the sample container.

Place each of the following solutions in a glass stoppered conical flask:

1.0 cm^3 of unknown solution,
4 cm^3 of CCl_4 or $CHCl_3$ (by a graduated cylinder),
10 cm^3 of 0.0021 M $KBrO_3$ (by a pipette),
2.5 cm^3 of 0.8% KBr (by a graduated cylinder),
5 cm^3 of 1% HCl (by a graduated cylinder).

Put a wet stopper in the flask immediately after you have added HCl. Mix thoroughly and allow to stand in the dark for 30 min. Mix the contents of the flask by vigorous shaking 2 or 3 times during the course of the reaction. Then measure 2.5 cm^3 of 5% KI in the flask, shake well and titrate the released iodine with 0.010 M $Na_2S_2O_3$ in the presence of starch indicator. Shake the flask after the addition of each drop of the standard solution. Vigorous shaking is advised since iodine needs to be transferred from the organic phase to the aqueous phase to react with $Na_2S_2O_3$. At the end point, the blue color of the aqueous phase changes to colorless and no iodine color can be detected in the organic phase.

Prepare a blank in a similar way except that use 1.0 cm^3 of CCl_4 or $CHCl_3$ instead of the lipid solution. Supplement the blank with 2.5 cm^3 of 5% KI and titrate the sample immediately as described above. **Discard the titrated solutions in an organic waste bottle!**

Calculation

1 mol of I_2 (253.8 g/mol) reacts with 2 mol of $Na_2S_2O_3$.

1.0 dm^3 of 0.010 M $Na_2S_2O_3$ contains 0.010 mol of $Na_2S_2O_3$. Hence, 1.0 cm^3 of 0.010 M $Na_2S_2O_3$ contains 0.00001 mol of $Na_2S_2O_3$ which reacts with

$$\left(0.00001\,\text{mol Na}_2\text{S}_2\text{O}_3\right)\left(\frac{1\,\text{mol I}_2}{2\,\text{mol Na}_2\text{S}_2\text{O}_3}\right)\left(\frac{253.8\,\text{g}}{1\,\text{mol}}\right)\left(\frac{1000\,\text{mg}}{1\,\text{g}}\right) = 1.27\text{ mg I}_2$$

Thus, 1.0 cm^3 of 0.010 M $Na_2S_2O_3$ reacts with 1.27 mg of I_2.

Calculate the volume of $Na_2S_2O_3$ equivalent to the iodine uptake of the diluted sample by subtracting the volume used for the sample from the volume used for the blank

$$V = V_{blank} - V_{sample}$$

Find the number of grams of iodine that would be taken up by 100.0 g of undiluted sample. The density of the lipid solution is about 1 g/cm^3. The iodine values of some commercial oils and fats are given below.

Lard	50-65
Sunflower oil	122-135
Cod-liver oil	140-170
Linseed oil	160-190

DUPress

Sample calculation

1.0 g of an oil was dissolved in $CHCl_3$ to obtain 200.0 cm^3 of solution. 1.0 cm^3 of the diluted oil sample was analyzed for its iodine value. Bromine that remained after the addition oxidized iodide ions to iodine. 5.1 cm^3 of 0.010 M $Na_2S_2O_3$ was required to completely react with the iodine. In the blank determination using 1.0 cm^3 of $CHCl_3$ instead of the oil sample, 10.3 cm^3 of 0.010 M $Na_2S_2O_3$ was used. Calculate the iodine value of the oil sample.

The volume of 0.010 M $Na_2S_2O_3$ equivalent to the iodine taken up by 1.0 cm^3 of 200-fold diluted oil sample is

$$V = V_{blank} - V_{sample} = 10.3\ cm^3 - 5.1\ cm^3 = 5.2\ cm^3.$$

1.0 cm^3 of 0.010 M $Na_2S_2O_3$ reacts with 1.27 mg of I_2 (see *Calculation* in *Experiment 1*). Therefore, 5.2 cm^3 of 0.010 M $Na_2S_2O_3$ reacts with

$$\left(5.2\ cm^3\right)\left(\frac{1.27\ mg}{1.0\ cm^3}\right) = 6.6\ mg\ of\ I_2.$$

6.6 mg of I_2 was taken up by 1.0 cm^3 of 200-fold diluted oil sample that contained $\frac{1}{200}$ g of the original oil. Therefore, the mass of iodine taken up by 1.0 g of undiluted oil is

$$200 \times 6.6\ mg = 1320\ mg = 1.32\ g$$

The iodine value of the sample refers to the iodine uptake (in grams) of 100.0 g of sample, thus it is 100 x 1.32 = 132.

Questions

Define the iodine value of fats and oils.

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How is the iodine value determined? Write the equations.

Why is it necessary to perform a blank assay during the determination of the iodine number?

Exercises

1. 1.0 g of an oil sample was dissolved in $CHCl_3$ to give 16.0 cm^3 of diluted solution and 1.0 cm^3 of this solution was used to determine the iodine value. For the addition, bromine was liberated in excess from a 0.017 M $KBrO_3$ solution with excess KBr and HCl. Bromine left over was equivalent to 3.5 cm^3 of 0.100 M $Na_2S_2O_3$. In the blank determination, 10.1 cm^3 of 0.100 M $Na_2S_2O_3$ was used. Calculate the iodine value of the original oil sample.

2. 2.0 cm^3 of a 10-fold diluted oil sample was analyzed for its iodine value. Bromine was liberated in excess from 10.0 cm^3 of 0.050 M $KBrO_3$ with KBr in the presence of HCl. The remained bromine oxidized iodide ions to iodine which reacted with 2.5 cm^3 of 0.305 M $Na_2S_2O_3$. In the blank assay, 10.1 cm^3 of 0.305 M $Na_2S_2O_3$ was used. Calculate the iodine value of the original (undiluted) oil sample.

DUPress

Results

Date:

Experiment 1. *Determination of iodine value of fats and oils*

Unknown sample No.:
Dilution of original sample: -fold
Titrated volume:
Molarity of $Na_2S_2O_3$:

	Titration of	
	Blank	Sample
Initial buret reading (cm^3)		
Final buret reading (cm^3)		
Volume of $Na_2S_2O_3$ (cm^3)		
Blank – sample (cm^3)		

Calculation

The iodine value of sample No. is .

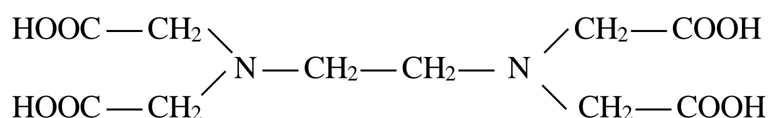
DUPress

Complexometric titrations

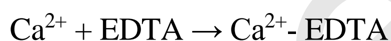
Complex formation reactions that can be used for titrimetric determination of metal ions have to meet the following criteria.

(i) Metal ions must form a stable complex with the titrating agent.
(ii) The resulting complex must be of stoichiometric composition.
(iii) The rate of complex formation must be high enough for the titration to be fast.
(iv) A suitable means of end-point detection must be available.

The most commonly used reagent in complexometry is EDTA (ethylenediaminetetraacetic acid).



The disodium salt of EDTA is commercially available under various names (e.g. Selecton B_2, Titriplex III). EDTA is a sexidentate ligand forming highly stable 1:1 chelates with most di- and trivalent metal ions, for example, with Ca^{2+}:



$$Ca^{2+} + EDTA \rightarrow Ca^{2+}\text{- EDTA}$$

The structure of the complex formed in the reaction is shown in *Fig. 10*.

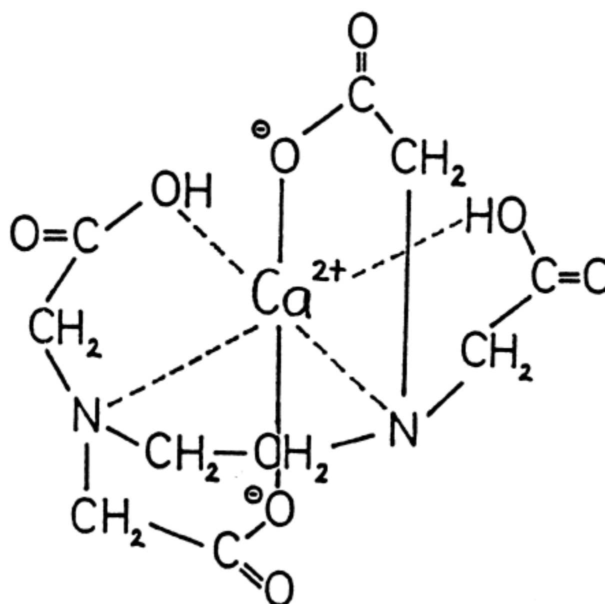


Figure 10. Structure of the Ca^{2+}-EDTA complex

During titration of a metal ion with EDTA, the free metal ion concentration gradually decreases. The decrease is sharp in the vicinity of the equivalence point (*Fig. 11*).

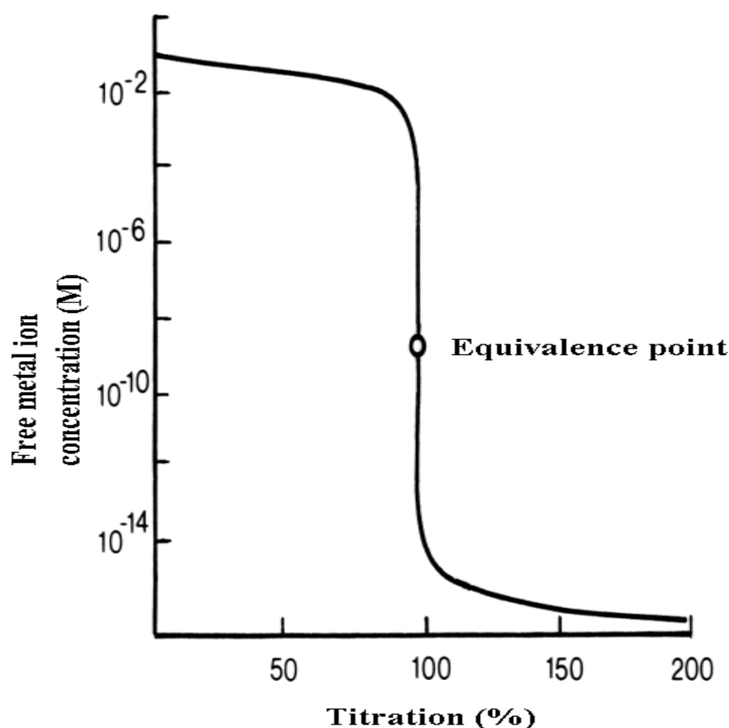
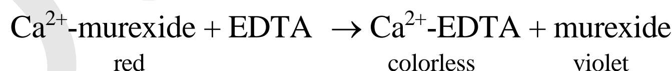


Figure 11. Complexometric titration curve of a metal ion

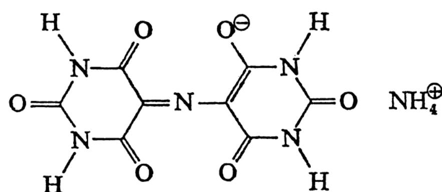
The end point of complexometric titrations is detected by metallochromic indicators. Metallochromic indicators are organic chelating ligands. A metal-metallochromic indicator complex differs in color from the free indicator and it is less stable than the EDTA-complex of the same metal. For example, in the titration of calcium ions with EDTA, murexide is used as an indicator. At the beginning of titration, calcium ions form complexes with both EDTA and murexide. Free calcium ions are also present. EDTA reacts with the free calcium ions first and then with Ca^{2+} in the Ca^{2+}-murexide complex. As a result, the color of free metallochromic indicator appears.



$$\underset{\text{red}}{Ca^{2+}\text{-murexide}} + \text{EDTA} \rightarrow \underset{\text{colorless}}{Ca^{2+}\text{-EDTA}} + \underset{\text{violet}}{\text{murexide}}$$

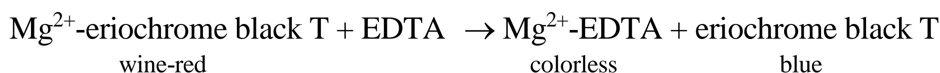
Therefore, the end point is indicated by a color change to permanent violet.

Murexide is the ammonium salt of purpuric acid, a weak acid.

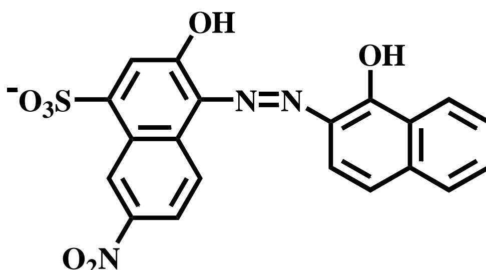


The color of murexide also depends on the pH.

The mechanism of action of eriochrome black T indicator during the titration of Mg^{2+} with EDTA is similar.



Eriochrome black T is an azo dye, 1-(1-hydroxy-2-naphthylazo)-6-nitro-2-naphthol-4-sulfonic acid.



Experiment 1. *Determination of calcium ions*

Pipet 10.0 cm^3 of an unknown Ca^{2+} solution into a conical flask. Add 2 cm^3 of 10% NaOH (using a graduated cylinder) and 5 drops of murexide indicator. Titrate the resulting red solution with 0.020 M EDTA until the color changes to a permanent violet. Repeat titration with two additional 10.0 cm^3 aliquots of the unknown Ca^{2+} solution.

Calculate how many mg of Ca^{2+} are equivalent to 1.0 cm^3 of 0.020 M EDTA. Ca^{2+} and EDTA react in a 1:1 mole ratio. The molar mass of Ca^{2+} is 40.1 g/mol. From the average volume of EDTA consumed, calculate the mass (in mg) of Ca^{2+} present in 100.0 cm^3 of the solution. Express the concentration of Ca^{2+} in terms of molarity.

Experiment 2. *Determination of calcium and magnesium ions in admixture*

EDTA forms stable complexes with a large number of metal ions. Titration with EDTA at different pH values or in the presence of additional complex-forming ligands that suppress the influence of other metal ions permits the selective determination of a selected metal ion in a mixture. For example, both Ca^{2+} and Mg^{2+} form complexes with EDTA in a 1:1 mole ratio. However, the Ca^{2+}-EDTA complex is more stable. Above pH 12, only Ca^{2+} reacts with EDTA since Mg^{2+} is precipitated as $Mg(OH)_2$. Ca^{2+} is determined first by titration with EDTA in the presence of murexide at pH > 12. Then the mixture is acidified to break down murexide (the decomposition can be accelerated by gentle warming). The pH is adjusted to 10 by an NH_3/NH_4Cl buffer and Mg^{2+} is titrated in the presence of eriochrome black T indicator.

Procedure

Pipet 10.0 cm^3 of an unknown solution containing $CaCl_2$ plus $MgCl_2$ into an Erlenmeyer flask. Add 2 cm^3 of 10% NaOH (using a graduated cylinder) and 5 drops of murexide. Titrate with 0.020 M EDTA until the color changes from red to violet. Then add 3 cm^3 of 5 M HCl (observe decoloration of murexide), 10 cm^3 of 5% aqueous ammonia (by a graduated cylinder) and 5 drops of eriochrome black T. Titrate Mg^{2+} with 0.020 M EDTA until the color changes from wine red to pure blue. Perform two more titrations with 10.0 cm^3 aliquots of the unknown solution.

Calculate how many mg of Mg^{2+} are equivalent to 1.0 cm^3 of 0.020 M EDTA. Mg^{2+} and EDTA react in a 1:1 mole ratio. The molar mass of Mg^{2+} is 24.3 g/mol. Give the Ca^{2+} and Mg^{2+} concentration of the unknown solution in mg/100 cm^3 and in mol/dm^3.

DUPress

Experiment 3. *Determination of the hardness of water*

The total hardness of water is generally due to dissolved calcium and magnesium salts. Waters containing HCO_3^- along with the metal cations exhibit carbonate or temporary hardness. Boiling hard water that contains HCO_3^- drives off carbon dioxide, leaving behind insoluble carbonate salts of the metal ions. Permanent or noncarbonate hardness is assigned to other water soluble salts of calcium and magnesium.

The hardness of water can be expressed in German degrees. The hardness in German degrees is numerically equal to the mass of CaO, in mg, that is equivalent to all of the calcium and magnesium salts dissolved in 100.0 cm^3 of water.

Calcium and magnesium ions are titrated together with EDTA in the presence of eriochrome black T. The titration allows the determination of combined amounts of the two ions.

Procedure

Place 50.0 cm^3 of water to be analyzed in a conical flask. To eliminate hydrogen carbonates add the volume of 0.1 M HCl which exceeds the half alkalinity of water by 0.5 cm^3. (The alkalinity of water is equal to the volume of 1.000 M HCl, in cm^3, which is required to neutralize 1 dm^3 of water. The alkalinity of the water sample will be given by the instructor.) Boil the solution for about a minute to drive off carbon dioxide, cool to about 50°C, then add 2 cm^3 of NH_3/NH_4Cl buffer, pH 10 (containing 12% of aqueous NH_3 plus 12% of NH_4Cl) and 6 drops of eriochrome black T indicator. Titrate with 0.020 M EDTA standard solution until the color changes from wine red to pure blue. Run triplicate titrations.

Calculate how many mg of CaO are equivalent to 1.0 cm^3 of 0.020 M EDTA. From the volume of EDTA consumed calculate the hardness of water in German degrees. The molar mass of CaO is 56.1 g/mol.

Sample calculations

1. A 10.0 cm^3 sample of a Ca^{2+} solution was titrated with 0.010 M EDTA in the presence of murexide. 8.0 cm^3 of EDTA was required to change the color of the indicator from red to violet. (a) Calculate the milligrams of Ca^{2+} equivalent to 1.0 cm^3 of 0.010 M EDTA. The molar mass of Ca is 40.1 g/mol. (b) Give the mass of Ca^{2+} present in 100.0 cm^3 of the solution. (c) Calculate the molarity of the solution.

(a) 1.0 cm^3 of 0.010 M EDTA contains

$$\left(1.0\ \text{cm}^3\right)\left(\frac{0.010\ \text{mol}}{\text{dm}^3}\right)\left(\frac{1\ \text{dm}^3}{1000\ \text{cm}^3}\right) = 1.0\ \text{x}10^{-5}\ \text{mol of EDTA.}$$

Since Ca^{2+} reacts with EDTA in a 1:1 molar ratio, 1.0 x10^{-5} mol of EDTA is equivalent to 1.0 x10^{-5} mol of Ca^{2+} the mass of which is

$$\left(1.0\ \text{x}10^{-5}\ \text{mol}\right)\left(\frac{40.1\ \text{g}}{\text{mol}}\right)\left(\frac{1000\ \text{mg}}{1\ \text{g}}\right) = 0.401\ \text{mg}$$

(b) 8.0 cm^3 of 0.010 M EDTA reacts with 8.0 x 0.401 mg of Ca^{2+} = 3.208 mg of Ca^{2+} which is present in 10.0 cm^3 of solution. Hence, 100.0 cm^3 of the solution contains 32.1 mg of Ca^{2+}.

DUPress

(c) 8.0 cm^3 of 0.010 M EDTA contains 8.0 x10^{-5} mol of EDTA which reacts with 8.0 x10^{-5} mol of Ca^{2+}. The volume of the solution is 10.0 cm^3 = 0.010 dm^3, thus the molarity of Ca^{2+} is

$$c_{Ca2+} = \frac{8.0 \text{ x } 10^{-5} \text{ mol}}{0.010 \text{ dm}^3} = 0.008 \ \frac{\text{mol}}{\text{dm}^3}.$$

2. A 10.0 cm^3 sample of a solution containing Ca^{2+} and Mg^{2+} was titrated with 0.020 M EDTA at pH > 12 in the presence of murexide and eriochrome black T indicators. Permanent color change of murexide was observed when 7.0 cm^3 of 0.020 M EDTA was added. Then the pH was adjusted to 10 and titration was resumed. Eriochrome black T exhibited a color change when the total volume of EDTA added was 15.3 cm^3. Calculate the concentration of Ca^{2+} and Mg^{2+} (a) in mg/100 cm^3 and (b) in molarity units (molar masses: Ca^{2+}, 40.1 g/mol; Mg^{2+}; 24.3 g/mol).

(a) 1.0 cm^3 of 0.020 M EDTA contains

$$\left(1.0 \text{ cm}^3\right)\left(\frac{0.020 \text{ mol}}{1 \text{ dm}^3}\right)\left(\frac{1 \text{ dm}^3}{1000 \text{ cm}^3}\right) = 2.0 \text{ x } 10^{-5} \text{ mol EDTA which reacts with}$$

2.0 x 10^{-5} mol of Ca^{2+} or 2.0 x 10^{-5} mol Mg^{2+}. Thus, 1.0 cm^3 of the solution reacts with

$$\left(2 \text{ x} 10^{-5} \text{ mol}\right)\left(\frac{40.1 \text{ g Ca}^{2+}}{1 \text{ mol}}\right)\left(\frac{1000 \text{ mg}}{1 \text{ g}}\right) = 0.802 \text{ mg of Ca}^{2+} \text{ or}$$

$$\left(2 \text{ x} 10^{-5} \text{ mol}\right)\left(\frac{24.3 \text{ g Mg}^{2+}}{1 \text{ mol}}\right)\left(\frac{1000 \text{ mg}}{1 \text{ g}}\right) = 0.486 \text{ mg of Mg}^{2+}.$$

Therefore, 1.0 cm^3 of 0.020 M EDTA is equivalent to 0.802 mg of Ca^{2+} and 0.486 mg of Mg^{2+}. In the titration, 7.0 cm^3 of 0.020 M EDTA reacted with Ca^{2+} and 15.3 cm^3 of EDTA reacted with Ca^{2+} plus Mg^{2+}. Hence, the volume of EDTA required for the reaction with Mg^{2+} was

$$15.3 \text{ cm}^3 - 7.0 \text{ cm}^3 = 8.3 \text{ cm}^3.$$

The mass of Ca^{2+} in 10.0 cm^3 of solution is

$$\left(7.0 \text{ cm}^3\right)\left(\frac{0.802 \text{ mg Ca}^{2+}}{1.0 \text{ cm}^3}\right) = 5.614 \text{ mg Ca}^{2+}$$

The mass of Mg^{2+} in 10.0 cm^3 of solution is

$$\left(8.3 \text{ cm}^3\right)\left(\frac{0.486 \text{ mg Mg}^{2+}}{1.0 \text{ cm}^3}\right) = 4.034 \text{ mg Mg}^{2+}$$

100.0 cm^3 of the solution contains 56.14 mg of Ca^{2+} and 40.34 mg of Mg^{2+}, therefore the concentrations are:

56.1 mg Ca^{2+}/100 cm^3 and
40.3 mg Mg^{2+}/100 cm^3

(b) 7.0 cm^3 of 0.020 M EDTA contains 7.0 x 2.0 x 10^{-5} mol of EDTA = 1.4 x 10^{-4} mol of EDTA equivalent to 1.4 x 10^{-4} mol of Ca^{2+}. The volume of the solution is 10.0 cm^3 = 0.010 dm^3. Therefore, the molarity of Ca^{2+} is

$$c_{Ca2+} = \frac{1.4 \text{ x } 10^{-4} \text{ mol}}{0.010 \text{ dm}^3} = 0.014 \text{ mol/dm}^3.$$

DUPress

8.3 cm^3 of 0.020 M EDTA contains 8.3 x 2.0 x 10^{-5} mol of EDTA = 1.66 x 10^{-4} mol of EDTA equivalent to 1.66 x 10^{-4} mol of Mg^{2+}. The volume of the solution is 10.0 cm^3 = 0.010 dm^3. Therefore, the molarity of Mg^{2+} is

$$c_{Mg2+} = \frac{1.66 \times 10^{-4}\ \text{mol}}{0.010\,\text{dm}^3} = 0.017\ \text{mol/dm}^3.$$

3. The hardness of a water sample was determined by titration with 0.020 M EDTA in the presence of eriochrome black T. Give the hardness of the water in German degrees, if 50.0 cm^3 of the sample required 7.8 cm^3 of 0.020 M EDTA to reach the end point. The molar mass of CaO is 56.1 g/mol.

