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Wrocław University of Technology

Medicinal Chemistry

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SYNTHETIC ORGANIC DRUGS LABORATORY

DRUG ANALYSIS

Wrocław 2011

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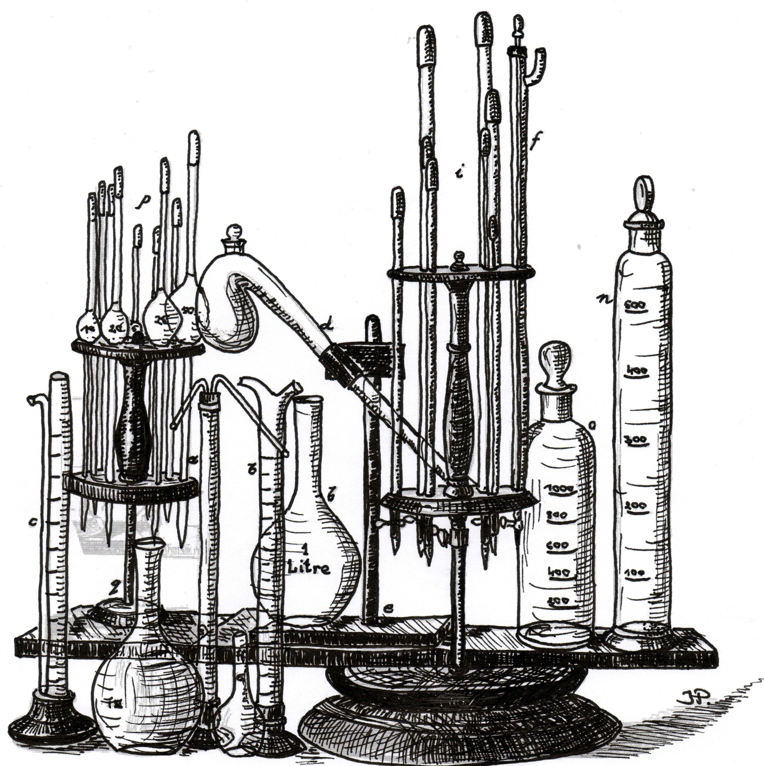

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Preface

"Synthetic Organic Drugs Laboratory" is the new version of the book "Chemia Organiczna. Chemia Leków Laboratorium. Analiza Substancji Organicznych", which was written in Polish, and printed in 2009. There was a real need to write a book in English, not only for students of Medicinal Chemistry specialization, in Chemistry Department, but also for abroad students, visiting Wrocław University of Technology. This book is intended for students who want to know more about identification methods of organic compounds, also of synthetic organic drugs. It is possible to find here some bases of an identification process of an unknown organic compound, like preliminary examination procedures, what is described in chapter no 2, as well as separation techniques, helpful to receive a pure compound, which will be ready to identify using the reactions of functional groups detection – chapter no 3. We decided to change the chapter no 4, about chemical characterization of functional groups in the analyzed substances. The new version of this chapter contains easy to do in a small scale procedures, with schemes of the reactions, and the descriptions of the expected results. We decided not to write about spectroscopic data of organic compounds. These information are easy to find in the other students book, of the same printing series. Chapter no 5 contains the instructions of the course for students of Medicinal Chemistry specialization – "Synthetic Organic Drugs – Laboratory". Nine various experiments describe how to analyze, qualitatively as well as quantitatively, the biologically active compounds of some popular medicines, possible to buy without a prescription. There are procedures of isolation these synthetic organic substances from different forms of medicines: from a tablet, from an ointment, and from a liquid form like drops or a suspension. This book is also dedicated for students attending at the different organic chemistry courses like laboratories, exercises as well as lectures, also natural product chemistry courses. We hope that information and the advices in this book will be helpful many times.

Izabela Pawlaczyk

Roman Gancarz

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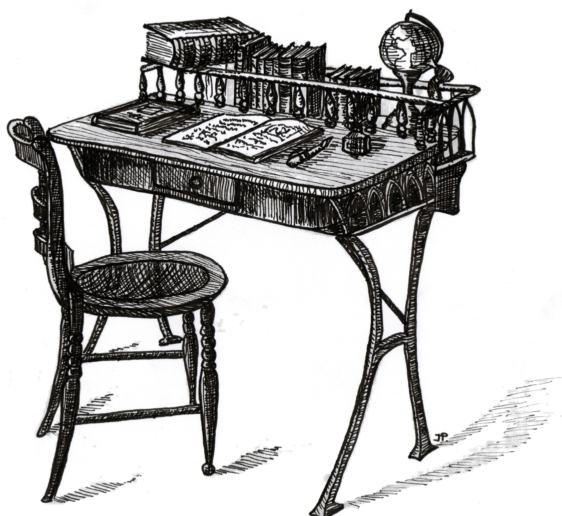


1

Safety Rules in the Organic Chemistry Laboratory

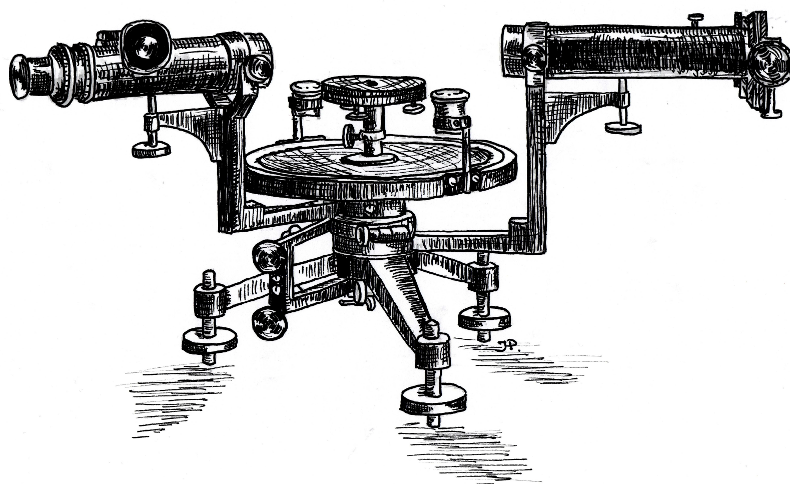
The standards establish the borders of behavior in a laboratory of chemistry there are **safety rules** and **care for health**. To realize these aims it is necessary to obey **the rules of law** and **safety principles**. They are described in many legislation and regulation acts of Polish Law as well as in European Union directives.
Working in a chemistry laboratory is connected with a potential danger, but the probability and consequences of an accident could be reduced to the minimum, if the basic precautions, great responsibility and deliberation are preserved.

There are main rules which must be obey in an organic chemistry laboratory makes it far less dangerous:

- Before start working it is obligatory to know the statute of the laboratory.
- Each student is obligated to know the current safety rules, what will be confirmed with personal signature on a declaration of compliance.
- Only students participated in the tutorials, the teacher and technical support are entitled to be in the laboratory.
- During the laboratories it is obligatory to keep calm and not to assemble because of do not expose yourself and the others to danger.
- It is necessary to be careful during tutorials, and in case of an accident it is very important to report the teacher about it as quickly as possible.
- Protective clothing – apron or lab coat, must be appropriate to work in the laboratory, made of natural based fiber such as cotton, to button up in front. Goggles or safety glasses must be worn all the time. When it is necessary, safety gloves also should be worn. Opened-toes shoes should not be worn. Long hair should be tied back or covered, especially in the vicinity of open flame. Jewelry that might a present hazard, such as dangling necklaces, chains, medallions or bracelets should not be worn in the laboratory. Make-up cosmetics could be the cause of some skin allergies, when their compounds react with some chemicals.
- Eating, drinking nor smoking in a laboratory of chemistry is strictly prohibited. Testing nor smelling any chemical as well.
- Students are responsible for keeping the place of working in order. Work area must be kept clean and tidy all the time. Only lab manuals and notebooks may be brought to the work area. It is necessary to use only the proper instrument for handling apparatus or other equipment. Student is obligatory to keep clean the glassware, to check it before the experiment, not to use broken, chipped or cracked glassware, and to clean it up and dry after.
- Sharp and contaminated waste should be placed in the appropriate place or container. Reagents are never poured down the sink.
- Working alone is strictly prohibited. In case of an accident no one will able to help.
- Before start an experiment students are encouraged to obtain the physical and chemical properties of the chemicals used to the experiment as well as these received as semi-products, products and wastes. The safety, health, and fire precautions are the most important information to locate.
- Every chemicals used in the laboratory must have labels. It is necessary to read and double check the information about the reagent before removing it, especially if it is dangerous. Returning unused chemical to stock bottles nor to the original jars is prohibited.
- The hazardous experiments with compounds which are particularly dangerous must be provided in a fume-hood area.
- The work area must be clean up after the experiment. It is very important to wash hands with soap and water during and especially after all lab activities.
- All accidents should be reported.