1 mol of EDTA binds 1 mol of Ca^{2+} + Mg^{2+} which is equivalent to 1 mol of CaO.
1.0 cm^3 of 0.020 M EDTA contains 2.0 x10^{-5} mol EDTA (see *Example 2*) which is equivalent to 2.0 x10^{-5} mol of CaO. The mass of CaO is

$$\left(2.0\,\text{x}10^{-5}\ \text{mol}\right)\left(\frac{56.1\,\text{g}}{1\,\text{mol}}\right)\left(\frac{1000\,\text{mg}}{1\,\text{g}}\right) = 1.12\ \text{mg}$$

The mass of CaO equivalent to Ca^{2+} and Mg^{2+} present in 100.0 cm^3 of the sample is

$$\left(7.8\,\text{cm}^3\right)\left(\frac{1.12\ \text{mg}}{1.0\,\text{cm}^3}\right)\left(\frac{100.0\,\text{cm}^3}{50.0\,\text{cm}^3}\right) = 17.5\ \text{mg}$$

The hardness of water is 17.5 German degrees.

Questions

Write the structural formula for EDTA.

What is the molar ratio of EDTA to the metal ion in its complexes with Ca^{2+} and Mg^{2+}?

What causes the hardness of water?

How is the hardness of water determined?

Excercises

1. Give the molarity of Ca^{2+} in the solution if titration of 20.0 cm^3 of the solution required 5.6 cm^3 of 0.05 M EDTA to reach the end point. How many milligrams of Ca^{2+} are present in 1.0 dm^3 of the sample? (The molar mass of Ca^{2+} is 40.1 g/mol.)
2. A 10.0 cm^3 sample of a solution containing Ca^{2+} (40.1 g/mol) and Mg^{2+} (24.3 g/mol) was titrated with EDTA as in *Experiment* 2 except that the concentration of EDTA was 0.050 M. The color change of murexide became permanent upon addition of 2.6 cm^3 of EDTA. Eriochrome black T exhibited a permanent color change when the total volume of EDTA added was 5.9 cm^3. (a) Calculate molarity of Ca^{2+} and Mg^{2+}. (b) How many milligrams of Ca^{2+} and Mg^{2+} are present in 100.0 cm^3 of the sample? (Molar masses: Ca^{2+}, 40.1 g/mol; Mg^{2+}, 24.3 g/mol)
3. Calculate the hardness of the water sample in German degrees if titration of 40.0 cm^3 of the sample required 6.5 cm^3 of 0.010 M EDTA for complete reaction.
4. Give the hardness of the water sample which was made by dissolving 500 mg of $CaCl_2$ in 1 dm^3 of deionized water. Assume that the volume of the solution is exactly 1 dm^3. The molar mass of $CaCl_2$ is 111.0 g/mol.

DUPress

Results

Date:

Experiment 1. *Determination of calcium ions*

Unknown Ca^{2+} solution No.:
Volume of unknown solution titrated:
Molarity of EDTA standard solution:

	Titration		
	1.	2.	3.
Initial buret reading (cm^3)			
Final buret reading (cm^3)			
Volume of EDTA consumed (cm^3)			
Average volume of EDTA consumed (cm^3)			

Calculation

100 cm^3 of solution No. contains mg of Ca^{2+}.

The concentration of Ca^{2+} is mol/dm^3.

DUPress

Experiment 2. *Determination of calcium and magnesium ions in admixture*

Unknown solution No.:
Volume of unknown solution titrated:
Molarity of EDTA standard solution:

	Titration		
	1.	2.	3.
Initial buret reading (cm^3)			
Buret reading at the transition of murexide (cm^3)			
Buret reading at the transition of eriochrome black T (cm^3)			
Volume of EDTA consumed by Ca^{2+} (cm^3)			
Average volume consumed by Ca^{2+} (cm^3)			
Volume of EDTA consumed by Mg^{2+} (cm^3)			
Average volume consumed by Mg^{2+} (cm^3)			

Calculation

The composition of unknown solution No. is as follows,

Ca^{2+} content: mg/100 cm^3, mol/dm^3,

Mg^{2+} content: mg/100 cm^3, mol/dm^3.

DUPress

Experiment 3. *Determination of the hardness of water*

Unknown water sample No.:
Volume of water titrated:
Molarity of EDTA standard solution:

	Titration		
	1.	2.	3.
Initial buret reading (cm^3)			
Final buret reading (cm^3)			
Volume of EDTA used (cm^3)			
Average volume used (cm^3)			

Calculation

The hardness of water is German degrees.

DUPress

INSTRUMENTAL METHODS OF CHEMICAL ANALYSIS

Instrumental methods of analysis are widely used to assay components or parts of a substance by measuring some of its physical or physico-chemical properties with the help of an instrument. Instrumental measurements are more general methods in quantitative analysis than in qualitative ones. Spectroscopy is an example of qualitative application. Components of a mixture can be analyzed by measuring some optical properties; this is the basis of colorimetry, photometry and polarimetry. Potentiometry, conductometry, electrogravimetry, polarography are based on the electrical properties of the system. In thermal analysis, conclusions are derived from heat effects.

The advantages of instrumental analysis are accuracy, speed, selectiveness and the fact that they can be easily automated. This is the reason why they are routinely applied in medical laboratories.

SPECTROPHOTOMETRY

Photometry is based on light absorption of substances. Analytical applications of the absorptive behavior of substances can be either qualitative or quantitative. The qualitative applications of absorption spectrometry depend on the fact that a given molecular species absorbs light only in specific regions of the spectrum, which is characteristic of the particular species. If we monitor a beam of light shining through a sample containing a substance that can absorb one of the beam's wavelength, we can obtain a plot of the amount of light observed versus the wavelength (as shown in *Fig. 13*). This plot is known as an *absorption spectrum*, and shows which particular wavelength of light a chemical species can absorb. The absorption spectrum for a substance can be used to identify the presence of that substance, since every chemical species has a specific set of energy levels that it can absorb depending on its unique electronic configuration.

In the case of quantitative measurements, the change in intensity of light passing through a system of absorbing species is measured by means of colorimetry, photometry or spectrophometry.

When monochromatic light of known frequency passes through a solution containing absorbing particles the intensity of light decreases. The decrease in intensity is characterized by transmittance (T) or absorbance (A). The latter is also termed extinction (E) or optical density (O.D.).

Absorbance is the base-ten logarithmic ratio of the intensity of the incident light (I_o) to that of the transmitted light (I) (*Fig.12*).

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

Its value varies from 0 (no absorption) to ∞ (full absorption).

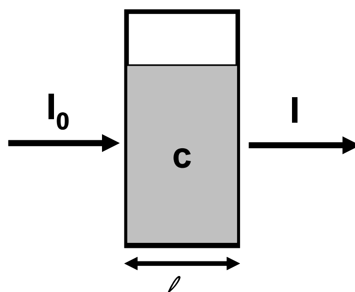


Figure 12. Absorbance of a solute with concentration c

Transmittance is defined as the percent transmission capacity of a substance

$$T = 100\frac{I}{I_0}$$

The value of T may vary between 0 and 100%.

The *fundamental law of photometry*, the Bouguer-Lambert-Beer (BLB) law, states that the absorbance of a solution is directly proportional to the concentration of the absorbing substance and to the thickness of the sample

$$A = \log\frac{I_0}{I} = \varepsilon \cdot c \cdot l$$

where A is the absorbance of the solution, c is the concentration of the solution, l is the thickness of layer (path length) in cm (see *Fig.12*), and ε is the absorption coefficient.

The *absorption coefficient,* also designated as specific absorption, is the absorbance of a solution of unit concentration and unit thickness of layer. If the concentration of the solution is 1 mol/liter, ε is termed molar absorption coefficient. $\varepsilon_{1cm}^{1\%}$ is the absorbance of a solution of 1 g/100 cm^3 (w/v) concentration if the thickness of layer is 1 cm.

According to the BLB law, the value of absorbance is dependent only on the concentration of the solution if the thickness of layer is constant. The BLB law can be used only for dilute solutions illuminated with monochromatic light.

To investigate biochemical samples, photometric determinations are usually carried out in the ultraviolet and visible region of the spectrum. A relatively simple instrument is needed for photometry in the visible range (400-800 nm), where colored solutions can be analyzed. A great number of compounds is colored or is convertible into a colored product with the help of a chemical reaction. The color intensity of their solution is proportional to the concentration of the compound to be analyzed.

Nucleotides and their derivatives, as well as amino acids with aromatic side chains (tyrosine, tryptophan, phenylalanine) have characteristic absorption maximum in the ultraviolet range (200-400 nm). For example, the concentration of proteins containing tyrosine and tryptophan can be determined by photometry at 280 nm (*Fig. 13*). The concentration of nucleotides can be measured at 260 nm.

DUPress

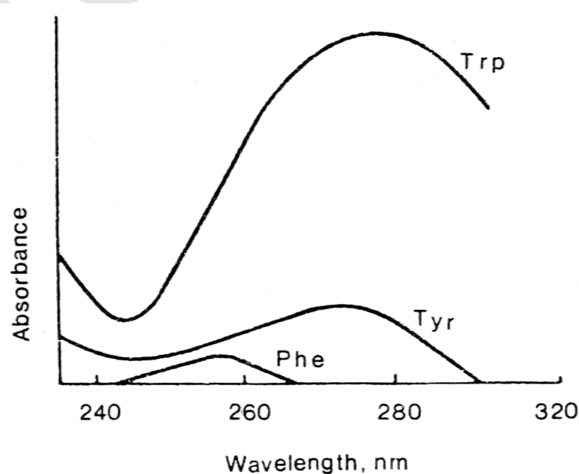


Figure 13. Absorption spectra of aromatic amino acids

The photometric determination of a substance requires the knowledge of its absorption maximum where the measurement is of the highest sensitivity. The absorption maximum is obtained from the absorption spectrum measuring absorbance as a function of wavelength (*Fig. 13*).

When determining the absorbance of a solution, we measure relative absorbance, i.e. we compare the absorbance of the solution with the absorbance of the reagent blank. (The reagent blank contains every component except the examined substance.) So, the error caused by non-specific absorption of the solvent and additional reagents can be avoided.

In chemical analysis, the following two methods utilize BLB law: the absolute and the working curve analysis. The *working curve analysis* is a widespread method that requires the construction of a calibration curve for the constituent being determined. A series of standard solutions with various concentrations is prepared from the constituent, and treated in the same way as the sample solution to develop the color. The absorbance at the absorption maximum is measured and plotted against the concentration (*Fig. 14*). The calibration curve can then be used to determine the concentration of the unknown solution after having measured its absorption. This method is more commonly used than the absolute calculation, because experimental error will average out over the number of standards.

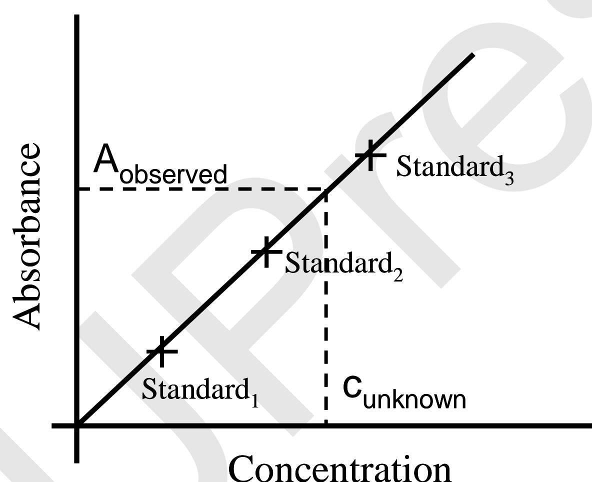


Figure 14. Calibration curve

In the *absolute calculation methods* it is enough to measure the absorbance of a single standard solution of a known concentration. Knowing that the absorbance is directly proportional to the concentration, the concentration of the unknown sample can be calculated.

$$\frac{C_{sample}}{C_{standard}} = \frac{A_{sample}}{A_{standard}}$$

Therefore, $$C_{sample} = \frac{A_{sample}}{A_{standard}} \times C_{standard}$$

The spectrophotometer

The instruments used for spectrophotometry are called spectrophotometers. They all include optical components that allow for the selection of illumination wavelength. This component can be either a set of filters, or a prism, or a grating. The sample is placed into a transparent container (called cuvette) and placed into the light beam to absorb a portion of the incident radiation; the remainder is transmitted on to a detector where it is changed into an electrical signal and displayed, usually after amplification, on a meter, chart recorder, or some other type of readout device. The theoretical construction is demonstrated in *Fig. 15*.

Basic functions of radiation (light) sources in absorption spectrophotometry are to provide sufficient radiant energy over the wavelength region, where absorption is to be measured and to maintain a constant light intensity over the time interval during which absorption measurements are made. Work in ultraviolet regions is done mainly with hydrogen or deuterium discharge lamps, while in visible regions tungsten lamps are used. The beam of the *light-source*

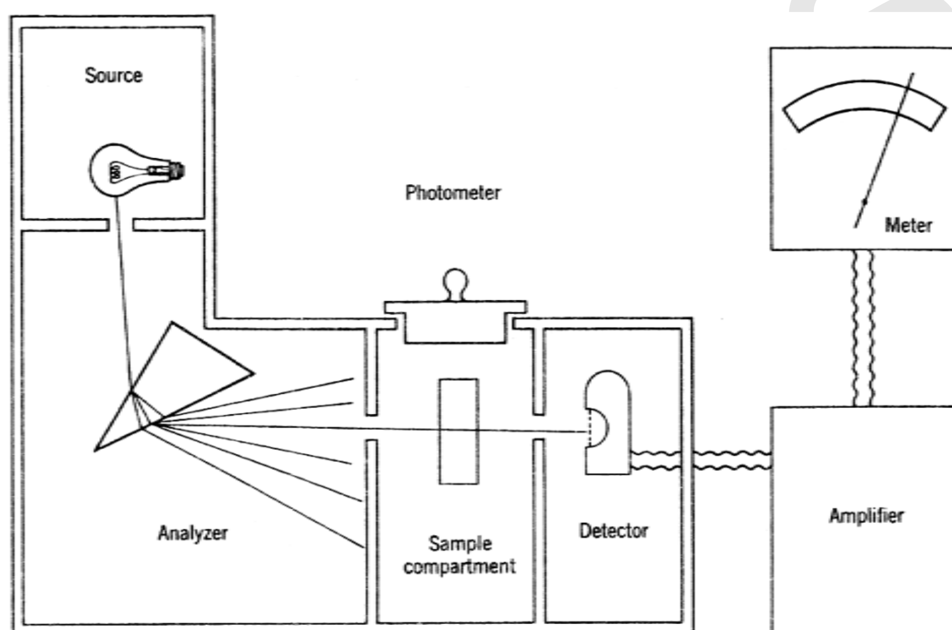


Figure 15. Building blocks of a spectrophotometer

is broken up into different wavelengths by some dispersion optics e.g. by a *grating or a prism.* A slit is used to select a certain (usually quite narrow) wavelength range from this dispersed beam, which results in projecting monochromatic light through the sample solution, hence this part of the optics is called the *monochromator*.

In case of single-beam instruments, this beam passing through the sample is the only one. In double-beam instruments, the monochromatic light is split into two beams, usually of equal intensity. One beam passes through the sample, while the other goes through a reference (reagent blank); the difference between the two components is determined. When using a single-beam instrument, the absorbance of the reference is obtained in a separate measurement. The sample to be analyzed is in a *cuvette*, the size of which is well-defined. Cuvettes made of glass or plastic can be used in the visible light, while quartz cuvettes should be used for measurements in the ultraviolet range. The *detector* converts electromagnetic radiation into electrons and the induced photocurrent is measured after amplification by a current voltage converter. The voltage can be directly displayed on a voltmeter. The scale usually has a double calibration, both in T (transmittance) and in A (absorption) units.

Spectronic 20 (*Fig. 16*) is a single-beam spectophotometer. After turning it on, the so-called dark-current will be compensated automatically. For using the spectrophotometer, the absorption maximum of the analyte (substance to be analyzed) must be known in advance. The wavelength is set to the absorption maximum, the reference (blank) is then positioned into the light path and the transmittance of the blank is adjusted to 100% (zero absorbance). After the adjustment has been made, the sample is placed in the light path and its absorbance is read.

Figure 16. Front-plate of the Spectronic 20 spectrophotometer

Instruction manual for the Spectronic 20 spectrophotometer

1. Turn on the main switch at the backside of the instrument.

2. Select wavelength by pushing the nm button to increase or decrease the values to reach the desired wavelength. The display shows the actual wavelength.

3. Select mode. The display mode can be set at absorbance (A), transmittance (T) or concentration (C). The last is obtained by multiplying the absorbance with a calculated factor. Mostly the absorbance mode is selected.

4. Insert blank (reagent without sample) into the cuvette housing. The cuvette should be filled with the blank to about ¾th and wiped dry on the outside carefully. The correct light path is marked with an arrow.

5. Close the cuvette holder.

6. Push the „0 ABS” (zero absorbance) button *to adjust the absorbance of the blank to zero,* corresponding to the value of 100 % transmittance. (The display will show the value of absorbance and the button should be pushed until it reaches 0).

7. Remove the blank and insert the sample, (the cuvette should be filled similarly to about ¾ volume.

8. Read the absorbance of the sample. After closing the cuvette house, the monitor displays the absorbance.

***Note*. The cuvette should be cleaned and dried from outside before placing into the holder. To prevent the contamination of the holder, the cuvette should be filled only to about 3/4th of its capacity. Touch only the opalescent side of the cuvette!**

When measuring several samples, it is advisable to test them in the order of increasing optical densities. For exact measurements, rinse the cuvette once with a small amount of the sample before filling it with the same sample for reading the absorbance. Finally, the spectrophotometer should be turned off only at the end of the working day, or when no further measurements are expected.

DUPress

Photometric determination of inorganic phosphate

The determination of inorganic phosphate (P_i) is indispensable both in clinical diagnostics and in biochemical research. A number of methods have been developed for the determination of the P_i concentration. The common principle of these methods is as follows. Inorganic phosphate and ammonium molybdate react in acidic solution to yield yellow phosphomolybdic acid, which upon reduction produces a blue color, due to molybdenum blue. The intensity of the blue color is directly proportional to the amount of P_i.

In the classical Fiske-Subbarow method, the molybdate-sulfuric acid solution and the reducing reagent (amino-naphtol-sulfonic acid plus sodium hydrogen sulfite) is added separately to the sample solution. The advantage of the Taussky-Shorr's procedure is the use of molybdate, sulfuric acid and iron(II) ions as reductant in a single solution termed P-reagent*. It is also favorable that the color develops in 2 minutes and it remains stable for 30 minutes. Maximal absorption is at 720 nm.

Experiment 1. *Construction of a calibration curve*

Pipette into test tubes the components of the color reaction as given in the table below.

Additions (cm^3)	Blank	Standard 1	Standard 2	Standard 3
P_i standard solution (10 μg phosphorus/cm^3)	-	0.25	0.5	1.0
Distilled water	2.0	1.75	1.5	1.0
P-reagent	1.4	1.4	1.4	1.4

Wait 5-10 min after mixing, and measure the absorbance of the standards against the blank at 720 nm. Construct the calibration curve by plotting the absorbance values on the ordinate (y axis) against the amount of P_i (in μg phosphorus) on the abscissa (x axis).

Experiment 2. *Determination of the phosphate concentration in a sample*

Pipette the following components into a test tube:

1 cm^3	solution of unknown P_i concentration
1.0 cm^3	distilled water
1.4 cm^3	P-reagent.

Measure the absorbance of the sample against the blank prepared in Experiment 1. After measuring the absorbance of a sample with unknown P_i concentration, its P_i content can be read off from the calibration curve. To obtain the amount of phosphate in μmoles, divide the μg values of P by 31, i.e. the relative atomic mass of phosphorus. Calculate the P_i concentration of the solution in mmol/dm^3 (mM).

* *P-reagent*: 5 g of $FeSO_4 \cdot 7\ H_2O$ or 5 g of Mohr salt [$Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$] is dissolved in about 70 cm^3 of water and 10 cm^3 of 5 M H_2SO_4, containing 10% $(NH_4)_2MoO_4$, is added. The solution is made up to 100 cm^3 in a volumetric flask. The light-sensitive reagent should be stored in a dark coloured bottle and refrigerated.

DUPress

Sample calculation

Calculate the P_i concentration of a human serum in mmol/dm^3 if the measured P_i content is 25 μg/cm^3 and the dilution of serum is 2.5-fold. The way of measurement is identical to the experiment written above. Is the P_i concentration of serum low, high or normal (the normal range is: 1.0-1.5 mmol/ P_i/dm^3 serum)?

$C_{serum\ Pi} = 25\ \mu g/cm^3$
$V = 1\ cm^3 = 10 \times 10^{-4}\ dm^3$
Dilution: 2.5-fold
P_i content of serum: 25 μg/cm^3 x 2.5 = 62.5 μg/cm^3.

$$n = \frac{m_{Pi}}{M_P} = \frac{6.25 \times 10^{-5}\ g}{31\ g/mol} = 2 \times 10^{-6}\ mol$$

$$C_{Pi} = \frac{n_{Pi}}{V} = \frac{2 \times 10^{-6}\ mol}{10 \times 10^{-4}\ dm^3} = 2 \times 10^{-3}\ mol/dm^3 = 2\ mmol/dm^3\ \mathbf{(mM)}$$

The P_i concentration in the human serum is above the normal range (1-1.5 mmol/dm^3).

Questions

Give the definition of absorbance.

Give the definition of transmittance.

Write the fundamental law of photometry.

What is an absorption spectrum? Give an example by drawing a sketch!

What is the “reagent blank” and what is it used for?

What is the principle of the photometric determination of inorganic phosphate according to Taussky-Shorr?

Excercise

A solution of P_i has an absorbance of 0.2 at 720 nm in a 1 cm cuvette. ε is 10 $M^{-1}cm^{-1}$. What is its concentration?

DUPress

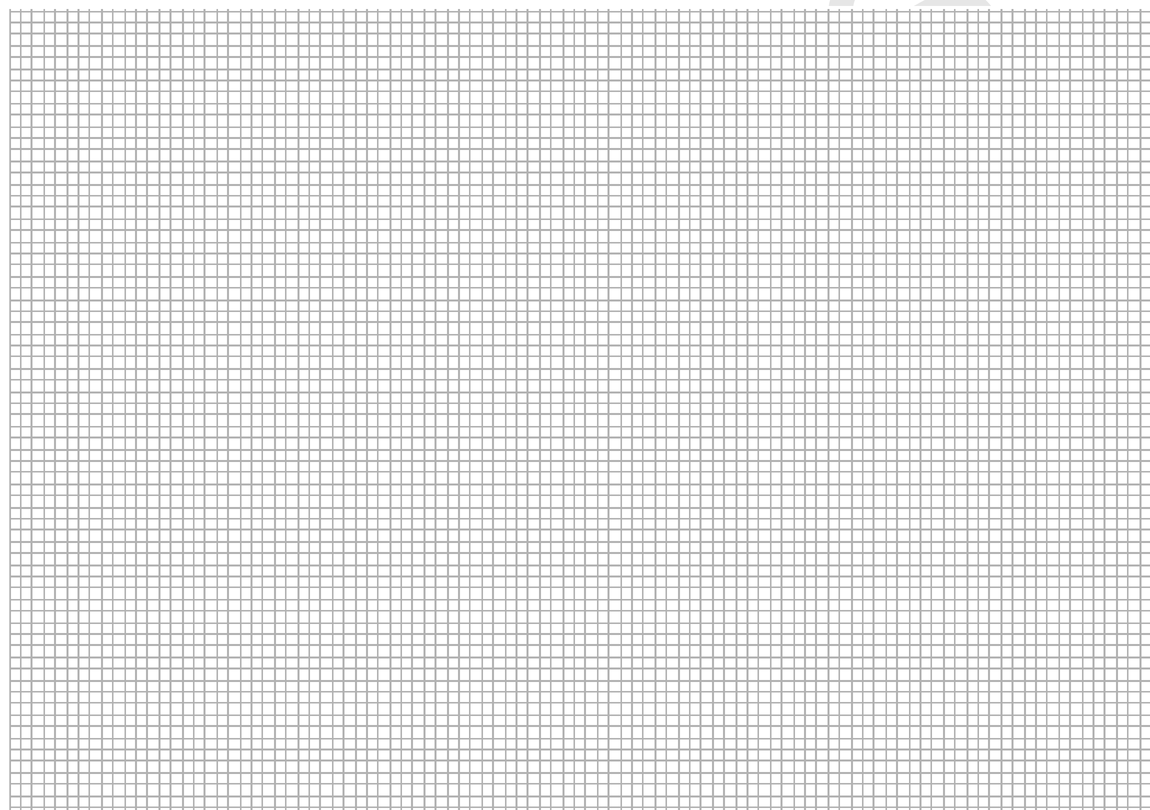
Results

Date:

Experiments 1. and 2. *Construction of a calibration curve and determination of the phosphate concentration in a sample*

	Standard 1	Standard 2	Standard 3	Unknown sample No. ….
P-content [μg]	2.5	5	10	
Absorbance				

Calibration curve

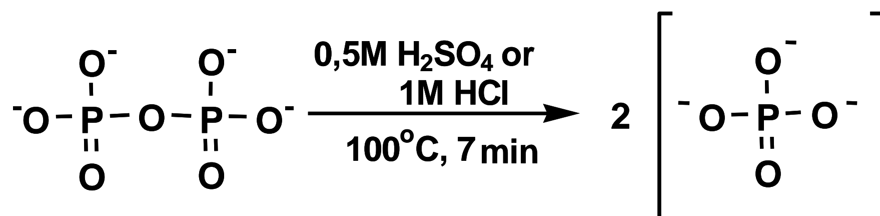


Calculation

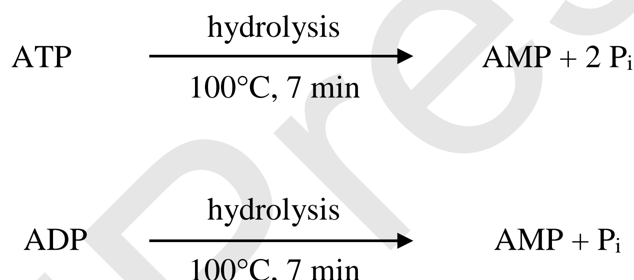
The phosphate content of No. unknown sample is μg/cm^3, which is equal to mmol/dm^3 concentration.

Determination of acid labile phosphate in organic compounds

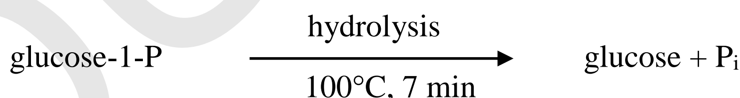
The pyrophosphate bond is a typical inorganic anhydride bond - it is susceptible to hydrolysis.



This fact is of practical importance since much of the organic phosphate commons present in cells contain pyrophosphate linkages estimated as the inorganic phosphate released by brief acid hydrolysis (7 min at 100°C in 1 M monoprotic mineral acid). The term acid labile phosphate, designated also as P_7, is used for phosphate groups hydrolyzed upon the above treatment. Most, although not all, monophosphate esters are quite stable under these conditions. Triphosphates, such as adenosine triphosphate (ATP), are more easily hydrolyzed than diphosphates, such as adenosine diphosphate (ADP), although the difference in rate is not large.



In the case of glucose-1-P (G-1-P), the phosphate group is attached to the C-1 carbon atom via glycosidic linkage that is also susceptible to hydrolysis:



Experiment 1. *Determination of acid labile phosphate in ATP, ADP and glucose-1-phosphate (G-1-P)*

Determine the P_7 content of the unknown ATP, ADP and G-1-P samples and calculate the concentration of the solutions in µmol/cm^3 (mM) and mg/cm^3 units. The molecular masses of ATP, ADP and G-1-P are 551 g/mol, 472 g/mol and 336 g/mol for G-1-P, respectively.

DUPress

The general scheme of the hydrolytic procedure is the following:

Additions (cm^3)	Blank	P_7
sample solution	-	1.0
2.25 M H_2SO_4	-	1.0
Distilled water	2.0	-
hydrolysis 100 °C 7 min cool down and add P-reagent	1.4	1.4
Total volume	3.4	3.4

Allow the solutions to stand for 5 minutes at room temperature and determine the absorbance of samples against the reagent blank (2.0 cm^3 H_2O + 1.4 cm^3 P-reagent). From the measured values of absorbance read the μg amount of phosphate from the calibration-curve.

Sample calculation

Calculate the ATP concentration of a solution (in mg/cm^3) based on its P_7 content (25.2 $\mu g/cm^3$). The molecular mass of ATP is 551 g/mol.

The P_7 content is determined according to the calibration curve. Since two of the three phosphates of ATP can be liberated in the form of P_7 by acid hydrolysis.

$$n_{P_7} = \frac{m_P}{M_P} = \frac{25.2\ \mu g/cm^3}{31\ \mu g/\mu mol} = 0.8\ \mu mol/cm^3$$

$$n_{ATP} = \frac{n_{P_7}}{2} = 0.4\ \mu mol/cm^3$$

$$m_{ATP} = n_{ATP} \times M_{ATP} = 0.4\ \mu mol/cm^3 \times 551\ \mu g/\mu mol = 220.4\ \mu g = 0.22\ mg/cm^3$$

Questions

Give the definition of the term acid labile phosphate (P_7).

Draw the structure of ATP showing the anhydride bonds. Write the equation for the hydrolysis of ATP in the presence of a mineral acid.

Draw the structure of α-D-glucose 1-phosphate and write the equation for the hydrolysis of glucose 1-phosphate.

Exercise

Calculate the concentration of an unknown glucose-1-phosphate solution in mM if the P_7 concentration is 16.5 $\mu g/cm^3$. The molecular mass is 336 g/mol for G-1-P.

DUPress

Results

Date:

Experiment 1.

Fill in the following table using the calibration curve constructed in the experiments entitled *Photometric determination of inorganic phosphate.*

Sample	Number of unknown sample	Absorbance after hydrolysis	P_7 μg
ATP			
ADP			
G-1-P			

(a) Determination of acid labile phosphate in ATP

Calculation

Concentration of the ATP solution: μmol/cm^3, that is mg/cm^3.

(b) Measurements with ADP

Calculation

The ADP concentration of the unknown sample No is μmol/cm^3, 1 cm^3 solution contains mg ADP.

(c) Measurements with G-1-P

Calculation

The G-1-P concentration of the unknown sample No. is μmol/cm^3, 1 cm^3 solution contains mg G-1-P.

DUPress

Photometric determination of iron

Iron is an important component of haemoglobin, myoglobin and of some enzymes (citochromes, peroxidase, catalase etc.). The iron content of the human body is 4-5 g. In clinical chemistry, photometric determination of iron in urine and in serum is a common method. The abnormal values of iron content of urine or serum give information indicate diseases.

The iron in serum is bound to the protein transferrin. This bonded iron is called serum iron. Under physiological conditions, in serum about one third of transferrin is saturated with iron. The additional quantity of iron which can be taken up by the transferrin is termed the unsaturated iron-binding capacity (UIBC). The total quantity of iron in 1 dm^3 serum after the complete saturation of all transferrin molecules is the total iron-binding capacity (TIBC). The difference between TIBC-UIBC gives the value of serum iron. Normal ranges for iron-binding capacities are given below.

Concentration ($\mu mol/dm^3$)	Women	Men
Serum iron	18 ± 6	20 ± 5
Total iron-binding capacity (TIBC)	54 ± 9	60 ± 10
Unsaturated iron-binding capacity (UIBC)	36 ± 9	40 ± 10

The normal value of serum iron for women is by about 10% lower since the iron depots are not sufficiently filled up.

Iron deficiency (due to bleeding, iron absorption troubles, eg. after gastrectomy or deficient intake) results in an increase in the TIBC first, followed by a decrease in serum iron. Iron deficiency can be cured effectively by administration of iron compounds which are absorbed at a great speed.

The basis of photometric determination of iron is that iron(II) ions react with bathophenanthroline disulfonate to give a red compound. The sensitive color reaction permits the quantitative determination of iron even in serum. For this method, the use of clean glass equipment is of special importance.

Experiment 1. *Construction of a calibration curve*

Pipette into four plastic test tubes the following components in the order given in the table below. (To add approximately 5 mg of ascorbic acid use the micro-spatula! ~5 mg is the amount of solid at the tip of the spatula.)

Substance to be added	Reagent blank	Standard 1	Standard 2	Standard 3
Ascorbic acid	appr. 5 mg	appr. 5 mg	appr. 5 mg	appr. 5 mg
Distilled water	1.0 cm^3	0.8 cm^3	0.5 cm^3	-
Iron standard solution (1 $\mu g/cm^3$) *	-	0.2 cm^3	0.5 cm^3	1 cm^3
Reagent**	1.0 cm^3	1.0 cm^3	1.0 cm^3	1 cm^3

* Iron standard solution: 860 mg of $FeNH_4(SO_4)_2 x 12 H_2O$ is dissolved in 16 cm^3 of 6 M HCl and made up to 1000 cm^3 with distilled water. The standard solution of 1 $\mu g/cm^3$ iron concentration is obtained from it by 100-fold dilution.

** Reagent: 0.68 $mmol/dm^3$ bathophenanthroline disulfonic acid disodium salt in 0.4 M sodium phosphate buffer (pH 5.5).

DUPress

Mix well and allow to stand at room temperature for 10 min. During this time, ascorbic acid reduces iron(III) ions to iron(II) ions, which form a red complex with the reagent. Measure the absorbance of the colored solutions against the reagent blank at 535 nm in a spectrophotometer. If the color becomes red already before adding the Iron standard solution, prepare a new mixture in a new test tube.
Plot the absorbance as ordinates against the μg of iron on the abscissa using a graphpaper.

Experiment 2. *Determination of the iron concentration in a solution*

Pipette into two plastic test tubes the following components in the order given in the table below. (To add approximately 5 mg of ascorbic acid use the micro-spatula! ~5 mg is the amount of solid at the tip of the spatula.)

Substance to be added	Reagent blank	Unknown sample
Ascorbic acid*	appr. 5 mg	appr. 5 mg
Distilled water	1.0 cm^3	-
Reagent **	1.0 cm^3	1.0 cm^3
Unknown sample	-	1.0 cm^3

Mix well and allow to stand at room temperature for 10 min. Measure the absorbance of the colored solutions against the reagent blank at 535 nm in a spectrophotometer. Calculate the iron concentration of the solution in μg Fe/cm^3 using the calibration curve.

Experiment 3. *Analysis of the iron content of a commercial drug*

Open up a capsule and suspend its content in about 20 cm^3 of 0.01 M HCl in a beaker. Transfer the mixture into a 100 cm^3 volumetric flask quantitatively by washing the beaker thoroughly with HCl. Make up the flask to the mark and mix well.

Dilute this solution further by pipetting 0.1 cm^3 of it into a 50 cm^3 volumetric flask and make up the flask with 0.01 M HCl to the mark. Determine the iron concentration using 1.0 cm^3 portions of the resulting solution. Pipette into one plastic test tube the following components in the order given in the table below. (To add approximately 5 mg of ascorbic acid use the micro-spatula! ~5 mg is the amount of solid at the tip of the spatula.)

Substance to be added	Sample
Ascorbic acid*	appr. 5 mg
Distilled water	-
Reagent **	1.0 cm^3
50000x diluted solution	1.0 cm^3

Mix well and allow to stand at room temperature for 10 min. Measure the absorbance of the colored solutions against the reagent blank prepared in **Experiment2**. Calculate the iron concentration of the solution in μg Fe/cm^3 using the calibration curve.
Calculate the iron content of a capsule in mg, using the following equation:

$$Fe - content = \frac{\mu g Fe}{1000} \times \frac{100}{0.1} \times \frac{50}{1.0} \ (mg\ /\ capsule)$$

The value of μg Fe can be read off the calibration curve.

* Ascorbic acid may be added by a micro spatula. One tip of spatula is about 5 mg.
** Reagent: 0.68 nmol/dm^3 bathophenanthroline disulfonic acid disodium salt in 0.4 M sodium phosphate buffer (pH 5.5)

DJ press

Experiment 4. *Determination of the iron concentration in serum*

In serum, the iron bound to the protein transferrin should be liberated by dissociation in weakly acidic buffer (pH 5.5) or upon addition of a detergent. The liberated iron(III) ions are reduced by ascorbic acid to give iron(II) ions which form a red complex with bathophenanthroline disulfonate. Serum proteins do not interfere with the determination. Deproteinisation with TCA should be carried out only if the serum has an intensive color (due to haemolysis).

Procedure

Add into four plastic test tubes the components given in the table below:

Substance to be added	Reagent blank	Serum blank	Standard	Serum (sample)
Ascorbic acid	5 mg	5 mg	5 mg	5 mg
0.4 M Phosphate buffer (pH 5.5)	-	1 cm^3	-	-
Distilled water	1 cm^3	-	-	-
Serum	-	-	-	1 cm^3
Fe standard solution (1µg/cm^3)	-	1 cm^3	1 cm^3	-
Reagent	1 cm^3	-	1 cm^3	1 cm^3

Mix well and let it stand for 30 minutes at room temperature. Measure the absorbance of serum and standard against the reagent blank using cells of 1 cm light path at 535 nm. Serum blank should be measured against distilled water.

Calculation

The iron concentration (c) in µg/cm^3 can be calculated on the basis of the Bouguer-Lambert-Beer law

$$\frac{A_s - A_{sb}}{A_{st}} = \frac{c}{1}$$

where A_s is the absorbance of the serum (sample) against the reagent blank, A_{sb} is the absorbance of the serum blank against distilled water, and A_{st} is the absorbance of the iron standard solution against the reagent blank. The denominator on the right is 1 because the iron standard is of 1 µg/cm^3 concentration.

Calculate the iron concentration of serum in µmol/dm^3 by multiplying c by 1000/55.8, where 55.8 is the value of relative atomic mass for iron.

DUPress

Sample calculation

Determine the iron concentration of a human serum sample in μmol/dm^3 if the measured absorbances at 535 nm are as follows:

A_s= 0.354
A_{sb}= 0.026
A_{st}= 0.658
and M_{Fe}= 55.8 g/mol

The concentration of the standard solution is 1 μg Fe/cm^3.

$$m_{Fe} = \frac{0.354\text{-}0.026}{0.658} = 0.45\ \mu g\ \text{Fe in 1 cm}^3$$

$$n_{Fe} = \frac{m_{Fe}}{M_{Fe}} = \frac{4.5 \times 10^{-7}\ g}{55.8\ g/mol} = 8.6 \times 10^{-9}\ mol$$

$$c_{Fe} = \frac{n_{Fe}}{V} = \frac{8.6 \times 10^{-9}\ mol}{10 \times 10^{-4}\ dm^3} = 8.6 \times 10^{-6}\ mol/dm^3 = 8.6\ \ mmol/dm^3$$

Questions

Define the term serum iron.

Describe the method for the photometric determination of iron.

Give the definition of TIBC and UIBC.

Exercise

The absorbance of an unknown iron solution is 0.5 at 535 nm. When measured under identical conditions, a 1.0 x 10^{-4} M iron is found to have an absorbance of 0.2. Determine the concentration of the unknown solution!

DUPress

Results

Date:

Experiments 1. and 2. *Construction of the calibration curve and determination of the iron concentration in a solution*

	Standard 1	Standard 2	Standard 3	Unknown sample No......
Iron content [μg]	0.2	0.5	1.0	
Absorbance				

Calibration curve

Calculation

The iron content of No. unknown sample is μg/cm^3.

Experiment 3. *Analysis of the iron content of a drug*

Name of the drug:
Volume of the solution containing one capsule:
Dilution:

Volume of sample used for the determination	Absorbance	μg Fe	Iron content of one capsule
1.0 cm^3			

Calculation

One capsule contains mg of iron.

Experiment 4. *Determination of the iron concentration in serum*

A_s =
A_{sb} =
A_{st}=

Calculation

The iron concentration in serum is μg/cm^3; μmol/dm^3.

DUPress

Quantitative protein analysis

A fast and easy way of quantitating proteins is spectrophotometry. The absorption maximum of proteins is in the ultraviolet region of the spectrum at 280 nm. It is due to the aromatic amino acid (mainly tyrosine) side chains. The Tyr content of proteins is significantly different, so the absorbance of a protein mixture at 280 nm gives only a rough estimate of its concentration. To determine the exact protein concentration from the absorbance value the knowledge of absorption coefficient of the given protein is necessary. This method can be applied only in the case of purified protein fractions.

Though proteins consisting exclusively of amino acids do not absorb visible light, they can be converted into colored products by chemical reactions.

One of the most often used methods is the *Biuret assay* that based on a pink to purple colored complex formed in the reaction of peptide bonds with copper(II) ions (Cu^{2+}) in alkaline solution. The biuret color reaction is not quite specific for proteins. The reaction is given by organic compounds containing at least two carbamyl ($-CONH_2$) groups, for example 2 peptide bonds. Even the color is named after the non-protein compound, biuret, because it yields the same color reaction. The absorbance maximum of the complex is at 545 nm. This assay can be used if protein concentration ranges between 1-10 mg/cm^3.

The *Bradford assay* is based on the observation that the dye Coomassie Brillant Blue G-250 exists in two different color forms, red and blue. The red form is converted to the blue form upon binding to basic amino acid side chains. The protein-dye complex has a high absorption coefficient at 595 nm that allows the determination of 0.1-1 mg/cm^3 of protein. The binding of protein to the colloidal dye particles is a fast process and the protein-dye complex remains dispersed in the solution for approximately 1 hr, thus the procedure is very rapid and yet does not require critical timing for the assay.

In fast protein quantitation, instead of recording the entire calibration curve, the absorbance of the sample is compared to the absorbance of a standard protein solution assuming that protein concentration is directly proportional to the absorbance. Usually bovine serum albumin (BSA) is used as a standard.

Experiment 1. *Biuret assay*

In this experiment the concentration of an unknown sample and of serum will be determined. In four test tubes, prepare the reaction mixtures given in the following table.

Additions (cm^3)	Reagent blank	Standard	Unknown solution	Serum
Unknown protein-solution	-	-	0.5	-
Serum	-	-	-	0.05
BSA standard (5 mg/cm^3)	-	0.5	-	-
0.9% NaCl	0.5	-	-	0.45
Biuret-reagent*	2.0	2.0	2.0	2.0

To avoid infections use either automatic pipette or pipette with ballon for measuring the serum!

* Biuret-reagent is prepared by dissolving 1.5 g of $CuSO_4 \times 5 H_2O$ and 6.0 g of sodium potassium tartrate ($NaKC_4H_4O_6 \times 4 H_2O$) in 500 cm^3 of distilled water. To this solution 300 cm^3 of 10 w/v % NaOH containing 0.1 w/v % KI is added with thorough mixing, and the mixture is diluted to 1 dm^3 with distilled water. If a precipitate is formed the reagent must be discarded. KI is used to minimize the formation of precipitate.

DUPress

After the addition of the biuret-reagent, shake the tubes well and allow to stand for 30 min at room temperature. Read the absorbance (A) of protein solutions against the reagent blank at 545 nm. Calculate the concentration of the unknown protein sample and of human serum in g/dm^3.

Calculation

$$Protein_{unknown}\ (mg) = \frac{A_{unknown}}{A_{standard}}\ x\ 5\ mg$$

Note that 0.5 cm^3 of the unknown protein solution and 0.05 cm^3 of the human serum contain the measured quantity.

Interpretation

The protein concentration of sera from normal human adults ranges between 60-80 g/dm^3. The major function of plasma proteins is to assist in the normal distribution of water between blood and tissues. Increased plasma protein levels are noted in dehydration and when there is an increase in immunoglobulin content, e.g. in malignancy and certain chronic infections. Decrease in serum protein concentration is found after loss of plasma through the kidneys (albuminuria) or when the synthesis is impaired by malnutrition, vitamin deficiencies or diseases of the digestive organs and liver.

Experiment 2. *Bradford assay*

Prepare the following reaction mixtures in three test tubes.

Additions (cm^3)	Reagent blank	Standard	Unknown sample
Unknown solution	-	-	0.05
BSA standard (500 $\mu g/cm^3$)	-	0.05	-
Distilled water	0.05	-	-
Bradford's reagent*	2.5	2.5	2.5

Shake the tubes well and after 2 min read the absorbance against the reagent blank at 595 nm. Calculate the concentration of the tested protein solution.

Calculation

$$Protein_{unknown} = \frac{A_{unknown}}{A_{standard}}\ x\ 500\ (\mu g/cm^3)$$

* Bradford's reagent is composed of 0.01 w/v % Coomassie Brillant Blue G-250; 4 v/v % ethanol, 8.5 v/v % H_3PO_4 and is filtered off at a rough paper.

DUPress

Sample calculation

Calculate the protein concentration of 0.05 cm^3 human serum sample, which has an absorbance of 0.36 at 545 nm measured by biuret assay. 1 cm^3 of 5 mg/cm^3 BSA solution was used as a standard under the same conditions, and the absorbance of the standard was 0.11. Is your result in the normal range?

The protein content of the serum is

$$Protein_{serum}\,(mg) = \frac{A_{serum}}{A_{standard}}\; x\; 5\; mg = \frac{0.36}{0.11}\; x\; 5\; mg = 16.36\ \text{mg}$$

The volume of the serum sample is 0.05 cm^3**.**
The protein concentration of the sample is

$$16.36\ \text{mg} : 0.05\text{cm}^3 = 327.3\ \text{mg/cm}^3 = 327.3\ \text{g/dm}^3$$

The protein concentration of serum is above the normal range (60-80 g/dm^3).

Questions

List spectrophotometric methods available for the determination of protein concentration

Describe the principle of the biuret assay.

Describe the principle of Bradford's assay.

Give the normal value of protein concentration of human serum!