The users of chemicals, also students, who are working in the organic chemistry laboratory are **strictly obligated**:

- **to know and obey** the law in force and safety rules,
- **to prevent** the hazardous situations,
- to apply the **material safety data sheets** of the dangerous compounds,
- **to give first aid** an injured person in an accident.



2

Identification Techniques of Organic Compounds

Each unknown compound, especially an organic substance could be characterized using simple or/and advanced techniques of identification. It is important to determine such elements as:

- physical properties: state, colour, odour, pH value, melting point, boiling point, refraction coefficient for liquids, density or viscosity, solubility in different solvents,
- ignition test results,
- molecular formula in elemental analysis method,
- functional groups present in an analyzed structure identified in some characteristic reactions,
- advanced structural analysis by spectroscopic methods like infrared spectroscopy (FT-IR), nuclear magnetic resonance analysis (NMR), mass spectroscopy techniques (MS), UV/Vis spectrophotometry, as well as chromatographic techniques, i.e. thin layer chromatography (TLC), high performance liquid chromatography (HPLC), or gas chromatography (GC).

On the basis of this information it is possible to propose a chemical structure which should be verified in comparison with some reference data.

2.1. Preliminary Examination

2.1.1. Physical State

Physical state of an analyzed compound, its phase, may give a lot of important information, to reduce the range of search. To be sure that the analyzed substance is pure, free from some contaminations, it is good to purify it. Liquids are usually purified by distillation or by gas chromatography, whereas solids are purified by recrystallization or by sublimation.

2.1.2. Colour

The colour of the analyzed compound should be determined after the purification process. The colour is sometimes changed by impurities, frequently produced by the slow oxidation process of the organic substance in the air. Aniline is a good example here. It is a colourless liquid in room temperature when freshly distilled, but in time it becomes yellow, orange and then even reddish brown.
Lots of organic compounds have a defined colour because of chromophoric groups in their structures. Many nitro compounds, quinones, azo compounds, stable carbocations, carbanions, and complex substances have colour. In conclusion, if an analyzed, purified substance is a stable, colourless liquid or white crystalline solid, it probably contains a/no? chromophoric functional group. If a compound becomes colourful after slow oxidation in the air, its oxidation products became chromophoric residue? in this reaction.

2.1.3. Odour

The odour of an identified compound gives very important information. However, in this examination it is important to be careful because of the toxicity of many organic substances. It is not recommended to check the odour by direct inhalation.
Lots of organic compounds have smell characteristic for some functional groups present in their structures, i.e. homologues of lower esters, ketones, aldehydes, alcohols, nitriles, aliphatic hydrocarbons, aromatic hydrocarbons, phenols. This typical odour may be helpful in the identification process.
Amines usually have a distinctly fishy smell and thus they are easily identified. Thiols, named also mercaptans, and thioesters – sulfides, smell like rotten egg or hydrogen sulfide. Carboxylic acids with low molecular weight are usually unpleasant and pungent in their odour, i.e. acetic acid is a strong vinegar acidic, and *iso*-butyric acid smells like rancid butter. On the other hand esters are generally pleasant and smell fruity. A good example is a banana aroma of *iso*-amyl acetate. Hydrocarbons have very different smells. Naphthalene because of its odour, which is unpleasant for clothes moths, is used as mothballs in wardrobes and drawers. Pinenes are components of turpentine, and benzaldehyde, nitrobenzene and benzonitrile all have odors frequently described as “cherry-like” or “bitter almonds”.
The theory of the smell of some organic compounds is closely connected to its stereochemical conformation. Carvone is a good example to confirm this theory: stereoisomer (+) is the main compound of caraway and dill seeds, whereas (–) form is the main component of spearmint.