Exercise

Calculate the protein concentration of a solution if an absorbance of 0.36 at 595 nm measured from 0.05 cm^3 by Bradford method. Under identical conditions, a 0.1 cm^3 of 500 $\mu g/cm^3$ BSA solution was found to have an absorbance of 0.2.

DUPress

Results
Date:

Experiment 1. *Biuret assay*
Number of the unknown sample:

Sample	Absorbance	Concentration (mg/cm^3)
Standard		5.0
Unknown protein solution		
Human serum		

Calculation

The protein concentration of No. unknown sample is mg/cm^3.

The total protein concentration of serum is g/dm^3.
Compare your result to the normal value!

Experiment 2. *Bradford assay*
Number of the unknown protein solution:

Sample	Absorbance	Concentration ($\mu g/cm^3$)
Standard		500
Unknown protein solution		

Calculation

The protein concentration of No. unknown solution is $\mu g/cm^3$.

DUPress

Assay of glucose

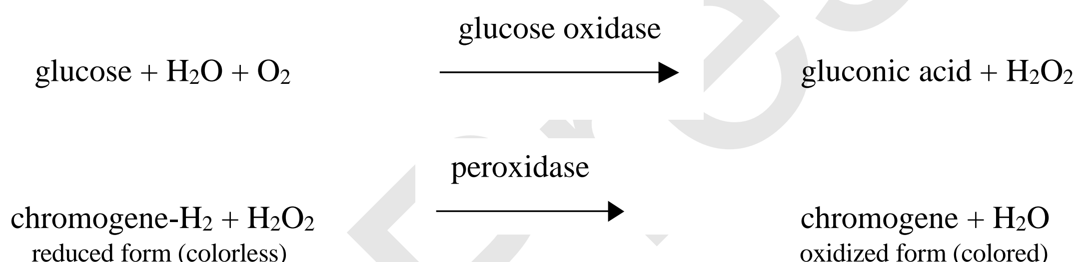
Amongst carbohydrate determinations, quantitative analysis of blood glucose is of special importance from a clinical point of view. The normal blood glucose level is 3.5-5.5 mmol/dm^3. It rapidly rises to 6.5-7 mmol/dm^3 following carbohydrate ingestion. In certain pathological conditions, blood sugar level increases above the normal level (*hyperglycemia*) e.g. in diabetes mellitus, while in others it decreases below normal level (*hypoglycemia*) e.g. in hyperinsulinism. In hyperglycemia, when blood sugar level exceeds renal threshold, glucose can also be detected in the urine. In hypoglycemia, when blood sugar level decreases below 1 mmol/dm^3, convulsions ensue.

There are general possible assays to determine glucose concentration in the blood.

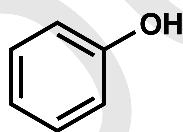
(i) *Methods based on reduction* give results higher than the real value because other substances having reducing power (creatin, creatinin, ascorbic acid, glutathion etc.) are also detected.

(ii) The *determination of glucose by o-toluidine* is based on the production of the Schiff's-base, which has a green color with an absorption maximum at 610 nm. Disadvantage of this method is that this is not specific for glucose, other aldoses give also positive reaction.

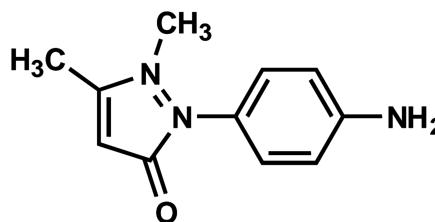
(iii) The *enzymatic determination* (Trinder's method) is based on the following series of reactions:



In the case of Trinder's method the chromogene is a mixture of phenol and p-amino phenazone. The intensity of the color is proportional to the concentration of glucose and can be detected at the 520 nm (the absorption maximum of the colored product).



phenol



p-amino-phenozone

The advantage of this method that it is specific for glucose. The chromogene of the Trinder's method does not react with compounds giving "residual reduction", therefore fructosaemia and galactosaemia can be easily distinguished by this method. The peroxidase is able to transfer its H_2O_2 not only to the chromogene but to other oxidisable compounds (f.e. bilirubine). This is the reason why this method gives lower glucose level in patients with jaundice (with high bilirubine concentration) than the effective value. Because of the simplicity of the glucose-oxidase method and the stability of its reagents, this is the most often used quantitative glucose determination assay.

Experiment 1. *Enzymatic determination of glucose in blood serum*

Prepare the following reaction mixtures in three test tubes.

Additions (cm^3)	Reagent blank	Serum	Glucose-standard
Glucose standard ($5\ mmol/dm^3$)	-	-	0.02
Serum	-	0.02	-
Trinder's reagent*	2.5	2.5	2.5

Incubate the test tubes at room temperature for 30 min. Determine the absorbance of the serum and glucose-standard samples against the blank at 520 nm. Calculate the glucose concentration of the serum.

$$c_{glucose} mM = \frac{A_{serum}}{A_{standard}} \cdot 5\ mM$$

Sample calculation

Calculate the glucose concentration of the serum which has an absorbance of 0.054 measured against the reagent blank at 520 nm. The absorbance of the 5 mM standard glucose solution is 0.12.

$$c_{glucose} = \frac{A_{serum}}{A_{standard}} \cdot 5\ mM = \frac{0.054}{0.12} \cdot 5\ mM$$

$$c_{glucose} = 2.25\ mM$$

Questions

List potential assays for the determination of blood glucose concentration!

What is the normal blood glucose concentration range?

Write the reactions of the enzymatic determination of glucose concentration! What is the advantage of this method?

Exercise

What is the absorbance of a 3.1 mM glucose solution if the absorbance of the glucose standard (5 mM) is 0.09 measured at 520 nm?

---

* The Trinder's reagent is prepared in Tris-phosphate-glycerol buffer. Dissolve 36.5 g Tris and 50 g NaH_2PO_4 x 2 H_2O in about 400 cm^3 of distilled water. Add 400 cm^3 of glycerol and water to 1 dm^3. Adjust the pH to 7.0 by the addition of solid $NaHPO_4$ x 2 H_2O. In 100 cm^3 of the buffer dissolve 30 mg glucose oxidase and 3 mg horseradish peroxidase and add 250 mm^3 of the solution containing 4 M phenol and 0.6 M p-amino phenazone. Stir vigorously, filter and store in cold in a darkened bottle.

DUPress

Results

Date:

Experiment 1. *Enzymatic determination of glucose in blood serum*

A_{standard}:

$A_{\text{human serum}}$:

Calculation

The glucose concentration of serum is mmol/dm^3.

Is the serum glucose concentration in the normal range?

DUPress

POLARIMETRY

Measurement of the change in polarization orientation of polarized light is called polarimetry. Light whose electric field oscillates in a particular way is said to be *polarized*. If the vibrations, perpendicular to the direction in which the light wave is travelling, occur in a single plane, the light is said to be *plane polarized.*

Optically active samples, such as solutions of chiral molecules exhibit rotation of the polarization of plane polarized light. The amount of optical rotation depends on the number of optically active species through which the light passes, and thus depends on the sample path length and the concentration of the optically active substance.

$$\alpha = [\alpha]_D^T \times l \times c$$

Where l is the layer thickness in dm, c is the concentration of solute in grams per cm^3 of solution, α is the observed rotation in degrees, and $[\alpha]_D^T$ is the specific rotation. The s*pecific rotation* is the angular rotation observed when the plane-polarized light is passed through a solution 1 dm in length and 1 g/cm^3 in concentration. The specific rotation also depends on temperature and the wavelength. *T* refers to the temperature (usually 25 ^oC) and *D* to the yellow *D* line of a sodium lamp (λ=589.3 nm).

The instrument that is suitable for the measurement of α, the angular rotation, is called *polarimeter* and is shown in *Fig. 17*.

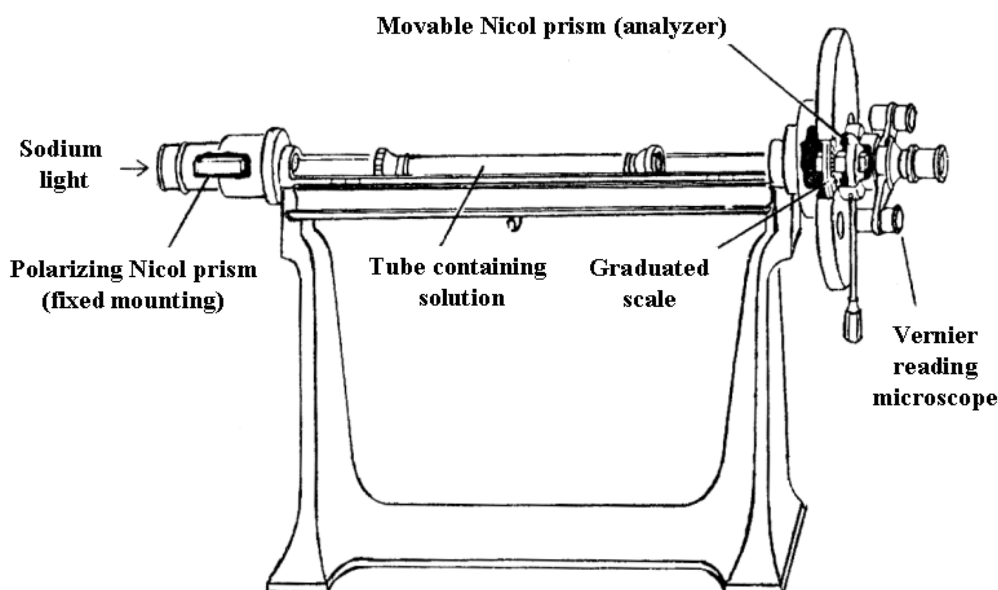


Figure 17. Polarimeter

The simplest polarimeter consists of the following basic parts:

1. light source (sodium light)
2. polarizer (fixed Nicol prism)
3. analyzer (movable Nicol prism)
4. graduated scale to read the angle of rotation, and
5. sample tube.

The sample tube into which the optically active substance is placed is a tube of well-defined length with flat glass ends. At each side of the sample tube is a Nicol prism or other kind of polarizer. The Nicol prism is made of calcite crystal which splits natural light into two polarized components perpendicular to each other. The crystal is cut and cemented in such a way that one component of the light is totally reflected, the second component passes through the crystal (polarizer). The other Nicol prism (analyzer) is oriented 90° to the polarizer so all of the light is reflected and no light will get to the detector. When an optically active substance is present in the beam, it rotates the polarization of the light reaching the analyzer so there is a component that can get through prism and make the visual field brighter. If we want to get total reflection again the analyzer should be adjusted by rotation. The angle that the analyzer must be rotated to return to the minimum detector signal is the optical rotation. Rotation can be either clockwise (in the case of dextrorotatory compounds) or counter clockwise (in the case of levorotatory compounds). The number of degrees and the direction of rotation can be read on a scale, on a graduated cycle which is connected to the analyzer Nicol prism.

Measuring optical rotation is useful for checking the purity of chiral mixtures, for determination of their concentration in solutions or for identification of an unknown pure compound.

Polarimetric analysis of carbohydrates

Carbohydrates (with the exception of dihydroxy acetone) are optically active. They contain at least one chiral center, an asymmetric carbon atom that has 4 different groups attached to it. The specific rotation for some of the common carbohydrates is summarized in *Table 6*.

Table 6. Specific rotation of common carbohydrates.

Carbohydrate	$[\alpha]_D^{25\,*}$
D-glucose	+52.7
D-fructose	-93.0
Sucrose	+66.5
Inverted sucrose (D-glucose: D-fructose, 1:1)	-20.2
Maltose	+136.0
D-mannose	+14.2
D-galactose	+80.5

*All values refer to a solution in the state of equilibrium (cf. mutarotation)

By measuring α of a well identified sugar, the concentration of its solution (expressed in g/cm^3) can be calculated.

$$c = \frac{\alpha}{l \times [\alpha]_D^{25}}$$

In clinical diagnostics polarimetry was one of the oldest instrumental procedures to determine glucose concentration in urine. Nowadays the more specific spectrophotometric methods are used for quantitative analysis of glucose because in some cases other optically active compounds (proteins, ß-hydroxybutanoic acid, lactose, galactose etc.) may be found in urine and their optical activity also modulates the angular rotation.

Experiment 1. *Determination of the concentrations of glucose and fructose solutions*

Check the 0 position of the polarimeter by filling the 1.00 dm long sample tube with distilled water and placing it into the polarimeter. The polarimeter should be adjusted to that position when in the visual field the disc is homogenous, no stripe can be seen in the middle of it and it is uniformly pale. Read the corresponding angular degree on the circular scale. That value is the zero position of the polarimeter which is not necessarily at 0.0° on the graduated cyclic scale.

Refill the polarimeter tube with the unknown carbohydrate (glucose or fructose) solution, and determine α, the optical rotation. Calculate the concentrations of D-glucose and D-fructose solutions, respectively using $[\alpha]_D^{25}$ values listed in *Table 6*.

Experiment 2. *Mutarotation of glucose*

Although fairly stable in crystals, glucose undergoes many changes in solution that are manifested in the change of its optical rotation. The change of optical rotation with time is known as *mutarotation*. Mutarotation may arise from interconversions between the α and ß forms of the sugar. The interconversions take place through the intermediate formation of the open-chain aldehydo form.

α-D-glucopyranose

ß-D-glucopyranose

The reaction is accelerated either by acids or by bases and obeys first-order kinetics. The optical activity of the glucose solution is changing until the equilibrium is reached. The $[\alpha]_D^{25}$ value of α-D-glucose is +112° while that of ß-D-glucose is +17.8°. In equilibrium the ratio of α : ß forms is 37% to 63%, respectively and the specific rotation is +52.7° (*Table 6*). Commercial glucose preparations contain α-D-glucose crystals, thus specific rotation of fresh glucose solution will decrease during mutarotation.

Procedure

Quickly dissolve 4 g of α-D-glucose crystals in 20 cm^3 of distilled water (c= 0.2 g/cm^3) and determine its optical rotation. After reading the value of α remove the solution from the polarimeter tube, add 0.2 cm^3 of 0.1 M NaOH to the glucose solution and mix it well. Start measuring the reaction time. Refill the tube with the glucose solution and determine α at 2, 4, 6, 8, 12, 16, and 20 min after the addition of NaOH solution. Calculate $[\alpha]_D^{25}$ at each time point. The initial $[\alpha]_D^{25}$ value (time=0 min) is calculated from the measurement of the freshly dissolved D-glucose in the absence of NaOH. The dilution caused by the addition of 0.2 cm^3 NaOH is negligible in the calculation. Depict $[\alpha]_D^{25}$ as a function of time. Estimate the specific rotation of D-glucose at equilibrium.

DUPress

Sample calculations

1. The angular rotation measured for a glucose solution was 2.64°. The length of the polarimeter tube is 1.00 dm. Calculate the concentration of the glucose solution.

$\alpha = 2.64^{o}$

$[\alpha]_D^{25} = 52.7$

l = 1.00 dm

$$c = \frac{\alpha}{l \times [\alpha]_D^{25}} \qquad c = \frac{2.64^{o}}{1.00 \times 52.7} = 0.05 \frac{g}{cm^3}$$

c = 0.05 g/cm^3

2. A solution of 2.0 g of (+)-glyceraldehyde in 10.0 cm^3 of water was placed in a 100 mm polarimeter tube. Using the sodium D line, a rotation of 1.74° was observed at 25°C. Calculate the specific rotation of (+)-glyceraldehyde.

$\alpha = 1.74^{o}$

$$l = 100\,mm \times \frac{1\ m}{1000\ mm} \times \frac{10\ dm}{1\ m} = 1.00\ dm$$

$$c = \frac{2.0\ g}{10.0\ cm^3} = 0.\ 20\ g/cm^3$$

$$[\alpha]_D^{25} = \frac{\alpha}{l \times c} \qquad [\alpha]_D^{25} = \frac{1.74}{1.00 \times 0.20} = 8.7$$

$[\alpha]_D^{25} = 8.7$

Questions

Give the formula for the calculation of optical/angular rotation (explain symbols).
Define specific rotation.
How could you determine the concentration of an optically active substance by polarimetry?
How could you identify an unknown carbohydrate by polarimetry?
Give the definition of mutarotation with structural explanation.
Draw the structure of α-D-glucose and mark the glycosidic -OH group.
Draw the structure of β-D-glucose and mark the glycosidic -OH group.
Draw the structure of the open chain form of D-glucose.

Exercise

Find the specific rotation and by the aid of *Table 6.* identify the name of that carbohydrate for which 10.54° rotation was observed with a solution of 0.2 g/cm^3 using 1 dm polarimeter tube.

DUPress

Results

Date:

The length of the polarimeter tube *(l)*: dm

0 position of the polarimeter: °

Experiment 1. *Determination of the concentration of glucose and fructose solution*

(a) Unknown glucose solution No:

$\alpha =$ ° $[\alpha]_D^{25} =$

Calculation

The glucose concentration of No. unknown solution is g/cm^3.

(b) Unknown fructose solution No:

$\alpha =$ ° $[\alpha]_D^{25} =$

Calculation

The fructose concentration of No. unknown solution is g/cm^3.

DUPress

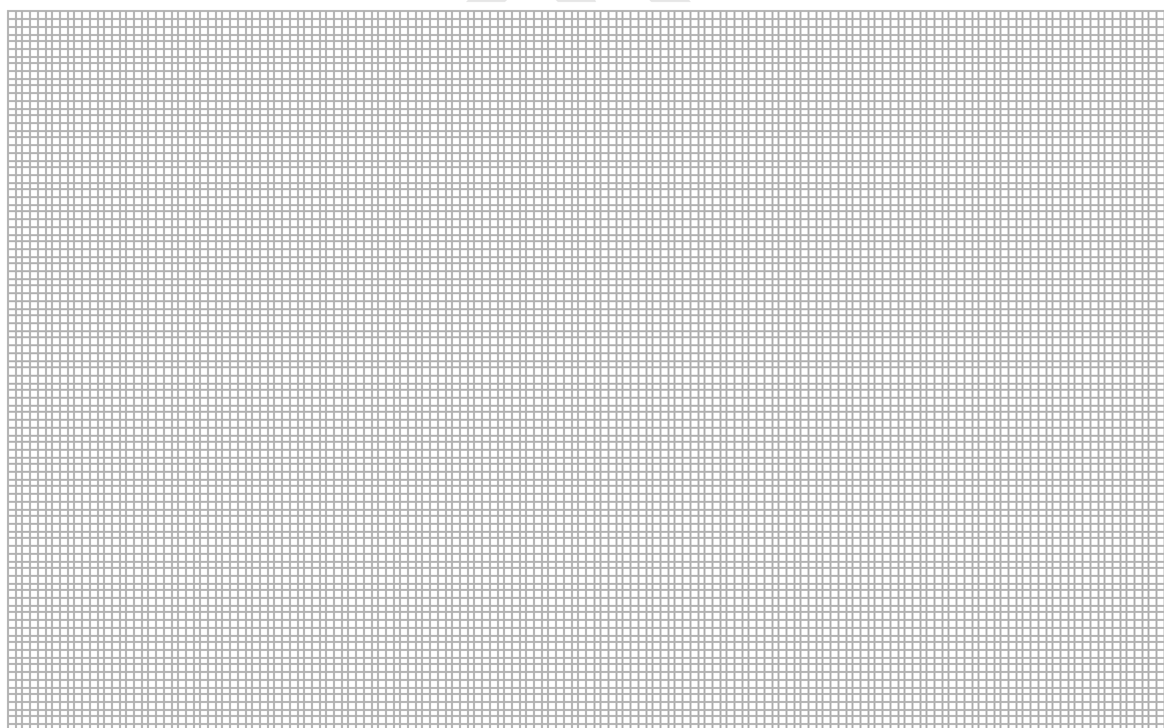
Experiment 2. *Mutarotation of glucose*

l = dm $c_{glucose}$= g/cm^3

Fill in the table

Time (min)	α (°)	$[\alpha]_D^{25}$
0		
2		
4		
6		
8		
12		
16		
20		

Depict the specific rotation as the function of time.



The specific rotation of glucose in the equilibrium is .

ELECTROMETRY

Electrometric methods of quantitative analysis are based upon a particular phenomenon occurring within a voltaic cell. Potentiometric (electrometric) methods involve two major types of analyses: the direct measurement of an electrode potential from which the activity (or concentration) of an ion may be calculated; and the continuous determination of changes in the electromotive force during titration.

According to the Nernst's- equation the electrode potential (ε) is equal to

$$\varepsilon = \varepsilon^{o} + \frac{RT}{nF}\ln c$$

where ε° is the standard reduction potential, R is the gas constant, T is absolute temperature, n is the number of electrons transferred, F is the Faraday constant and c is the concentration of metal ion. It is not possible to measure the electrode potential of a single electrode, we can measure only the potential difference between two electrodes. The difference between two electrode potentials is the electromotive force (e.m.f.) which is also known as cell potential (E_{cell}) of a voltaic cell.

$$E_{cell} = \varepsilon_{catode} - \varepsilon_{anode} = E^{o} - \frac{RT}{nF}\ln Q$$

where E^{o} is the standard reduction potential difference of cathode and anode, Q is the reaction quotient.

Electrometric pH measurement

By definition pH = -lg [H^+], that is the negative logarithm of H^+ concentration is called pH.

For pH determinations the so called *glass electrode* is used as a pH-responsive electrode. Its sensor bulb is made of special soft glass that is characterized by relatively high electric conductivity and high hygroscopicity. The thin-wall bulb is fragile and must be handled with care. The *reference electrode* is metal/metal ion precipitate electrode (e.g. Ag/AgCl). The electrode potential of the reference electrode remains constant because during operation of the voltaic cell its metal ion concentration is not changing considerably. The commonly used *combined electrode* has the reference electrode built in the glass electrode body. The ionic contact between the glass and reference electrodes is ensured by a porous ceramic plug (*Fig. 18.*)

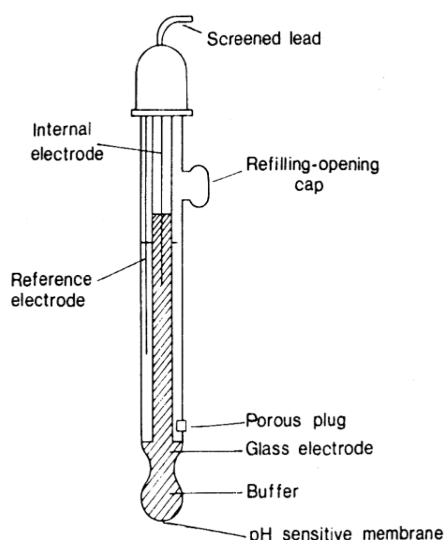


Figure 18. Combined electrode

The scheme of the combined electrode is

Ag | $AgCl_{(s)}$, Buffer | Glass | Test solution | | $KCl_{(aq)}$, $AgCl_{(s)}$ | Ag

Its cell potential (E_{cell}) is determined by the pH of the test solution:

$$E_{cell} = K' + 0.0592\ pH$$

where K' is a constant which is dependent on the reference electrode, the composition of the glass membrane and other external conditions. (The Nernst equation written in terms of base-10 logarithms, 2.303RT/F has a value of 0.0592 at 25 ºC)

A combined electrode can be used in the presence of strong oxidizing and reducing agents as well as proteins. It is also applicable in colored and viscous media. The electrode may give erroneous results only in the case of strong acids (pH<1) and strong bases (pH>10) and in ill buffered solutions.