2.1.4. Ignition Test

The ignition test allows for a preliminary classification of the analyzed organic compounds. Many of them burn with a characteristic flame that helps to determine the nature of the compound. The result of this test may give the information like strong oxidation properties of the compound, its explosive nature (sudden burning), the presence of a big amount of carbon atoms in the hydrocarbon structure, when soot remains after ignition, the presence of heteroatoms, especially in halide compounds, when the substance is dies down after taking it out from fire. When the analyzed compound is burning up with yellow smoky flame, it is a sign that the substance contains aromatic rings. More bluish flame indicates the presence of a big amount of oxygen atoms.
The ignition test should be made by burning of 0.02 – 0.10 g of the analyzed substance on a metal spoon or a platinum rod. At the beginning it should be heated slowly, to enable observation: if the compound melts easily, if it is flammable, if water is emited, and if the residue stays after ignition.

2.2.Physical Properties

A very important element of the identification analysis, very helpful in excluding the risk of a mistake, is the estimation of melting temperature and/or boiling temperature. Even some small amounts of contaminations in the analyzed sample may change the final results.

2.2.1. Melting Point

The melting point of an analyzed substance is the range of temperatures at which under the atmospheric pressure the compound in solid phase is changing to a liquid form.
For melting point determination, many instruments based on melting the analyzed substance in a capillary tube are available. There are apparatuses like Thomas-Hoover melting point apparatus, a Thiele tube, or Mel-Temp melting point instrument. They are simple to use. Another type of instruments are Fisher-Johns apparatus or Boëtius instrument. They have copper or aluminum heating plate with temperature regulation fitted with a thermometer. The plate with the melting substance is observed under a microscope with the help of the illuminator through a magnifying glass. The heating table has a growing temperature regulation.
Usually, the speed of heating of a melting substance is not faster than 2 – 4 °C per minute. As the melting point is obtained the range of temperatures is such that the borders of the observed solid state substance start to melt till the moment when the substance is melted totally.
It is recommended to repeat this experiment a few times to receive the average precise final result. The more contaminations in the analyzed compound, the wider the range of melting temperature measurements. Therefore the analyzed compound may be confirmed as chemically pure if after the repeating of the purification process the result of melting point does not change and is the same.