Electrode care

The combined electrode has to be thoroughly washed with distilled water after each measurement. The ceramic plug and the pH-responsive glass of the electrode should not be allowed to become dry, they must always be immersed in a solution. Store the electrode in saturated KCl solution when it is out of use for a longer period of time, otherwise it can be stored in distilled water.

Equipment

The cell potential of a voltaic cell can be determined with a valve potentiometer (voltmeter). The pH meter is a precise voltmeter which measures potential difference between the known reference electrode and the measuring glass electrode. It scaled in such a way that it displays not the measured potential, but the pH value. The pH scale is constructed on the bases of the following formula:

$$pH = \frac{E(V)}{0.0592}$$

Different pH meters may require slightly different operating procedures. You should consult the manual to be sure how to proceed.

Experiment 1. *pH determination of an unknown solution*

Standardize the electrode according to the manual of the pH meter. Pour about 50 cm^3 of the unknown solution in a beaker. Wash the combined electrode with distilled water and immerse it in the solution to be tested. Read the pH value after 1 min stabilization. Turn the *operation* mode selector in 0 (stand by) position, wash the electrode and store it in distilled water.

Experiment 2. *The effect of dilution on the pH of a strong and a weak acid*

Determine the pH of 0.1 M, 0.01 M and 0.001 M HCl as well as that of 0.001 M, 0.01 M, 0.1 M, and CH_3COOH as described in *Experiment 1.* Calculate the theoretical pH of the solutions. Remember that HCl is a strong acid that fully dissociates even in concentrated solutions.

$$pH_{HCl} = -\lg c_{HCl}$$

On the other hand CH_3COOH is a weak acid (pK_a= 4.76) and its dissociation is influenced by dilution.

$$pH_{CH_3COOH} = -\frac{1}{2}\lg(K_a \times [CH_3COOH]) = 2.38 - \frac{1}{2}\lg[CH_3COOH]$$

DUPress

Experiment 3. *Potentiometric titration of a strong acid*

Measure 100 cm^3 of unknown HCl solution into a 250 cm^3 beaker with a graduated cylinder. Titrate the solution with 0.1 M NaOH and measure the pH of the mixture electrometrically with a combined glass electrode after the addition of 0, 5, 7.5, 9, 10, 11, 12.5 and 20 cm^3 of 0.1 M NaOH.

Plot the pH values as a function of the volume of 0.1 M NaOH. Determine the equivalence point from the graph (i.e. the inflection point) and calculate the concentration of the HCl solution. Explain the pH changes during the course of titration.

Experiment 4. *Potentiometric titration of a weak acid*

Measure 100 cm^3 of unknown CH_3COOH into a 250 cm^3 beaker and titrate it with 0.1 M NaOH potentiometrically. Record the pH after addition of 0, 2, 5, 8, 9, 10, 11, 12, 15 and 20 cm^3 of 0.1 M NaOH. Construct the titration curve by depicting the pH values as a function of NaOH volume, and estimate the equivalence point. Calculate the concentration of the CH_3COOH solution.

Compare the titration curves of HCl and CH_3COOH and give an explanation for the observed differences.

Experiment 5. *Determination of buffering capacity*

A buffer can be defined as a solution which maintains a nearly constant pH value despite the addition of substantial quantities of acid or base. Generally it consists of a mixture of a weak acid and its conjugate base.

A buffer solution retains its buffering action as long as the quantity of acid or base added is much less than the quantities of the weak acid (HA) and its conjugate base (A^-) in the buffer solution. If the moles of H^+ added exceeds the moles of A^- in the buffer solution (or if the moles of OH^- added exceeds the moles of HA in the buffer solution), the buffer is exhausted and there is a large change in pH.

Buffering capacity is expressed by the volume in cm^3 of 1 M HCl or 1 M NaOH added to 1000 cm^3 of buffer solution shifting the pH of buffer by 1 pH unit. The capacity against HCl or NaOH is not necessarily equal.

(a) Buffering capacity against a strong acid

Measure 20 cm^3 of the unknown buffer into a 100 cm^3 beaker. Add 30 cm^3 of distilled water to obtain an appropriate volume and determine the pH of the solution electrometrically. Titrate the solution with 0.1 M HCl until its pH decreases by about 1 pH unit. From the volume of 0.1 M HCl utilized calculate the capacity of the buffer.

$$\textit{Buffering capacity}_{HCl} = \frac{Volume_{HCl}\left(cm^3\right)\times 5}{pH_{start} - pH_{end}}$$

(b) Buffering capacity against a strong base

Repeat the experiment with another 20 cm^3 portion of the unknown buffer using 0.1 M NaOH in the titration procedure. From the consumption of 0.1 M NaOH calculate the capacity of the buffer.

$$\textit{Buffering capacity}_{NaOH} = \frac{Volume_{NaOH}\left(cm^3\right)\times 5}{pH_{end} - pH_{start}}$$

Sample calculations

1. Calculate the pH of a 0.2 M HCl solution.

pH=-lg 0.2

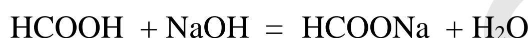
pH = 0.699

2. Calculate the pH of a 0.02 *M* acetic acid (CH_3COOH) solution. K_a= 1.8x 10^{-5}.

$$pH = -\frac{1}{2}\lg(K_a \times [CH_3COOH]) = -\frac{1}{2}\lg(1.8 \times 10^{-5} \times 0.02) = -\frac{1}{2}\lg 3.6x10^{-7} = 3.22$$

pH = 3.22

3. What is the pH when 10.0 mL of 0.10 M NaOH is added to 30.0 mL of 0.15 M HCOOH. K_a(HCOOH) = 1.7 x 10^{-4}.



$$HCOOH + NaOH = HCOONa + H_2O$$

0.15 mol/L x 0.03 L = 0.0045 mol HCOOH was to be titrated
0.1 mol/L x 0.01L = 0.001 mol NaOH was added

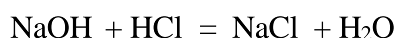
HCOOH is in excess: 0.0045 – 0.001 = 0.0035 mol in 30.0 + 10.0 = 40 mL = 0. 04 L solution
Concentration of HCOOH: 0.0035 mol/ 0.04 L = 0.0875 M
In the reaction 0.001 mol HCOONa has been formed.
Concentration of HCOONa: 0. 001mol/ 0.04 L = 0. 025 M
The weak acid and its conjugate base is present in the solution at this stage of the titration.
According to the Hendelson-Hasselbach equation:

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{\text{conjugate base}}{\text{conjugate acid}} \qquad \mathrm{pH} = \mathrm{p}K_a + \log\frac{[HCOONa]}{[\mathrm{HCOOH}]}$$

pH = 3.770 - 0.544 = 3.226

pH = 3.226

4. The volume of 0.1 M NaOH consumed in the titration of 10 cm^3 HCl solution at the equivalence point was 10.5 cm^3. Calculate the concentration of the HCl solution. What is the pH at the equivalence point?



$$NaOH + HCl = NaCl + H_2O$$

Amount of NaOH added: 0.1 x 0.0105 = 0.00105 mol
1 mol NaOH reacts with 1 mol HCl At the equivalence point none of the reactants is in excess.
So there was 0.00105 mol HCl in the titrated 10 cm^3 solution.
Molarity of the HCl solution is 0.105mol/1000cm^3 = 0.105 mol/L

The concentration of the HCl solution is 0.105 M.
The pH = 7.0 at the equivalence point since water and NaCl is present in the solution and NaCl will not react with water (no hydrolysis of the salt occurs).

DUPress

Questions

Give the composition and function of the two electrodes used in pH determination.

Draw a sketch of the titration curve expected for the titration of a strong acid with a strong base. Mark the position of the equivalence point.

Make a rough sketch of the titration curve expected for the titration of a weak acid with a strong base. Mark the position of the equivalence point.

Give the definition of the buffering capacity.

Give the equations for the reactions occurring when (a) a strong acid or (b) a strong base is added to an acetate buffer solution.

What are the components of a phosphate buffer? Write the Handerson-Hasselbalch equation for a phosphate buffer solution.

Exercises

1. Calculate the pH value of a 0.4 M HCl solution.

2. Calculate the pH value of a 0.2 M CH_3COOH solution. (pK_a= 4.76 for acetic acid)

3. Calculate the H^+ ion concentration and the pH of a 10^{-1} M HCl and of a 10^{-1} M CH_3COOH solution, respectively. (K_a for acetic acid is 1.8 10^{-5})

4. How much difference in pH values can be detected after (a) tenfold, (b) hundredfold dilution of hydrochloric acid?
How much difference in pH values can be detected after (a) tenfold, (b) hundredfold dilution of acetic acid solution?
Explain your answer.

5. What is the pH after the addition of 5.0 cm^3 of 0.10 *M* NaOH to 25 cm^3 of 0.10 *M* HCl?

6. What is the pH after the addition of 5.0 cm^3 of 0.10 *M* NaOH to 25 cm^3 of 0.10 *M* CH_3COOH? (pK_a= 4.76 for acetic acid)
What volume of NaOH is needed to get to the equivalence point?
What is the pH of the solution at the equivalence point?

DUPress

Results

Date:

Experiment 1. *pH determination of an unknown solution*

Unknown sample No:

pH =

Experiment 2. *The effect of dilution on the pH of a strong and a weak acid*

Acid concentration	HCl		CH_3COOH	
	Calculated pH	Measured pH	Calculated pH	Measured pH
0.1 M				
0.01 M				
0.001 M				

Calculations

DUPress

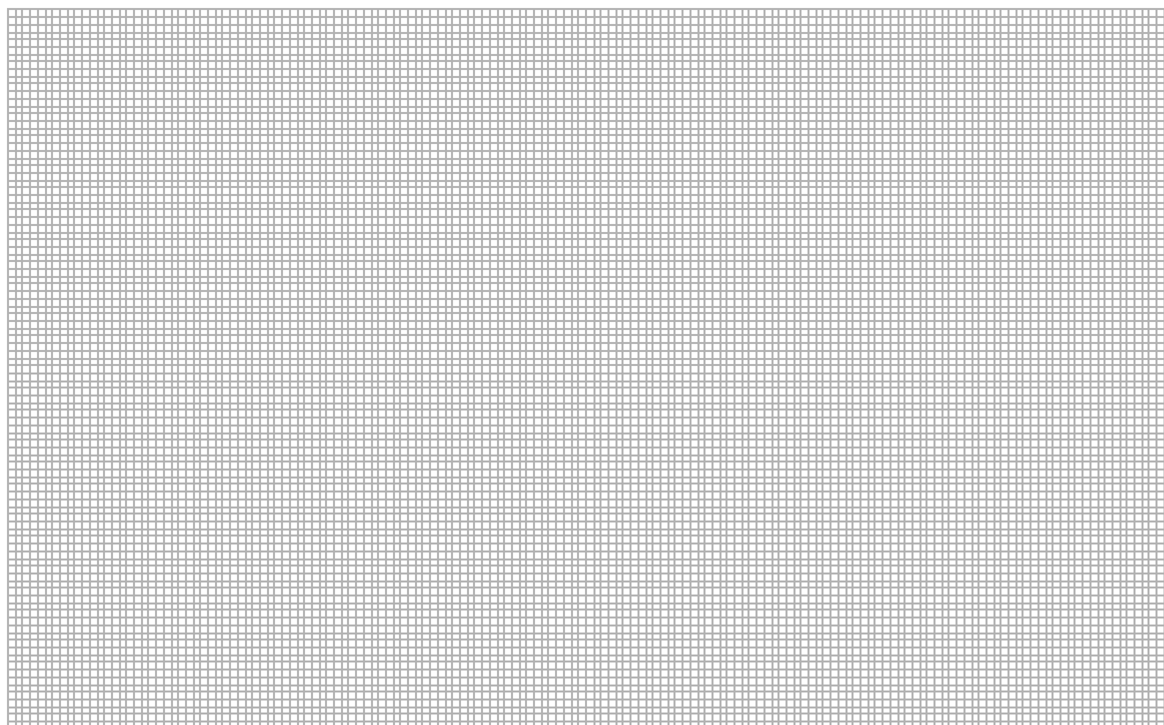
Compare the calculated and measured pH values.

Explain the differences between HCl and CH_3COOH.

Experiment 3. *Potentiometric titration of a strong acid*
Number of the HCl solution:
Volume of HCl solution: Concentration of NaOH: 0.1M

Added NaOH (cm^3)	pH
0.0	
5.0	
7.5	
9.0	
10.0	
11.0	
12.5	
20.0	

Depict pH as a function of volume (cm^3) of NaOH.



pH at the equivalence point:

Consumed 0.1 M NaOH at the equivalence point: cm^3.
Calculation

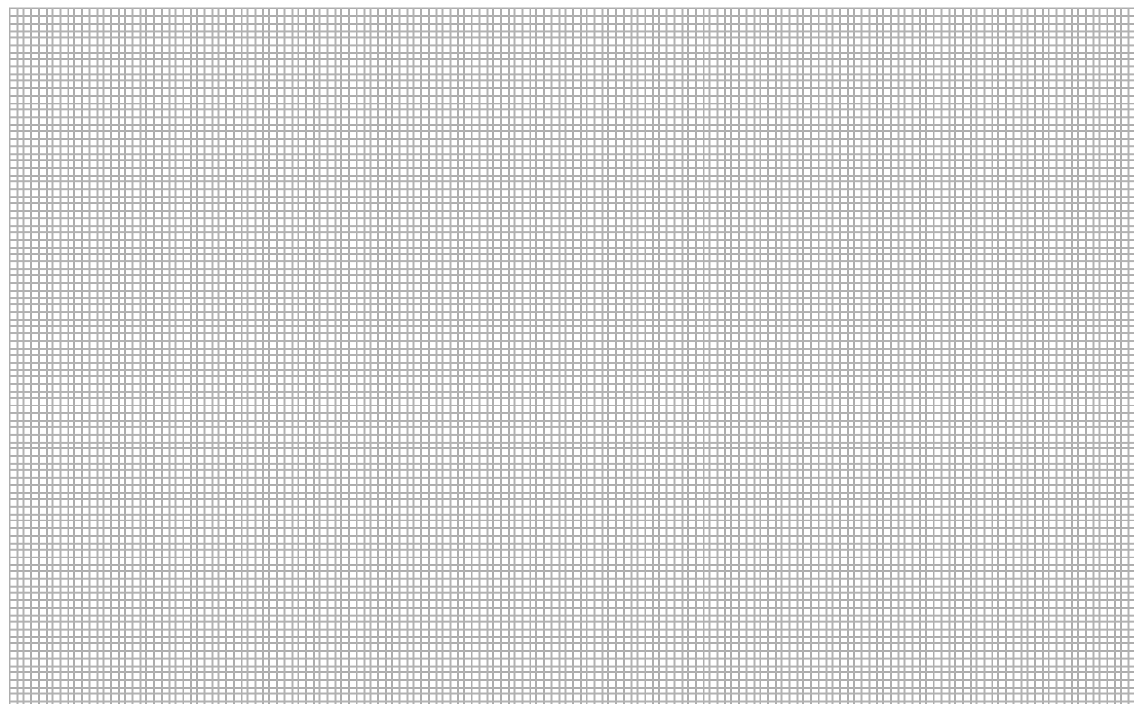
The concentration of HCl is M.

Experiment 4. *Potentiometric titration of a weak acid*
Number of CH_3COOH solution:
Volume of CH_3COOH solution: Concentration of NaOH: 0.1 M

Added NaOH (cm^3)	pH
0.0	
2.0	
5.0	
8.0	
9.0	
10.0	
11.0	
12.0	
15.0	
20.0	

ess

Depict pH as a function of volume (cm^3) of NaOH.



pH at the equivalence point:

Consumed 0.1 M NaOH at the equivalence point: cm^3
Calculation

The concentration of CH_3COOH is M.

Experiment 5. *Determination of buffering capacity*

Number of the unknown sample:

(a) Buffering capacity against a strong acid

Consumed 0.1 M HCl (cm^3)	pH_{start}	pH_{end}	ΔpH	Buffering capacity

Calculation

(b) Buffering capacity against a strong base

Consumed 0.1 M NaOH (cm^3)	pH_{start}	pH_{end}	ΔpH	Buffering capacity

Calculation

DUPress

CHROMATOGRAPHY

Chromatography (from Greek χρώμα: *chroma*, color and γραφειν: *grafein*, to write) is the collective term for a family of laboratory techniques for the separation of mixtures.

Chromatography terms

- The *analyte* is the substance which is to be purified, isolated, or analyzed during chromatography.
- The *mobile phase* is the analyte and solvent mixture which travels through the stationary phase.
- The *stationary phase* is the substance which is fixed in place for the chromatography procedure and is the phase to which solvents and the analyte travels through or binds to.
- The *retention time* is the characteristic time it takes for a particular molecule to pass through the system under set conditions.
- The *eluent* is the solvent mixture used to form the mobile phase.
- A *chromatogram* is the visual output of the chromatographic separation. Different peaks or patterns on the chromatogram correspond to different components of the separated mixture.

Chromatography theory

Chromatography is a method that exploits the differences in partitioning behavior between a mobile phase and a stationary phase to separate the components in a mixture. Components of a mixture may be interacting with the stationary phase based on charge, relative solubility or adsorption.

To understand the process of chromatography we consider that the mobile and stationary phases are in equilibrium. The partition coefficient K is based on this equilibrium, and is defined by the following equation:

$$K = \frac{\textit{concentration of the analyte in the stationary phase}}{\textit{concentration of the analyte in the mobile phase}}$$

K is assumed to be independent of concentration, and can change if experimental conditions are changed, for example temperature is increased or decreased. As K increases, the analyte is bound stronger to the stationary phase therefore it is more efficiently retarded as compared to the speed of the mobile phase.

The retention is a measure of the speed at which a substance moves in a chromatographic system. In paper chromatography the retention is measured as the *retention factor* R_f, the run length of the compound divided by the run length of the eluent front:

$$Rf = \frac{\textit{distance moved by the analyte}}{\textit{distance moved by the solvent}}$$

The R_f value may vary between 0 and 1 ($R_f = 1$ – the analyte moves together with the solvent, $R_f = 0$ – the analyte does not depart from its original point).

DUPress

In column chromatography, where the analytes elute together with the eluent, retention is defined as retention time. Retention time (t_R) is the time between sample load and detection.

A chromatographic separation begins with loading the samples that is followed by the running of the samples. In that step we apply the mobile phase and the chromatographic separation takes place. The endpoint of the process has to be monitored, either by following the movement of the analyte or by detecting the outflow of the analyte from the applied chromatographic system. The graphical representation of the running of analytes is called the chromatogram.

Qualitative evaluation of chromatograms means the identification of compounds in each peak (*Fig. 19*). This is done by the comparison of the position of the unknown peak(s) with that of well characterized standards. The quantity of a given compound can be determined after separation by measuring the peak height or by determining the area under the peak. Quantitative evaluation requires a calibration method using several known quantities of the compound to be tested.

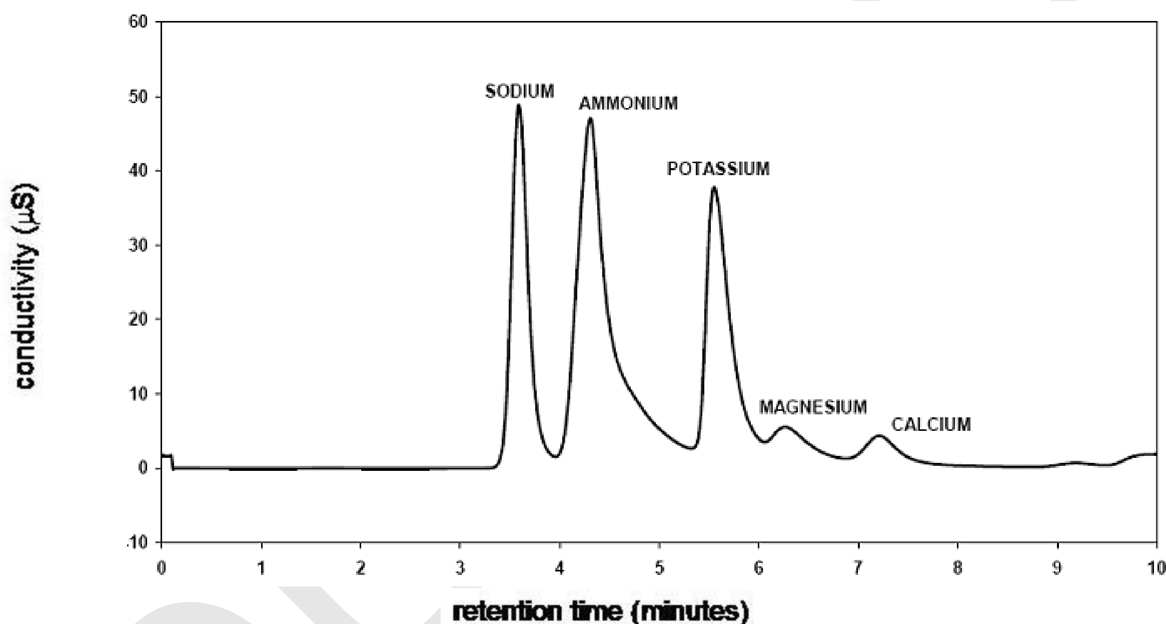


Figure 19. A typical chromatogram

The increase in the conductivity indicates the occurrence of cations. The identification of the respective cations and their corresponding peaks is the qualitative evaluation of the chromatogram. The area bellow a peak corresponds to the quantity of the particular analyte. The determination of the quantity in this fashion is called the quantitative evaluation of the chromatogram.

Paper chromatography

Paper chromatography (PC), together with thin-layer chromatography (TLC) belongs to the group of the planar chromatography techniques. TLC and PC differ fundamentally in the nature of the stationary phase whereas they are very closely related in their technical aspects: sample application, development and detection.

For PC, in practice, one can use the so-called standard cellulose papers (the products of Whatman, or Schleicher - Schüll), or glass-fiber papers containing silica gel. The stationary phase consists of the paper and the water actually bounded to the fibers of the paper. The mobile phase consists of organic solvent(s).

A PC separation takes place as follows. A drop of solution containing the analyte is placed on a piece of filter paper and is allowed to dry. Then the filter paper is placed in a reservoir of a solvent mixture (eluent) which flows along the paper by capillary action. When the eluent reaches the spot, some of the analytes dissolve in it. As the eluent containing the dissolved substances flows to a fresh paper surface some of the analytes reabsorb in an attempt to establish equilibrium at the location. At the same time an additional amount of pure eluent flows over and more of the originally deposited analytes redissolve. As the solvent flow continues the substances move along in the direction of the solvent at different rates. The rate at which a substance migrates depends on how strongly it is adsorbed on the cellulose and how great a tendency it has to dissolve in the solvent.

After a suitable length of time the paper is removed from the reservoir. The paper is dried and when necessary is treated with a reagent to make the substances visible. The general use of PC is to determine the components of mixtures (qualitative analysis).

IMPORTANT: In the following experiments use a soft pencil for marking. Do not scratch the fibers of the paper!

Experiment 1. *Separation of food dyes by ascending paper chromatography*

In this experiment we shall separate foodstuff dyes. These dyes are used for the coloration of foods (sweets, drinks). For alimentary use strict standards apply and only the allowed dyes can be used. Therefore it is of importance to identify them. The unknown substances can be identified on the basis on their mobility on paper as compared to the standard solution of the permitted dyes.