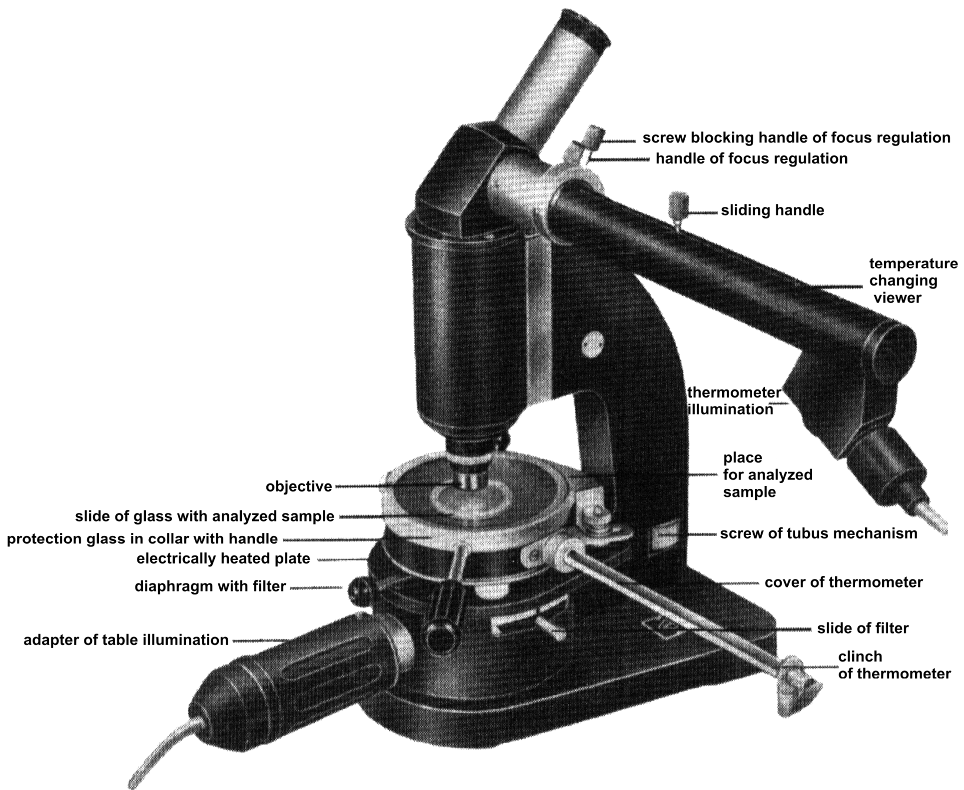


Fig. 2.2.1. Boëtius instrument (according to the instruction manual of Boëtius apparatus, Franz Küstner Nachf. KG, Dresden).

2.2.2. Boiling Temperature

The boiling point is the temperature or the range of temperature at which a substance changes its state from liquid to gas. A stricter definition of the boiling point is the temperature at which the liquid and vapour phases of a substance can exist in equilibrium. When heat is applied to a liquid, the temperature of the liquid rises until the vapour pressure of the liquid equals the pressure of the surrounding gases.

The boiling point of a liquid is lowered if the pressure of the surrounding gases is decreased. For example, water will boil at a lower temperature at the top of a mountain, where the atmospheric pressure on the water is smaller, than it will at sea level, where the pressure is greater. On the other hand, if the pressure is increased, the boiling point is raised. For this reason, it is customary when the boiling point of a substance is given to include the pressure at which it is observed, if that pressure is other than standard, i.e., 760 mm of mercury or 1 atmosphere equal to 1013 hPa. The boiling temperature under the atmospheric pressure is called **normal temperature**.

Usually the boiling point is determined by the measurement of the temperature of a boiling substance using a thermometer. A more proper method is to determine this parameter by the distillation under the atmospheric pressure, with the accuracy of 1 – 2 °C.
When the pressure of gas phase above the boiling liquid phase is reduced, the boiling temperature value is reduced as well, in accordance with Clausius – Clapeyron formula, which after some conversions forms the following dependence:

$$\ln p = \frac{L_V}{RT} + C,$$

where:

p – vapour tension above the liquid phase,
L_V – molar heat of evaporation,
T – absolute temperature,
R – gas constant.

Such property of liquids is helpful in the distillation process under the reduced pressure. When the substance could be decomposed or become self-burned before achieving the normal boiling temperature, the reduction of the pressure above the solution reduces boiling temperature, and then the effective safety distillation of the substance is possible.