The following foodstuffs-dyes are permitted by the FDA:

Amaranth (crimson)
Erythrozin (red)
Indigocarmine (blue)
New coccine (cerise)
Tartrazine (yellow)

Procedure

Take a 20 by 20 cm piece of Whatman No.1 filter paper. Draw a pencil line 2.5 cm from the bottom of the paper and make six vertical marks at every 3 cm as in *Fig. 20*. Mark the crosses by numbers 1 through 6.

DUPress

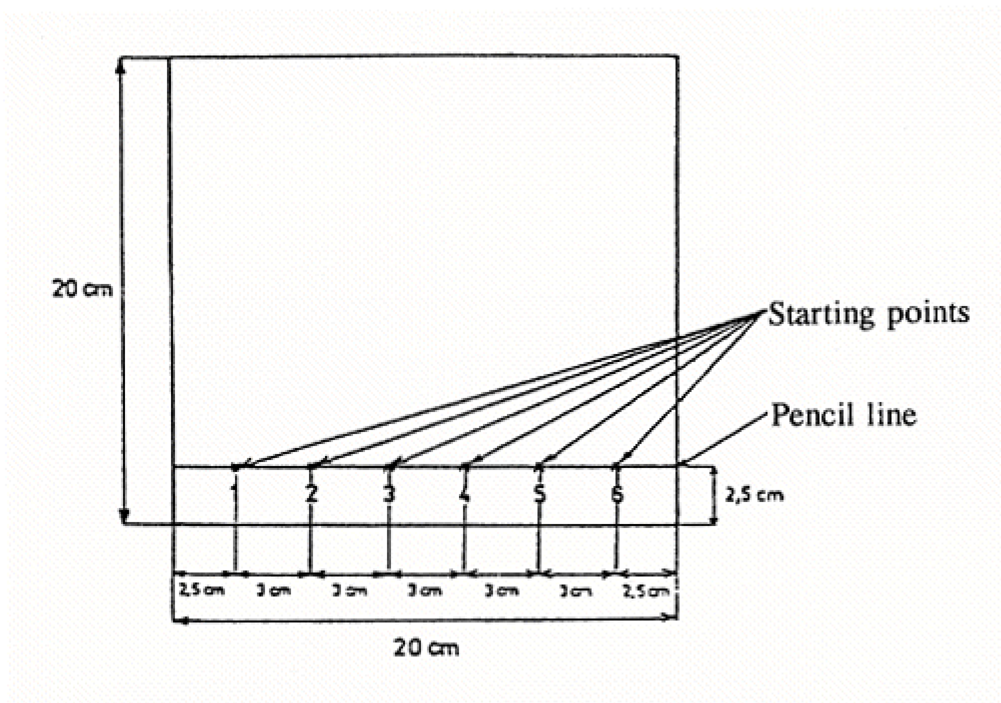


Figure 20. Layout of the chromatographic paper for separation of dyes

To the points apply a drop of the following samples by a micropipette tip:

Start point	Sample
1	Amaranth standard solution
2	Indigocarmine standard solution
3	New coccine standard solution
4	Tartrazine standard solution
5	Mixture of standard solution
6	Unknown

Dry the spots with a hairdryer, roll and staple the paper to form a cylinder and stand in a jar containing a mixture of 2.5% CH_3COONa : conc. NH_4OH : distilled water in a 2 : 5 : 93 volume ratio. Close the jar and develop the chromatogram at room temperature *(Fig. 21)*.

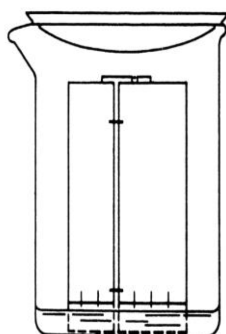


Figure 21. Vertical chromatographic chamber (ascending development)

When the solvent approaches the top of the paper stop the run, remove the paper and **after marking the solvent front** dry the chromatogram. Calculate the R_f values of the spots. Based on R_f values and the color of the spots identify the dyes in the unknown solution.

Experiment 2. *Separation of metal ions by horizontal paper chromatography*

In this experiment round Schleicher-Schüll or Macherey-Nagel filter papers and a horizontal set-up is used. Obtain a filter paper of 9 cm diameter and draw a circle in its middle using a 20 Forint coin. Divide the circle into six parts by fine pencil lines and mark the points by numbers 1-6 on the paper (*Fig. 22*). Pierce a hole in the middle of the paper.

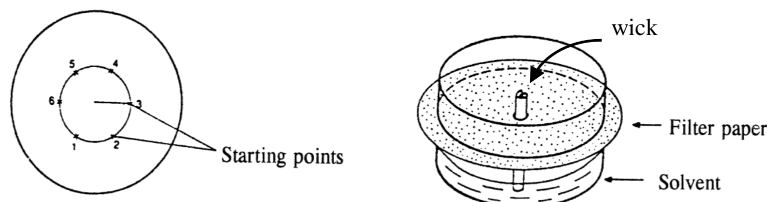


Figure 22. Layout of the round filter paper and the chromatographic chamber for separation of metal ions by horizontal paper chromatography

Apply a drop of the samples on the points as follows:

Start point	Sample
1	$NiSO_4$ solution
2	$Co(NO_3)_2$ solution
3	$CuSO_4$ solution
4	$FeCl_3$ solution
5	Mixture of 1, 2, 3, 4
6	Unknown

Form a filter paper piece roll a small column (wick) and insert it into the hole of the round-shaped filter paper. Place the round-shaped filter paper on the rim of a Petri-dish containing 10 cm^3 of solvent (acetone-20% HCl in 88:12 volume ratio) so that the wick should be dipped into the solvent. Cover the paper with another Petri-dish as seen in *Fig. 22*.
Develop the chromatogram until the solvent approaches the edge of the paper. Remove the paper from the chamber, discard the wick. Mark the front of the solute around the paper and dry the paper. Describe the color of the metal ions and note them in the table in page 22 (in the column "After first drying").
Although the metal ions have their own color for better visualization spray the whole paper with rubeanic acid solution and dry it again. The metal ions give colored spots:

Ion	Color
Ni^{2+}	blue
Co^{2+}	yellow
Cu^{2+}	greenish brown
Fe^{2+}	yellowish brown

Identify the ions of the unknown solution.

Questions

Describe the principle of chromatography.

What is the eluent?

What is the mobile phase?

What is a stationary phase?

What are the phases in PC?

What does "R_f" mean?

How is the Rf value calculated? (Empirical formula and explanation).

What is the range for R_f values?

Describe the process of the ascending chromatography for the food dye identification! (loading of the samples, way of the ascending chromatography, evaluation of data)

Describe the process of the horizontal chromatography for the metal ion identification! (loading of the samples, way of the ascending chromatography, evaluation of data)

How can one identify an unknown analyte by chromatography?

DUPress

Results

Experiment 1. *Separation of food dyes by ascending paper chromatography*

Date:

Number of unknown sample:

Paper: Whatman No.1

Solvent: 2.5% (w/v) Na-acetate - conc. NH_4OH - water (2:5:93)

Running time: min

Distance moved by the solvent: cm

Starting point	Dye(s)	Color of the spot	Distance moved by the spot (cm)	R_f
1.	Amaranth			
2.	Indigocarmine			
3.	New coccine			
4.	Tartrazine			
5.	Standard mixture 1st spot 2nd spot 3rd spot 4th spot			
6.	Unknown mixture 1st spot 2nd spot 3rd spot			

The unknown solution No. contains the following dyes:

DUPress

Experiment 2. *Separation of metal ions by horizontal paper chromatography*

Date:

Number of unknown sample:

Paper: Macherey-Nagel (9 cm diameter)

Solvent: acetone - 20% HCl (88:12)

Running time: min

Distance moved by the solvent: cm

Starting point	Metal ion(s)	Color of the spot		Distance of the metal ion spot (cm)	R_f
		after first drying	after rubeanic acid treatment		
1.	Ni^{2+}				
2.	Co^{2+}				
3.	Cu^{2+}				
4.	Fe^{2+}				
5.	Standard mixture 1st spot 2nd spot 3rd spot 4th spot				
6.	Unknown mixture 1st spot 2nd spot				

The unknown solution No. contains the following metal ion(s):

DUPress

Ion exchange chromatography

The term ion exchange means the exchange of ions bound on a solid matrix with ions in the solution in contact with the matrix.

The solid matrix is called the *ion exchanger*. An ion exchanger consists of an insoluble material (*matrix* or *resin*) with permeable molecular structure containing covalently bound charged groups (*fix ions*). There are mobile ions (also called *counter ions*) bound to the fix ions. The counter ions may be reversibly exchanged with other ions of the same charge. The counter ions may change place with the ions of the solution that surrounds the matrix.

Depending on the charge of the counter ions we distinguish between cation**-** and anion exchanger matrixes. If the matrix carries positive groups the counter ions will be negative, such an ion exchanger will exchange negative ions and is therefore termed an anion exchanger. If the matrix carries negative groups the counter ions will be positive. Since the positive ions are exchangeable the term cation exchanger is used.

The matrix is a high molecular mass cross linked durable polymer material since it has to provide physical scaffold. *Anion exchange resins* contain amino (usually quaternary ammonium) groups on their surface as integral parts of the polymer lattice. The quaternary amino group's positive charge is balanced by an equivalent amount of anions (e.g. OH^- ions). During the anion exchange the OH^- of the anion exchanger can be exchanged for Cl^- *(Fig. 23.A)*.

Cation exchange resins contain sulfonic, carboxylic or phenolic groups that provide a negative charge. This negative charge is counterbalanced by an equivalent amount of cations (e.g. H^+ ions). During the cation exchange the H^+ of the cation exchanger can be exchanged for Na^+ of the NaCl solution *(Fig. 23.B)*.

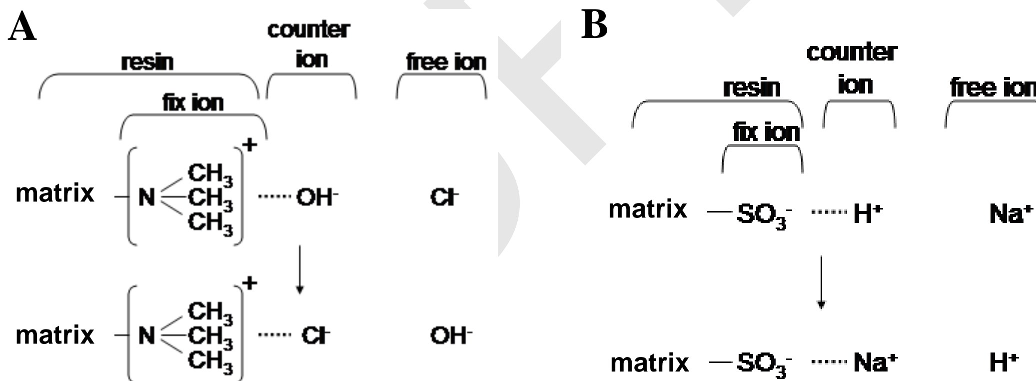


Figure 23. The schematic representation of anion exchange (A) and cation exchange (B)

Ion exchange chromatography is a frequently used technique in biochemistry in the purification of proteins or nucleic acids (cDNA or RNA). In a separation procedure the column is equilibrated with the buffer that will be used then the sample is loaded. From the sample the molecule of interest (analyte) bind to the surface of the matrix, replacing the counter ions. The non-bound molecules from the sample are removed by washing. The bound molecules can be removed (eluted) from the column by washing with a solution of high ionic strength (eluting ions) *(Fig. 24).*

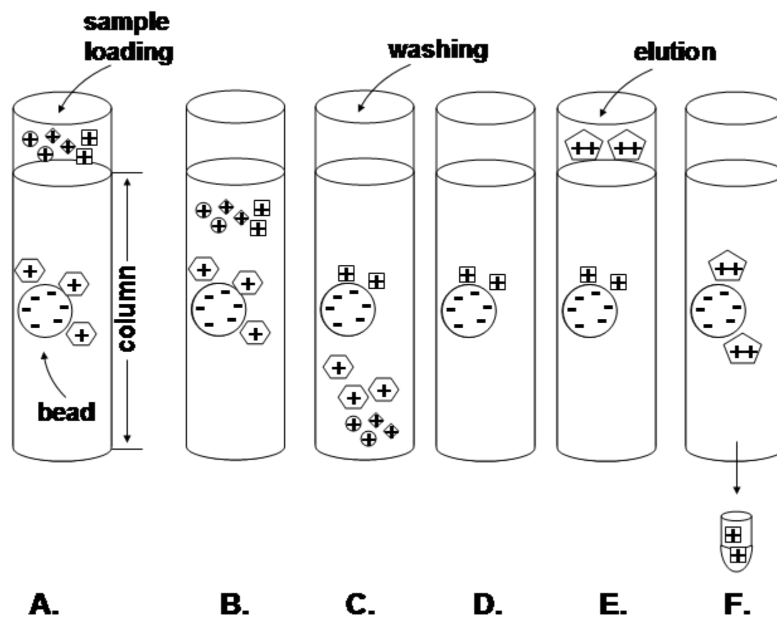


Figure 24. The principle of a cation exchange experiment

A. The beads are equilibrated with the counter ions and the samples are loaded onto the column.
B. The samples enter the column.
C. The analyte binds to the negatively charged beads displacing the counter ions. The non-bound ions are removed by washing.
D. The non-bound ions are completely removed by washing.
E. The elution begins by the loading of positively charged ions that bind stronger then the analyte.
F. The analyte is displaced by the ions used for elution and is washed out from the column. The eluted analyte is collected.

Experiment 1. *Cation exchange chromatography*

In this experiment the cation exchange chromatography of a NaCl solution will be performed. The Na^+ ions of the solution will be exchanged with the H^+ ions (these are the counter ions!) of the ion exchange resin. The cation exchanger resin beads used in this experiment have been packed in a chromatographic column.

IMPORTANT! The resin should never be dried, there should be always liquid on the resin! The flow rate of the column should not exceed 2 cm^3/min.

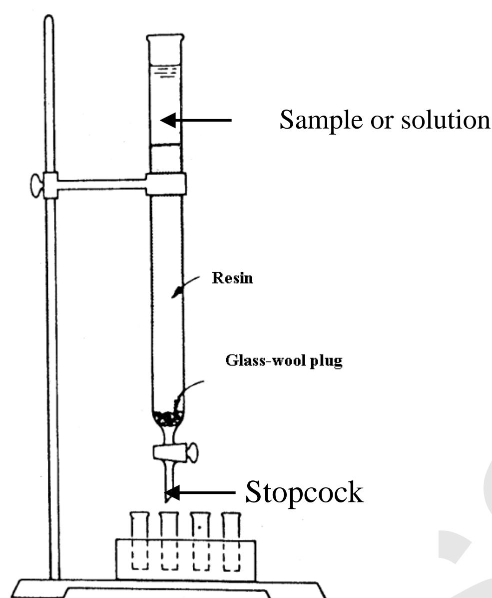


Figure 25. A chromatography column

Application of the NaCl sample

-Place an Erlenmeyer flask under the column. Run the excess of water out of the column. When the level of water has just reached the top of the resin bed, close the stopcock and apply with a pipette 5 cm^3 of unknown NaCl solution onto the column.
-Place a new, empty and clean Erlenmeyer flask bellow the column.
-Open the stopcock and let the salt solution enter the resin bed. Collect the effluent in an Erlenmeyer flask as it flows out of the column. When the level of NaCl solution has dropped to the top of the resin, stop the flow again.

Elution

-Apply 3 x 10 cm^3 of distilled water onto the column.
-Open the stopcock and collect the effluent (eluted solution) in the Erlenmeyer flask.
-Split the effluent into 2 x 10 cm^3 samples in two new Erlenmeyer flasks.

Measurement

Determine the HCl content of the two samples by titrating with 0.1 M NaOH in the presence of methyl orange indicator.

Calculation

Calculate the NaCl concentration (in terms mol/dm^3) of the applied sample assuming that Na^+ were quantitatively exchanged for H^+.

Experiment 2. *Anion exchange chromatography*

Apply 2 cm^3 of the remaining cation exchange effluent (from the Experiment 1.) onto the anion-exchange column. Wash the column with 10 cm^3 of distilled water. Collect the liquid that has passed through the column in an Erlenmeyer flask.

Check the pH with indicator paper as well as **the Cl^- content** by the addition of a few drops of $AgNO_3$ solution to a small sample aliquot: of the original unknown NaCl solution, of the cation exchanged effluent and of the anion exchanged effluent.

Sample calculation

The cation-exchange chromatography of 5 cm^3 NaCl solution had been performed. The NaCl solution was loaded onto the column and was washed with 30 ml of water resulting in 35 cm^3 of eluent. The eluent was split into 3 X 10 cm^3 samples that were titrated.
The three parallel titrations of the eluent (HCl in 10 cm^3) with 0.1 M NaOH gave the following results:

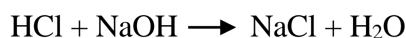
Titration 1: 9.9 cm^3
Titration 2: 10.2 cm^3
Titration 3: 10.2 cm^3

The average volume from the titrations is: $(9.9\ cm^3+10.2\ cm^3+10.2\ cm^3)/3 = 10.1\ cm^3$
10.1 cm^3 of 0.1 M NaOH solution contains:

1000 cm^3 of solution	X	0.1 mol of NaOH
10.1 cm^3 of solution		x mol of NaOH

$$x = \frac{0.1\ \text{mol NaOH} * 10.1\ \cancel{\text{cm}}^3}{1000\ \cancel{\text{cm}}^3} = 0.00101\ \text{mol NaOH} = 1.01\ \text{mmol NaOH}$$

The following equation describes the chemical reaction during titration:



The ratio between NaOH and NaCl is 1:1, therefore there was in the 10 cm^3 sample eluted from the column 1.01 mmol NaCl. The total elution volume was 35 cm^3 (5 cm^3 of the original NaCl + 30 cm^3 of water) that makes a total load of NaCl:

10 cm^3	1.01 mmol
35 cm^3	x mmol

$$x = \frac{35\ cm^3 * 1.01\ mmol}{10\ cm^3} = 3.535\ mmol$$

The total NaCl loaded on the column was 3.535 mmol.

The concentration of the NaCl solution can be calculated in the following way (we have loaded 5 cm^3 NaCl solution that contains 3.535 mmol of NaCl):

c (mol/dm^3) = moles of NaCl / volume of the samples in dm^3

3.535 mmol NaCl = 0.003535 mol NaCl
5 cm^3 = 0.005 dm^3

c = 0.003535 mol NaCl / 0.005 dm^3 = 0.707 mol/dm^3

The original NaCl concentration in the solution loaded to the column is 0.707 cm^3/dm^3.

DUPress

Questions

Sketch the structure of a cation-exchange resin particle and mark the fix- and the counter-ions.

Sketch the structure of an anion-exchange resin particle and mark the fix- and the counter-ions.

Give the chemical equation of cation exchange.

Give the chemical equation of anion exchange.

What will be the pH of the effluent of a NaCl solution after cation exchange?

Exercises

1. A cation-exchange chromatography of a NaCl solution had been performed. 10 cm^3 of solution was loaded onto the column and the column was washed with 20 ml of water. The 30 cm^3 effluent was titrated with 0.1 M NaOH in 3 X 10 cm^3 portions. The titrations gave the following results: 15.2 cm^3, 14.9 cm^3 and 15.0 cm^3. What was the original concentration of the NaCl solution?

2. An anion exchange chromatography had been performed. 5 cm^3 of solution was loaded onto the column and the column was washed with 30 cm^3 of water. The 35 cm^3 effluent was titrated with 0.1 M HCl in 3 X 10 cm^3 portions. The titrations gave the following results: 5.2 cm^3, 4.9 cm^3 and 5.0 cm^3. What was the original concentration of the NaCl solution?

DUPress

Results

Experiment 1. *Cation-exchange chromatography*

Date:

Number of unknown sample:
Bed material: Varion KS cation-exchange resin
Solvent: distilled water
Flow rate: cm^3/min
Applied volume of NaCl solution: cm^3
Volume of the effluent: cm^3

Titration of effluent by NaOH solution

Volume of effluent titrated:
Standard solution: 0.1 M NaOH

Titration 1: cm^3
Titration 2: cm^3

Average of titrations: cm^3

Calculation

The NaCl content of the applied sample was mmol.
The concentration of the NaCl solution was mol/dm^3

DUPress

Experiment 2. *Anion exchange chromatography*

Record your observations and write chemical equations of the cation and anion exchanging and for checking the Cl^- content.

	pH	Cl^- content
Unknown NaCl solution		
Effluent of cation-exchanger		
Effluent of anion-exchanger		

DUPress

Gel filtration

Gel filtration or gel chromatography is a technique of partition chromatography in which the partitioning is based on the molecular size of the substances to be separated. The mobile phase is the buffer used in the experiment, while the stationary phase is a porous *gel*. The pores of the gel are filled with the liquid of the mobile phase. The gel is composed of macromolecules (dextrane, agarose, or polyacrylamide) with a high affinity for the solvent. These gels usually have a covalent cross-linked structure which forms a three-dimensional insoluble network.

The separation in gel filtration depends on the different abilities of the various sample molecules to enter pores of the stationary phase. Very large molecules never enter the stationary phase (steric obstruction), they move through the chromatographic column the fastest, without hindrance. Smaller molecules, which can enter the gel pores, move more slowly through the column, since they spend a proportion of their time in the stationary phase. Molecules are, therefore, eluted in order of decreasing molecular size (*Fig. 26*).

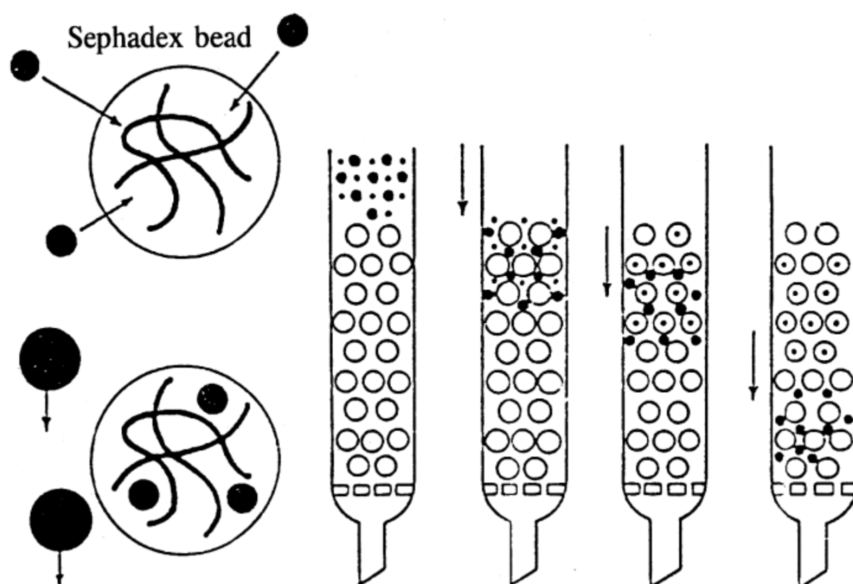


Figure 26. The principle of gel filtration
O *gel beads;* . ● *small and large molecules*

We will use a Sephadex G-25 coarse column in our experiments. Sephadex-G is a brand name defining the chemical composition of the gel, Sephadex columns are made up of dextrane. Each Sephadex G-type column has a different molecular weight range over which molecules can be fractionated. Molecules with molecular weight above the upper limit of this range, the *exclusion limit*, are totally excluded from the gel (they never enter the gel pores). These molecules are eluted from the column the first, but there is no separation between them! Molecules smaller than the lower limit are eluted at last, equally there is no separation between them. Fractionation range (molecular weight) for the Sephadex G-25 type is 1000-5000 Da and the exclusion limit is 5000 Da.

In gel chromatography the bed usually needs no regeneration and can be used over and over again.

Experiment 1. *Desalting of egg-white solution*

- Number two series of test tubes from 1 to 12.
- Place an empty beaker bellow the chromatography column.
- Before use wash the gel bed in the column with 5 cm^3 of the buffer solution. Adjust the flow rate to 0.5-1 cm^3/min using a timer or your watch and a graduated cylinder. The volume of the effluent measured by the graduated cylinder under a minute defines the flow rate. Stop the flowthrough when the level of the buffer reaches the level of the gel.
- Apply 1 cm^3 of a sample containing diluted egg-white and $(NH_4)_2SO_4$ onto the gel. Let the solution enter into the gel, then wash it in with 2 cm^3 of Na-ß-glycerophosphate (buffer solution).
- Place one series of numbered tubes bellow the column and collect 1 cm^3 of eluent sequentially into each. (1 cm^3 ~ the thickness of a finger.) Naturally, wash the column with the buffer solution.
- Divide the fractions roughly into two equal parts using the other test tube series. Assay one part with the Biuret reagent for the presence of proteins while assay the other half with $BaCl_2$ for the presence of the SO_4^{2-} ions. To the half of a fraction add 1 cm^3 of Biuret reagent and to the other half add 0.5 cm^3 of 5% $BaCl_2$. Identify the tubes containing protein or salt by crosses in the table on the following page.

Questions

What is the principle of gel separation?

What is the mobile and what is the stationary phase in gel chromatography?

What is the meaning of the exclusion limit?

How can you desalt a mixture of egg-white and ammonium sulfate solution using gel filtration (list steps)?

DUPress

Results

Experiment 1. *Desalting of egg-white solution*

Date:

Gel-type: Sephadex G-25 coarse

Flow rate: 0.5 cm^3/min

Sample to be separated: 1 cm^3 of egg-white containing $(NH_4)SO_4$

Buffer: 0.01 M Na-β glycerophosphate (pH 6.8)

Fraction volume: 1 cm^3

Fraction number	1	2	3	4	5	6	7	8	9	10	11	12
Protein content												
SO_4^{2-} content												

(After the detection of protein with Biuret reagent and SO_4^{2-} with $BaCl_2$ solution, mark the fractions by +++, ++, + and - symbols according to the intensity of color or amount of precipitation.)

No. fractions contain salt free protein.

No. fractions contain the salt.

DUPress

REACTION KINETICS

Reaction kinetics is the study of *reaction rates* in a chemical reaction. Analyzing the influence of different reaction conditions on the reaction rate gives information about the *reaction mechanism* of a chemical reaction. The *rate of a reaction* is defined as the *change of the concentration of reactants or products in time*. For the unidirectional reaction

$$A + B \rightarrow C$$

the rate (v) can be expressed as follows

$$v = -\frac{d[A]}{dt} = -\frac{d[B]}{dt} = \frac{d[C]}{dt};$$

where [A], [B] and [C] are the concentrations of the reactants and the product; t is the time of the reaction.

The initial rate of the above reaction is determined by the concentrations of the reactants

$$v = k[\mathrm{A}]^n[\mathrm{B}]^m;$$

where k is the rate constant, and $(n+m)$ is called the *overall order of the reaction*. k is independent of concentrations and is a function of the temperature.

The rate of a *first order chemical reaction* depends on the concentration of a single compound.

$$v=k[A]$$

The half-life of the reaction (i.e. the time required to reduce [A] to the half of the initial value) is independent of the concentration of the reactant.

$$t_{1/2} = \frac{\ln 2}{k}$$

Thus a first order reaction can be characterized either by the k or by the $t_{1/2}$ values.

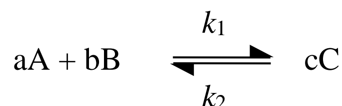
The product of two concentrations or the square of one concentration determines the rate of a *second order reaction.*

$$v = k[\mathrm{A}][\mathrm{B}] \quad \text{or} \quad v = k[\mathrm{A}]^2$$

The half-life is inversely related to the initial concentration, $[\mathrm{A}]_0$, in the latter case.

$$t_{1/2}= \frac{1}{k[A]_0}$$

When analyzing equilibrium reactions, one has to take into consideration two opposing processes. The forward and reverse reactions are characterized by the rate constants k_1 and k_2, respectively.



$$\mathrm{aA + bB} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{cC}$$

A and B are the reactants, C is the product, a, b, and c are stoichiometric coefficients. The rate of the overall reaction is determined by the reaction rates of the forward (v_1), and reverse (v_2) reactions.

$$v = v_1 - v_2$$

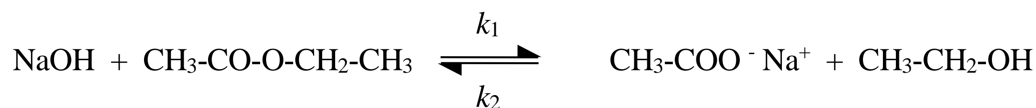
When equilibrium is attained, $v_1= v_2$, there is no detectable change in the concentrations and the overall reaction rate becomes zero, v=0.

The rate of a given chemical reaction can be controlled by changing the concentration of the reactants, by changing the temperature or by the addition of a catalyst. Catalysts are substances that increase the reaction rate without affecting the equilibrium, i.e. a catalyst accelerates both the forward and the reverse reactions equally.

DUPress

Kinetic study of the saponification reaction of ethyl acetate

The alkaline hydrolysis (saponification) of ethyl acetate results in the production of sodium acetate and ethanol.



$$NaOH + CH_3\text{-}CO\text{-}O\text{-}CH_2\text{-}CH_3 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} CH_3\text{-}COO^- Na^+ + CH_3\text{-}CH_2\text{-}OH$$

At the beginning of the reaction, high concentrations of ethyl acetate and sodium hydroxide are present whereas sodium acetate and ethanol are practically missing from the mixture. Under these circumstances the reaction proceeds fast in the direction of the upper arrow and the reverse reaction can be neglected, i.e. the reaction is unidirectional and its rate is defined by the concentration of the reacting materials

$$v_1 = k_1 [CH_3COOC_2H_5] [NaOH]$$

where k_1 is the rate constant of the forward reaction.

As the reaction proceeds, the rate of saponification gradually decreases for two reasons. First, the concentration of starting materials diminishes; second, the accumulation of products initiates the reverse reaction. The rate of the reverse reaction is defined as

$$v_2 = k_2 [CH_3COONa] [C_2H_5OH]$$

where k_2 is the rate constant of the reverse reaction. The overall rate of saponification is determined by the above two elementary reactions and can be expressed as

$$v = v_1 - v_2 = k_1 [CH_3COOC_2H_5] [NaOH] - k_2 [CH_3COONa] [C_2H_5OH]$$

Finally, v_1 and v_2 become equal and the conversion of the reactants virtually ceases, when the state of equilibrium is reached.

$$v_1 = v_2$$
$$v = v_1 - v_2 = 0$$

Since the reaction rate continuously changes until the equilibrium is attained, the reaction is best characterized by the rate constants k_1 and k_2. It is also a good approximation to determine the half-life of the reaction. The concentration of NaOH can be easily followed by a volumetric method. Thus, the half-life can be determined as the time required for decreasing the initial NaOH concentration to half of its original value. The initial phase of the saponification should be considered as a second-order reaction. The half-life of a second order reaction depends on the initial concentration of the reactants; consequently, saponification is characterized by the half-life *and* the initial concentrations of ethyl acetate and NaOH.

Experiment 1. *Determination of the half-life of saponification reaction at room temperature*

(a) Determine the initial concentration (concentration at 0 min) of NaOH in a separate experiment. Add 5 cm^3 of 0.25 M NaOH to 10 cm^3 of ice-cold distilled water and place it on ice. Pipette 5 cm^3 of ethyl acetate to the cold solution and titrate the NaOH content **immediately** with 0.1 M HCl in the presence of phenolphthalein. The equivalence point is indicated by the color change from faint red to colorless.

(b) Measure 10 cm^3 distilled water each into four Erlenmeyer flasks and place them on cracked ice. Using a graduated cylinder, measure 40 cm^3 of ethyl acetate into another flask with

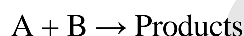
a glass stopper. Add 40 cm^3 of 0.25 M NaOH to the ester, start recording the reaction time and shake the mixture immediately. Plug the flask to avoid evaporation of ethyl acetate during the reaction. Withdraw 10 cm^3 samples each from the reaction mixture at 2, 5, 10, and 20 min after the start. Add the withdrawn samples to the cold distilled water in the Erlenmeyer flasks. The drop in temperature and the dilution ensure that the saponification reaction slows down significantly. Titrate the residual NaOH in the sample **immediately** with 0.1 M HCl solution using phenolphthalein indicator.

Experiment 2. *Effect of temperature on the half-life of the reaction*

Repeat the reaction at 50°C as described in *Experiment 1.b* with the following modifications. Incubate both 40 cm^3 of ethyl acetate in a stoppered flask and 40 cm^3 of 0.25 M NaOH in a separate Erlenmeyer flask in a water-bath at 50°C for 5 min. Mix the two solutions by adding the NaOH to the ethyl acetate, and start recording the reaction time. Withdraw 10 cm^3 samples each at 1, 4, 8, and 12 min and analyze them as above. The initial NaOH concentration determined in *Experiment 1.a* can be used for the construction of the NaOH decomposition curve.

Sample calculations

1. The following reaction is first order in A and first order in B:



$$A + B \rightarrow \text{Products}$$

Give the reaction rate equation.

$$Rate = k[A][B]$$

2. What is the half-life of a first order reaction, if $k = 5.7 \times 10^{-5}\ s^{-1}$?

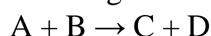
$$t_{1/2} = \frac{\ln 2}{k} = 0.693/5.7 \times 10^{-5}\ s^{-1} = 1.22 \times 10^{4}\ s$$

Questions

Define the rate of a chemical reaction.
Give a general formula for the reaction rate equation.
Define the rate constant and the order of the reaction.
Define the first order chemical reaction.
Define the second order chemical reaction.
What is the half-life of a reaction?
What are the three main factors that affect the rate of a given chemical reaction?
Give the chemical equation and the rate equation for the saponification reaction of ethyl acetate.

Exercises

1. The following reaction is second order in A and first order in B:



$$A + B \rightarrow C + D$$

Give the reaction rate equation.

2. What is the rate constant, k, for a first order reaction, if $t_{1/2} = 1600$ s?

DUPress

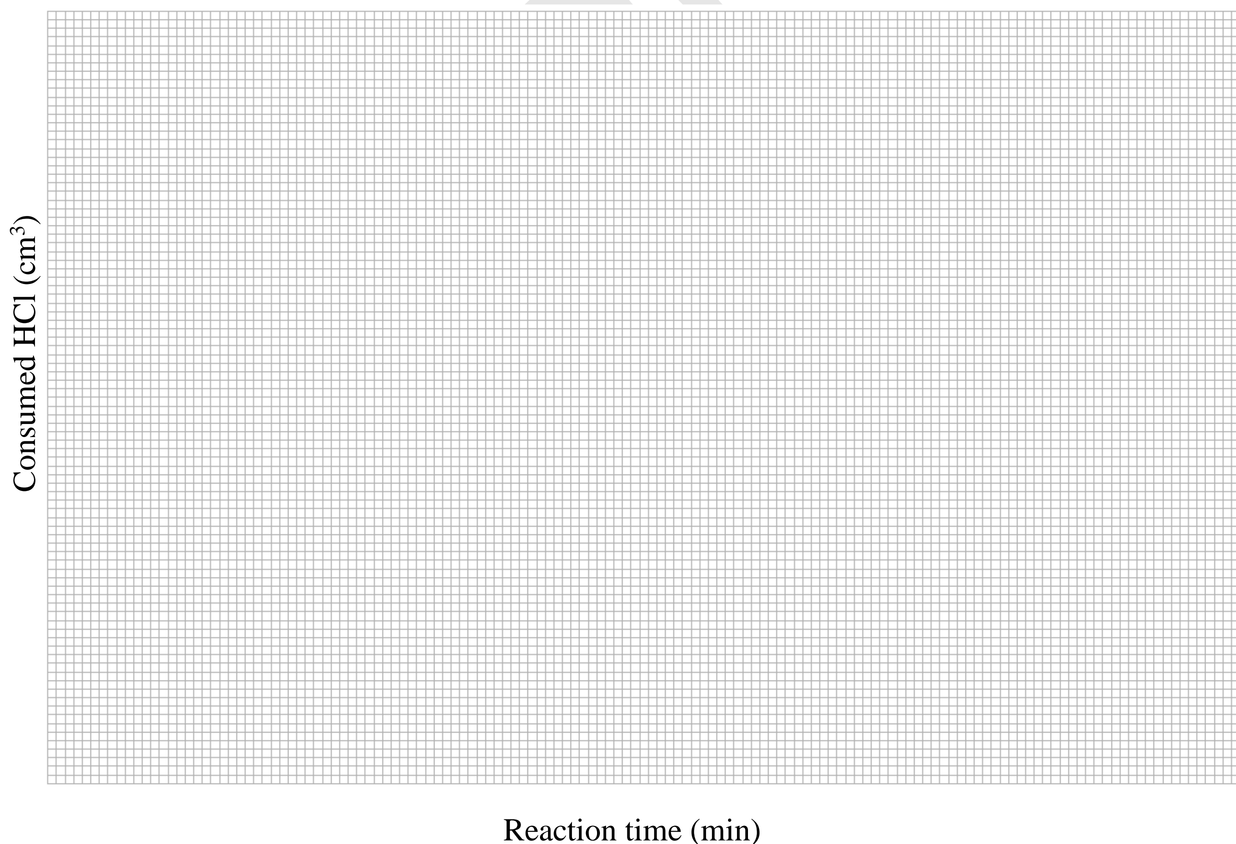
Results

Date:

Concentration of the ethyl acetate: %

Room temperature: …...°C		50 °C	
t (min)	consumed HCl (cm^3)	t (min)	consumed HCl (cm^3)
0		0	
2		1	
5		4	
10		8	
20		12	

The volume of 0.1 M HCl required to neutralize the samples is proportional to the concentration of NaOH. Draw the decomposition curve of NaOH by plotting the volume of 0.1 M HCl consumed (cm^3) as the function of reaction time (min) at room temperature and at 50 °C.



Analyse the kinetic curves and answer the following questions.

How does the NaOH concentration change with time?

How could one determine the order of the reaction by additional experiments?

How much time was needed to reach an equilibrium state

at room temperature:

at 50°C:

What is the half-life of the reaction

at room temperature:

at 50°C:

Compare the half-life values obtained at the two different temperatures. What is the effect of the temperature on the half-life?

What is the effect of the temperature on the reaction rate?

DUPress

Kinetic analysis of the oxidation of iodide ion using the Landolt-method

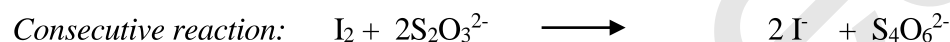
Under special conditions one can use the *Landolt-method* for the estimation of reaction rate. According to this method instead of measuring the concentration change as a function of time, the time required for the consumption of a given concentration (given amount) of reactant(s) is determined in a series of reactions. As the change in concentration (Δc) is constant, i.e. it is the same in each reaction, the reaction time (t) is inversely related to the reaction rate (v).

$$v = \frac{\Delta c}{t}$$

The specific criteria allowing to use the Landolt-method are: (*i*) one of the reaction products reacts rapidly (much faster than it is formed) in a consecutive reaction with another compound; (*ii*) this compound does not react with any other substances (reactants or intermediates) involved in the reaction; (*iii*) this compound must be the limiting reactant in the overall reaction. The oxidation of iodide ions with persulfate ions for example is suitable for the application of the Landolt –method.

Studied reaction: $2\,I^- + S_2O_8^{2-} \longrightarrow I_2 + 2\,SO_4^{2-}$

Thiosulfate ions react with I_2 in a specific and fast reaction.


Consecutive reaction: $I_2 + 2S_2O_3^{2-} \longrightarrow 2\,I^- + S_4O_6^{2-}$

The reaction of iodine with thiosulfate is much faster than the production of iodine in the first reaction. Therefore, iodine accumulates in the reaction mixture only after the whole amount of the thiosulfate has been used in the second reaction. The accumulation of iodine can be visualized easily with starch, since starch forms a blue complex with iodine. If the starting concentrations of the reactants are within a well defined range the reaction can be conveniently analyzed by the Landolt-method.

Experiment 1. *Effect of iodide ion concentration on the reaction rate*

Determine the temperature of the laboratory with a thermometer. Prepare the following mixtures in four separate 100 cm^3 glass beakers as outlined below.

Reaction	#1	#2	#3	#4
Distilled water (cm^3)	10	10	10	10
0.005 M $Na_2S_2O_3$ (cm^3)	10	10	10	10
0.2 % starch (cm^3)	5	5	5	5
0.2 M KI (cm^3)	12.5	9.4	6.3	3.1
0.2 M KNO_3 (cm^3)	-	3.1	6.2	9.4

Measure into each of four separate test tubes 12.5 cm^3 0.1 M $K_2S_2O_8$ solution. Pour the content of the test tubes into the beakers one by one and measure the time between the addition of $K_2S_2O_8$ and the appearance of the blue color of the iodine-starch complex (this is the so called Landolt-time of the reaction).

DUPress

Experiment 2. *Effect of a catalyst on the reaction rate*

Copper(II) ions catalyze the oxidation of iodide ions by persulfate ions. To analyze the effect of Cu^{2+} prepare one beaker containing all of the solutions described in the first column in *Experiment 1*. Measure 12.5 cm^3 of 0.1 M $K_2S_2O_8$ plus 0.4 cm^3 of 0.01 M $CuSO_4$ into a separate test tube and perform the reaction as in *Experiment 1* at room temperature. Determine the Landolt-time in the presence of the catalyst.

Sample calculation

Calculate the concentration of KI in molarity (mol/dm^3) in reaction mixture #1.

Volume of KI=12.5 cm^3
Original concentration of KI = 0.2 M
Volume of reaction mixture = volume of water + volume of $Na_2S_2O_3$ + volume of starch + volume of KI + volume of $K_2S_2O_8$ =
$10\ cm^3 + 10\ cm^3 + 5\ cm^3 + 12.5\ cm^3 + 12.5\ cm^3 = 50\ cm^3$

Dilution of KI = volume of KI : volume of reaction mixture = 12.5:50 = 1:4

Concentration of KI in the reaction mixture = 0.2 M : 4 = 0.05 M

Questions

How can one determine the rate of a reaction by the Landolt-method?

What is the relationship between the rate of a reaction and the Landolt-time?

How can the Landolt-time be determined in the iodide-persulfate reaction?

What is the effect of increasing the concentration of iodide on the rate of the iodide-persulfate reaction and on the Landolt-time?

What is the effect of Cu(II) ions on the rate of the iodide-persulfate reaction and on the Landolt-time. What is the role of Cu^{2+} in the reaction?

Exercises

1. Calculate the concentration of KI in molarity (mol/dm^3) in reaction mixtures #2-4.

2. Calculate the concentration of KNO_3 in molarity (mol/dm^3) in reaction mixtures #2-4.
   What is the function of KNO_3 in the reaction mixtures?

DUPress

Results

Date:

Experiment 1. *Effect of iodide ion concentration on the reaction rate*

The temperature of the laboratory is °C.

Beaker #	1	2	3	4
Concentration of KI (mol/dm^3) in the assay*				
Landolt-time (min)				

*For calculations see previous page.

What is the effect of changing the iodide ion concentration on the Landolt-time and on the reaction rate?

Experiment 2. *Effect of catalyst on the reaction rate*

Landolt-time of the reference reaction (#1 from *Exp. 1*): min

Landolt-time in the presence of Cu^{2+}: min

Compare the reaction rates of the catalyzed and non-catalyzed reaction. What is the role of the catalyst in the reaction?

DUPress

ENZYME KINETICS

Enzymes are proteins endowed with high catalytic power, substrate specificity and subtle regulatory mechanisms. They play a pivotal role in biochemical reactions taking place in living organisms. The Michaelis-Menten model for enzyme kinetics assumes that the following steps are involved in the reaction

where E stands for the enzyme, S for the substrate, P for the product, and ES represents the intermediate enzyme-substrate complex; k_1, k_2, and k_3 correspond to the rate constants for the individual steps.

The reverse reaction, P + E $\rightarrow$ ES, is neglected and only the initial reaction rate is considered. If k_1 is much larger than k_3, as can be expected for most of the reactions, the concentration of the enzyme-substrate complex, [ES], quickly reaches a steady-state level. Under these circumstances the overall reaction rate is determined by the rate of product formation

$$v = k_3[ES]$$

and the steady-state concentration of ES can be calculated. According to the steady-state assumption, the rates of ES formation, $v_1 = k_1[E][S]$, and its decomposition, $v_2 = k_2[ES] + k_3[ES] = (k_2 + k_3)[ES]$, are identical; and the overall ES concentration does not change significantly.

$$k_1[E][S] = (k_2 + k_3)[ES]$$

$$[ES] = \frac{[E][S]}{(k_2 + k_3)/k_1}$$

By the combination of the rate constants, a new term, the so-called *Michaelis constant* (K_M) is derived

$$\boxed{K_M = \frac{k_2 + k_3}{k_1}}$$

and the steady-state ES concentration can be expressed as

$$[ES] = \frac{[E][S]}{K_M}$$

It has been assumed that the total concentration of substrate is much greater than the total concentration of enzyme, thus the steady state substrate concentration is not much different from the total substrate concentration, $[S_T] \approx [S]$. On the other hand, the concentration of the free enzyme, [E], is much smaller than the total concentration of enzyme [E_T].

$$[E] = [E_T] - [ES]$$

DUPress

Taking into account the above considerations

$$[ES] = ([E_T] - [ES])\frac{[S]}{K_M}$$

$$[ES] = [E_T]\frac{[S]}{[S] + K_M}$$

and the reaction rate (v) is

$$v = k_3[ES] = k_3[E_T]\frac{[S]}{[S] + K_M}$$

The maximal reaction rate ($v_{\max}$) is attained when the enzyme is saturated with substrate. This situation is approached when [S]>>K_M, therefore $\frac{[S]}{[S] + K_M} \cong 1$, and

$$v_{\max} = k_3[E_T]$$

Substitution of v_{max} into the equation of v yields the *Michaelis-Menten equation* characteristic for enzyme-catalyzed reactions

$$v = v_{\max}\frac{[S]}{[S] + K_M}$$

The two constants in the Michaelis-Menten equation, $v_{\max}$ and K_M, are unique for each enzyme under specific conditions.

The graphical representation of the Michaelis-Menten equation is shown in *Fig. 27A*. The rate is directly proportional to the substrate concentration, $v = v_{\max}[S]/K_M$, at very low substrate concentrations, when [S]<<K_M. The rate is maximal, $v=v_{max}$, and independent of substrate concentration at high substrate concentrations, when [S]>>K_M. When the reaction rate is half maximal ($v = 1/2v_{\max}$)

$$\frac{1}{2}v_{\max} = \frac{v_{\max}[S]}{[S] + K_M}$$

and

$$[S] = K_M$$

Thus, K_M is equal to the substrate concentration at which the reaction rate is half-maximal.

DUPress

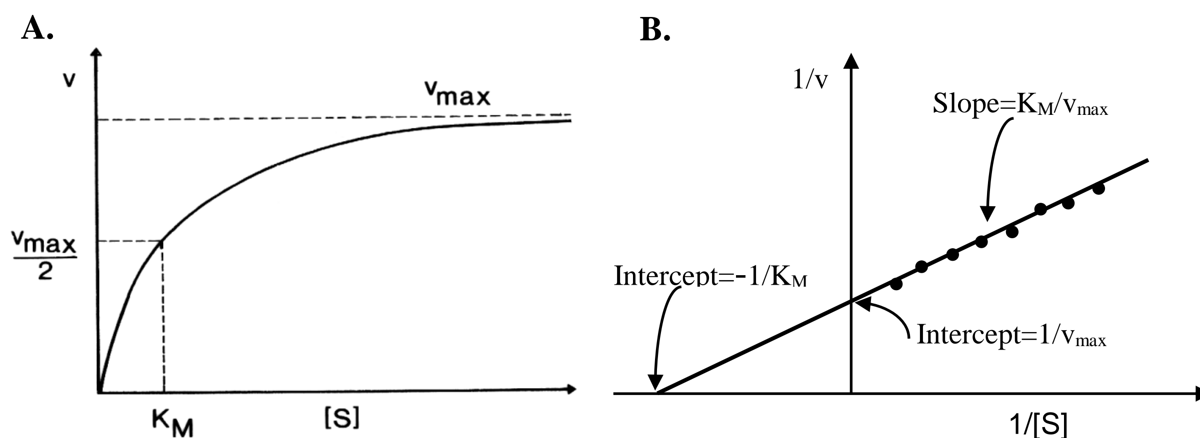


Figure 27. ***A.*** *Plot of reaction rate versus substrate concentration for an enzyme-catalyzed reaction. v: reaction rate, [S]: substrate concentration, v_{max}: maximal reaction rate, K_M: Michaelis-constant.* ***B.*** *Linewaver-Burk plot.*

It is difficult to estimate v_{max} and K_M values from the direct graphic representation of the equation with appropriate accuracy. There are several methods for the linearization of the originally hyperbolic function and obtaining more precise graphic readings. According to the most popular linearization technique, the *Linewaver-Burk plot*, we plot the reciprocal value of reaction rate ($1/v$) as a function of the reciprocal substrate concentration ($1/[S]$) (*Fig.27B*). A straight line is obtained which intercepts the $1/v$ axes at $1/v_{\text{max}}$ and the $1/[S]$ axes at $-1/K_M$. Consequently the v_{max} and K_M can be attained from the two intercepts.

The catalytic activity of an enzyme depends on many factors besides substrate concentration. Optimal temperature, ionic strength, and pH are required for the maximal activity. The determination of the maximal activity is of great importance from a practical point of view. If measurements are performed at substrate saturation (and preferably under optimal conditions) the reaction rate is directly proportional to the total enzyme concentration.

Activators and inhibitors, substances accelerating or decelerating the catalyzed reaction, respectively, can regulate the activity of an enzyme. The analysis of enzyme inhibition is of significance since in many cases toxic compounds and/or drugs inhibit enzyme reactions in living organisms.

Various units are available to express the activity of an enzyme. *Katal* (abbreviated as kat) is the approved SI unit. One katal is defined as that amount of the enzyme that catalyses the conversion of 1 mole substrate per second (1 kat = 1 mol/sec). The more traditional *enzyme unit* (U) of enzyme activity is defined as the amount of the enzyme that catalyses the conversion of 1 micro mole substrate per minute (1 U = 1 μmol/min). 1 U = 1/60 micro katal = 16.67 nano katal, or 1 kat = 60,000,000 U.

One can characterize the purity of an enzyme with its *specific activity* (kat/kg, or U/mg). The efficiency of an enzyme is expressed in terms of molecular activity or turnover number. The *molecular activity* specifies the number of substrate moles converted by 1 mole of the enzyme in one minute. The *turnover number* tells the number of substrate molecules converted by a single molecule of the enzyme in one minute.

DUPress

Assay of glycogen phosphorylase activity

Glycogen phosphorylase is an enzyme that catalyses the following reaction

$glycogen_{(n)} + P_i$ $glycogen_{(n-1)}$ + glucose-1-phosphate

where *n* is the number of glycosyl units in glycogen. In equilibrium, the ratio of glucose-1-phosphate (glucose-1-P) to P_i is 3.6 (pH 6.8; 30°C). Since in living cells P_i is always in great excess over glucose-1-P, glycogen phosphorylase catalyses glycogen breakdown *in vivo*. However, *in vitro,* the reaction is reversible; at high glucose-1-P and low P_i concentrations glycogen synthesis takes place. This fact is utilized in the activity assay of the enzyme. The liberated P_i is determined spectrophotometrically as the function of reaction time.

In the experiments described below, glycogen phosphorylase *b* isolated from skeletal muscle is used. This enzyme has the peculiarity that it may exist in three inter-convertible forms: *a* (fully active), *ab* (partially active) and *b* (inactive). The activity of all forms can be assayed in the presence of the allosteric activator adenosine 5'-monophosphate (AMP), which activates glycogen phosphorylase *b* and *ab*.

Experiment 1. *Assay of glycogen phosphorylase activity in the presence of AMP*

For the determination of total glycogen phosphorylase activity prepare the following reaction mixture *in triplicate* in 3 test tubes:

1.) 0.1 cm^3 diluted glycogen phosphorylase *b*,
2.) 0.1 cm^3 0.05 M Na-glycerophosphate buffer, pH 6.8,
3.) 0.1 cm^3 4 % glycogen dissolved in water (first substrate).

After 5 min pre-incubation in a water-bath at 30 °C add the pre-warmed (30 °C) second substrate and activator solution to each of the test tubes:

4.) 0.1 cm^3 64 mM glucose-1-P containing 4 mM AMP.

Start recording the reaction time immediately, shake the test tubes vigorously and incubate them at 30°C. Stop the reaction at 5 (tube 1), 10 (tube 2), and 15 (tube 3) min by the addition of

5.) 1.6 cm^3 5 % trichloroacetic acid (TCA).

Measure the liberated P_i concentration according to the method of Taussky and Shorr by adding

6.) 1.4 cm^3 of P-reagent.

3.4 cm^3 total volume

After shaking the test tubes, wait 5 min and read the absorbance of the mixtures at 720 nm against a *reagent blank*. The reagent blank is prepared as follows. Please note, that the sequence of addition of the components is important!

1.6 cm^3 5 % TCA,
0.1 cm^3 diluted glycogen phosphorylase *b*,
0.1 cm^3 0.05 M Na-glycerophosphate buffer, pH 6.8,
0.1 cm^3 4 % glycogen,
0.1 cm^3 64 mM glucose-1-P containing 4 mM AMP,
1.4 cm^3 P-reagent.

3.4 cm^3 total volume

DUPress

Read the P_i content of the samples in μg from the P_i calibration curve prepared previously. Calculate glycogen phosphorylase activity of the undiluted glycogen phosphorylase *b* using the following formula:

$$\text{Activity } (\mu\text{kat/cm}^3) = \frac{P_i\,(\mu g)}{31\text{g/mol} \times 0.1\,\text{cm}^3 \times \text{reaction time}\,(s)} \text{ x dilution}$$

Where 31 g/mol is the molar mass of phosphorus, 0.1 cm^3 is the volume of the diluted glycogen phosphorylase *b* in the assay. The dilution factor is given on the container of the enzyme.
Compare the values obtained in the 3 assays. The average activity thus obtained represents the glycogen phosphorylase activity of the undiluted glycogen phosphorylase *b* sample.

Experiment 2. *Effect of glucose-1-P concentration on glycogen phosphorylase activity*

Determine the dependence of phosphorylase activity on the concentration of one of the substrates, glucose-1-P, while the concentration of glycogen and AMP is kept constant. Perform 5 activity assays as described in *Experiment 1* with the following modifications. Instead of 0.1 cm^3 of 64 mM glucose-1-P containing 4 mM AMP use the concentration series of glucose-1-P (each containing 4 mM AMP) given below. The suggested reaction times are also shown in the table.

Test tube number	Concentration of glucose-1-P (mM)		Reaction time (min)
	original	in the assay	
1	8	2	15
2	16	4	15
3	32	8	10
4	64	16	5
5	128	32	5

After completing the reactions, measure the absorbance at 720 nm against reagent blank and calculate the enzyme activity at each substrate concentration. Use the blank prepared in *Experiment 1*.

Sample calculation

0.1 cm^3 of a 100-fold diluted glycogen phosphorylase *b* sample was assayed for glycogen phosphorylase activity for 10 min as described in *Experiment 1*. The amount of the liberated P_i (determined using P_i-calibration curve) was 15 μg. Calculate

a) the activity of the 100-fold diluted glycogen phosphorylase *b* in μkat/cm^3, and

b) the activity of the undiluted glycogen phosphorylase in μkat/cm^3, if M_r = 31 for phosphorus.

a) During 10 minutes

0.1 cm^3 100-fold diluted glycogen phosphorylase *b* catalyzed the liberation of 15 μg phosphorus.

Consequently

1.0 cm^3 100-fold diluted glycogen phosphorylase *b* catalyzes the liberation of

1.0 cm^3/0.1 cm^3x15μg =150 μg phosphorus.

31 g phosphorus corresponds to 1 mole phosphorus

31 μg phosphorus corresponds to 1 μmole phosphorus

150 μg phosphorus corresponds to 150μg /31μg x1μmole = 4.83 μmole phosphorus

Thus; 4.83 μmole phosphorus was liberated during 10 min.

BUPress

One katal is defined as the amount of the enzyme that catalyses the conversion of 1 mole substrate per second under well defined conditions. 1 kat = 1 mol/sec and 1 μkat = 1 μmol/sec

Reaction time: 10 min = 10x60 sec = 600 sec
During 600 sec 4.83 μmole phosphorus was liberated (substrate was converted)
During 1 sec 4.83/600 = 0.008 μmole phosphorus was liberated (substrate was converted)

Consequently the glycogen phosphorylase activity of the 100-fold diluted glycogen phosphorylase *b* is 0.008 $\mu kat/cm^3$.

b) If the activity of 100-fold diluted glycogen phosphorylase *b* is 0.008 $\mu kat/cm^3$, then the activity of undiluted glycogen phosphorylase *b* is 100x0.008 $\mu kat/cm^3$ = 0.8 $\mu kat/cm^3$.

Questions

What are the main properties of an enzyme?

Plot the activity of the enzyme as a function of substrate concentration. Label the Michaelis constant and maximal reaction rate in the plot.

Write the Michaelis-Menten equation and explain the symbols.

What is the meaning of K_M?

What is the meaning of v_{max}?

Define the two main units used for expressing enzyme activity.

What substrate(s) are used for the determination of the activity of glycogen phosphorylase?

How does the concentration of glucose-1-phosphate influence the activity of glycogen phosphorylase?

Exercise

0.1 cm^3 50-fold diluted glycogen phosphorylase *b* sample was assayed as described in the *Sample calculation* for 5, 10, and 15 min. The amounts of the liberated P_i were 21, 44, and 80 μg, respectively. Calculate
a) the activity of the 50-fold diluted glycogen phosphorylase *b* in $\mu kat/cm^3$ at each time point,
b) the average activity from the three experiments
c) the activity of the undiluted glycogen phosphorylase in $\mu kat/cm^3$
d) the activity of the same undiluted enzyme in U/cm^3.

DUPress

Results

Date:

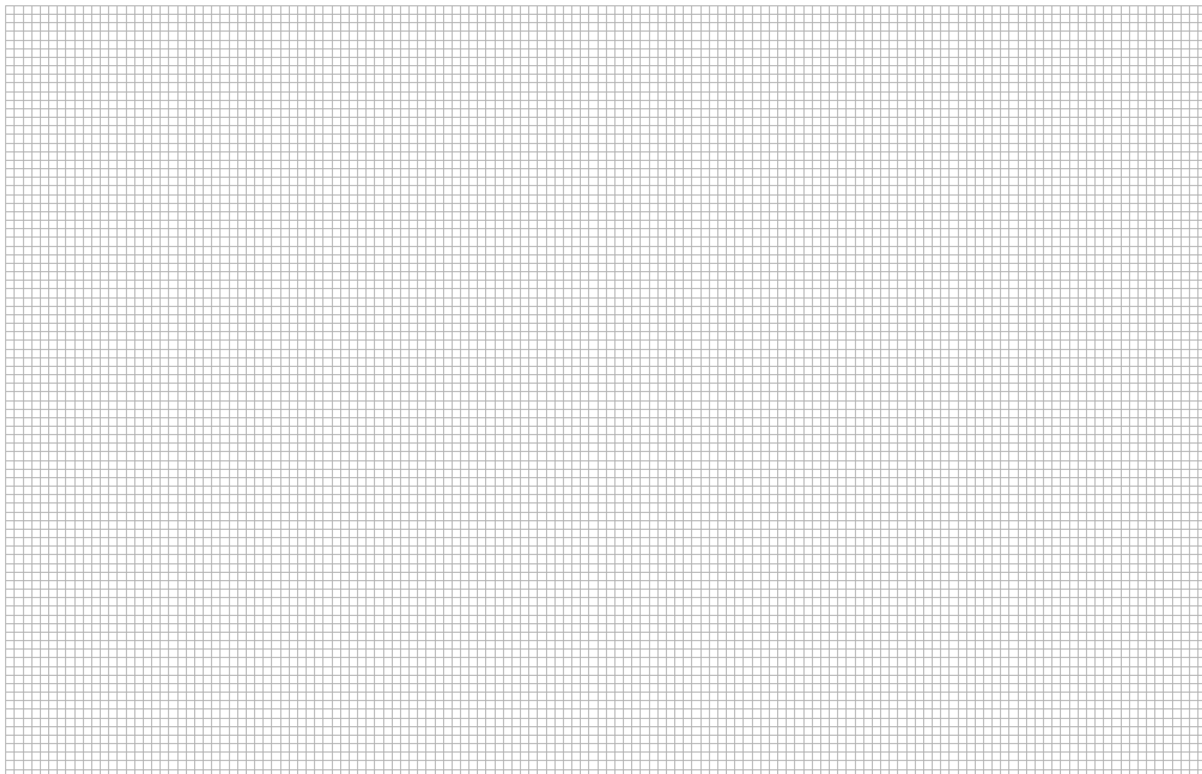
Experiment 1. *Assay of glycogen phosphorylase activity in the presence of AMP*
Record the results of absorbance measurements and calculate the enzyme activity in μkat/cm^3.

Tube	Time (min)	A_{720}	P_i (μg)	μkat/cm^3
1	5			
2	10			
3	15			

Calculate the average activity from the three experiments.

Average activity: μkat/cm^3

Plot the amount of P_i liberated (μg) as a function of reaction time.



Calculate the initial activity of glycogen phosphorylase from the graph, and compare the result with the average activity calculated previously.

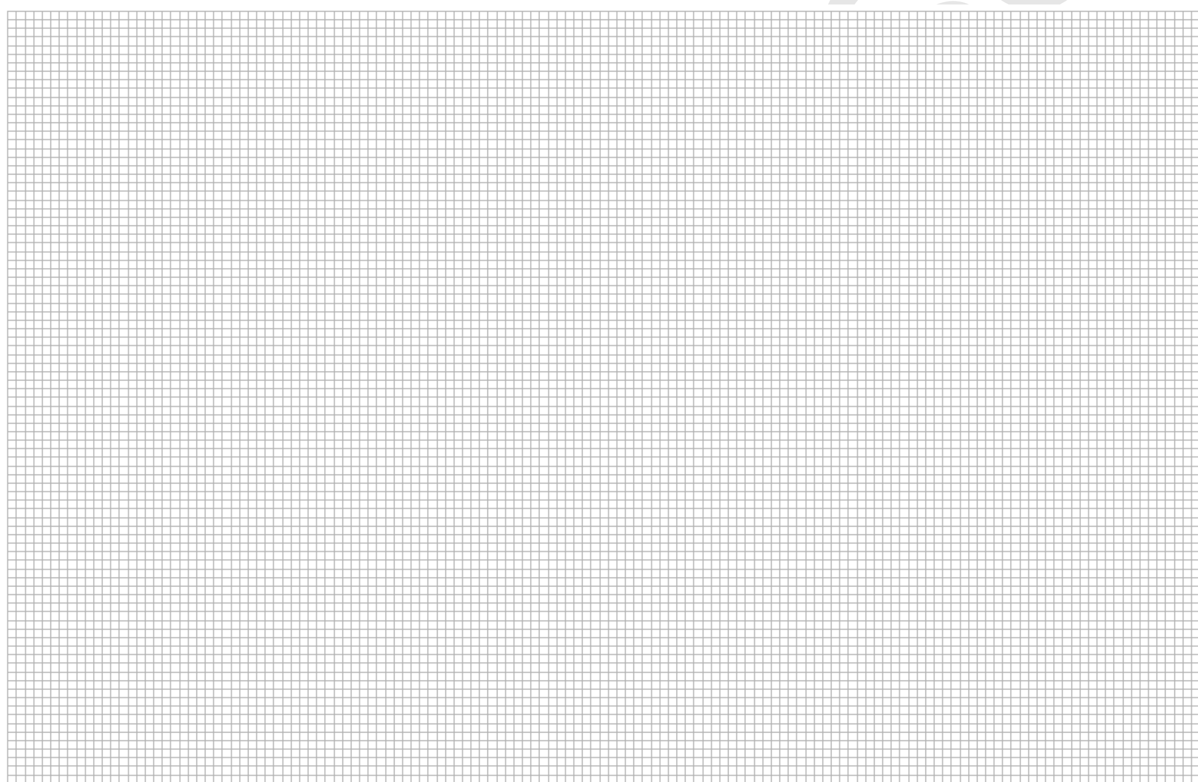
Graphically estimated initial activity: μkat/cm^3

Why does the rate of reaction decline with time?

Experiment 2. *Effect of glucose-1-P [G1P] concentration on glycogen phosphorylase avtivity*
Fill in the table below on the bases of your measurements and calculations.

[G1P] in the assay (mM)	1/[G1P] (1/mM)	Time (min)	A_{720}	P_i (µg)	Activity (µkat/cm^3)	1/activity (cm^3/ µkat)
2		15				
4		15				
8		10				
16		5				
32		5				

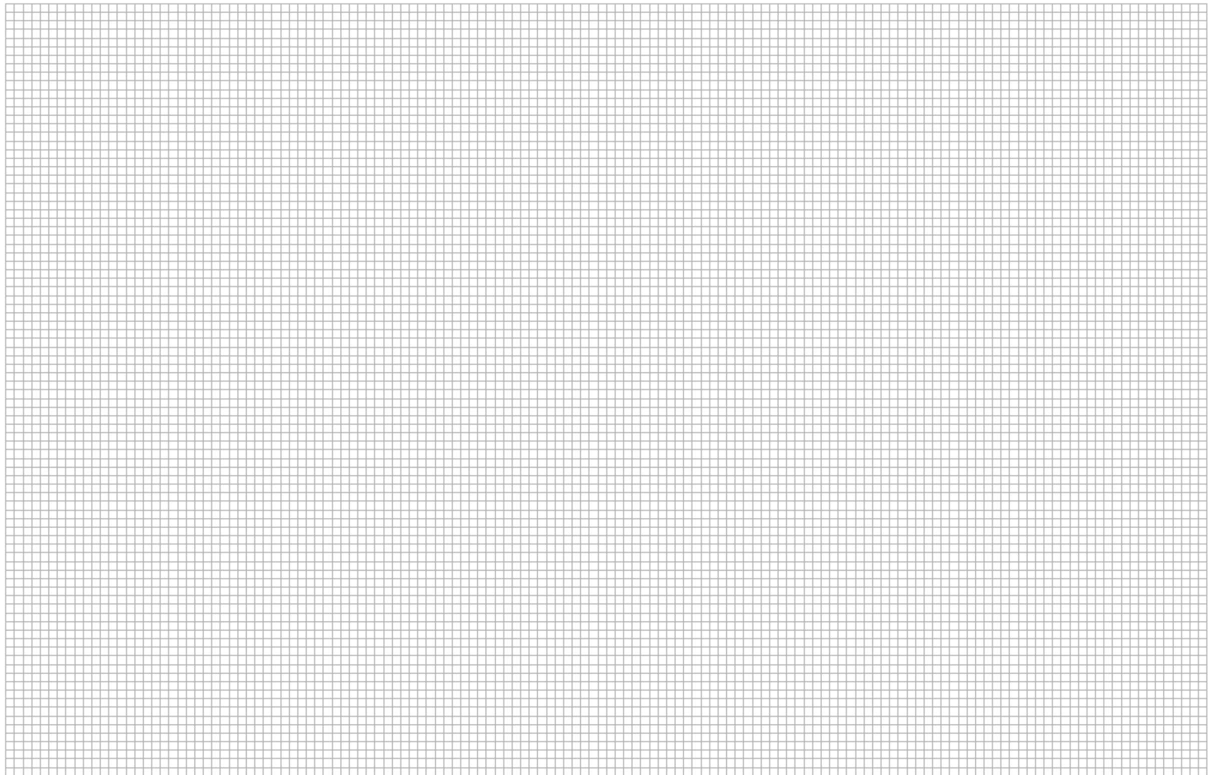
Plot the activity of the enzyme as a function of glucose-1-P concentration.



Estimate the values of v_{max} and K_M from the graph

v_{max}: K_M:

Prepare Linewaver-Burk graph by plotting the reciprocal activity of the enzyme as a function of reciprocal glucose-1-P concentration.



Determine the values of v_{max} and K_M from the Linewaver-Burk plot

v_{max}: K_M:

Compare the estimated values (previous page) with the ones determined according to Linewaver and Burk.

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APPENDIX

DUPress

MEDICAL CHEMISTRY EXPERIMENTS

Week	Date	Title of experiment	Signature of supervisor
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
Name: **Group number:**			

DUPress

CONTENTS

Preface 3

Laboratory safety instructions and fire regulations 4

Laboratory techniques 6

Qualitative analysis 15
Chemical analysis of drinking-water 15
Analysis of inorganic salts and complexes 21
Analysis of mono- and disaccharides 24
Quantitative analysis 30
Volumetric analysis 30
Acid-base titrations 30
Strong acid-strong base titrations 35
Weak acid-strong base titrations 40
Redox titrations 46
Iodometric titrations 47
Titration with potassium bromate 54
Complexometric titrations 58
Instrumental methods of chemical analysis 67
Spectrophotometry 67
The spectrophotometer 70
Photometric determination of iorganic phosphate 72
Determination of acid labile phosphate in organic compounds 75
Photometric determination of iron 78
Quantitative protein analysis 84
Assay of glucose 88
Polarimetry 91
Polarimetric analysis of carbohydrates 92
Electrometry 97
Electrometric pH measurement 97
Chromatography 106
Paper chromatography 108
Ion exchange chromatography 114
Gel filtration 121
Reactions kinetics 124
Kinetic study of the saponification reaction of ethyl acetate
125
Kinetic analysis of the oxidation of iodide ion using the Landolt-method 129
Enzyme kinetics 132
Assay of glycogen phosphatase activity 135

Appendix 141

DUPress

DUPress