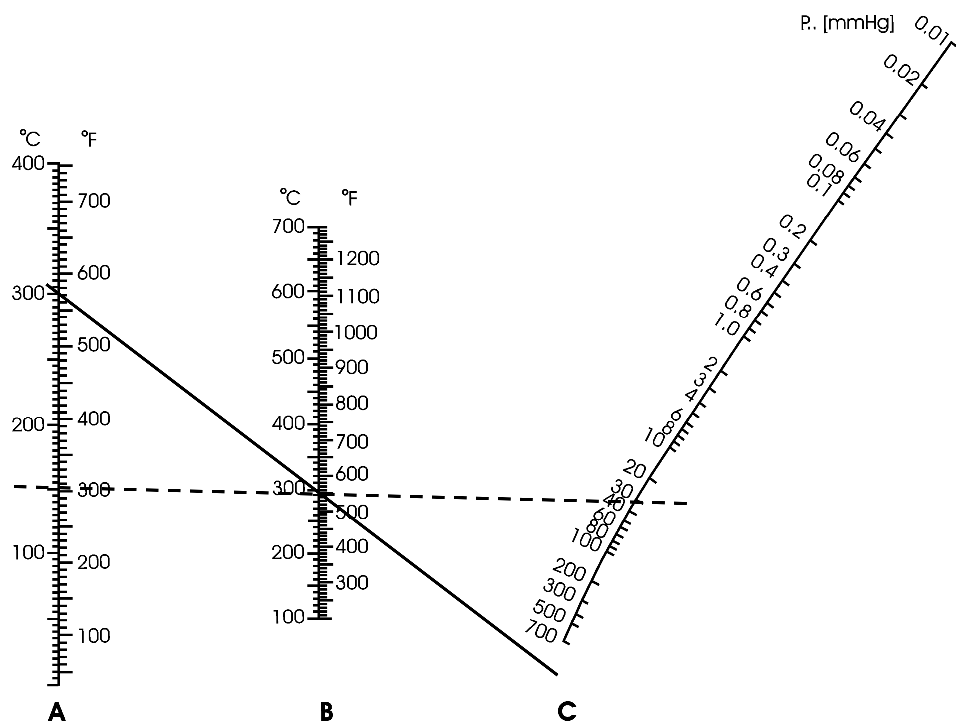


Fig. 2.2.2. The dependence diagram of temperature on pressure to establish the condition of distillation under the reduced pressure. **A** –boiling temperature axis of liquid under the reduced pressure, **B** – boiling temperature axis of liquid under atmospheric pressure (760 mm Hg), **C** – pressure changing curve (mm Hg).

The above diagram is helpful to establish the proper pressure to achieve the adequate temperature. The reduced boiling temperature should be marked on **A** axis, whereas the boiling temperature in normal pressure condition should be marked on **B** axis. Thereafter a straight line should be drawn between these two points (dashed line), and lengthened to be able to cross **C** curve. This point of crossing **C** curve is the pressure value [mm Hg] needed to be kept, not to exceed the established boiling temperature.

2.2.3. Density

Density, as a value describing the proportion of a mass of an examined substance to its volume, is an important parameter in the identification of the substance and in the evaluation of its chemical purity.
The device used to estimate the density of some liquid is an aerometer. Its working rules are based on the Archimedes principle that a solid suspended in a fluid will be buoyed up by a force equal to the weight of the fluid displaced. Pycnometer, sometimes called pyknometer, usually containing a thermometer, is also used to determine the density of a liquid. A pycnometer is made of glass, with a close-fitting ground glass stopper with a capillary tube through it.
The measurement is based on the estimation of mass of the analyzed liquid in a compartment with the mass of a standard liquid, when both of them occupy the same volume of pycnometer, in the same temperature. Usually water is used as a standard liquid, and the measurement is at 20 °C. This so-called **specific density** of a liquid is described by the formula:

$$d_{20}^{20} = \frac{m_p}{m_w},$$

where:
m_p – mass of analyzed substance,
m_w – mass of standard liquid.

For this measurement the analyzed sample must be pure. Sometimes it is necessary to determine the density with reference to water at the temperature of 4 °C. Then the formula must be created by means of the factor 0.99823:

$$d_{4}^{20} = \frac{m_p}{m_w} \times 0.99823.$$

The density of solid state compounds in organic chemistry laboratory is estimated infrequently.

2.2.4. Refraction Index of Liquids

The refraction index (n) describes the optic rotation parameter of the analyzed compound dissolved in the liquid phase. The index of refraction of a liquid is equal to the ratio of sine of the incidence angle of a light ray in air to sine of the refraction angle in the liquid phase:

$$n = \frac{v_1}{v_2} = \frac{\sin\alpha}{\sin\beta},$$

where:
ν_1 – speed of diffusion of the light in medium 1,
ν_2 – speed of diffusion of the light in medium 2,
α - angle of the light incidence,
β - angle of the light refraction.

The value of the refraction index of organic compounds oscillate between 1.3 and 1.8, and when the tested substance is chemically pure it could be important information in the process of identification of this compound. On the basis of this measurement it is possible to assess the level of purity of the analyzed substance.

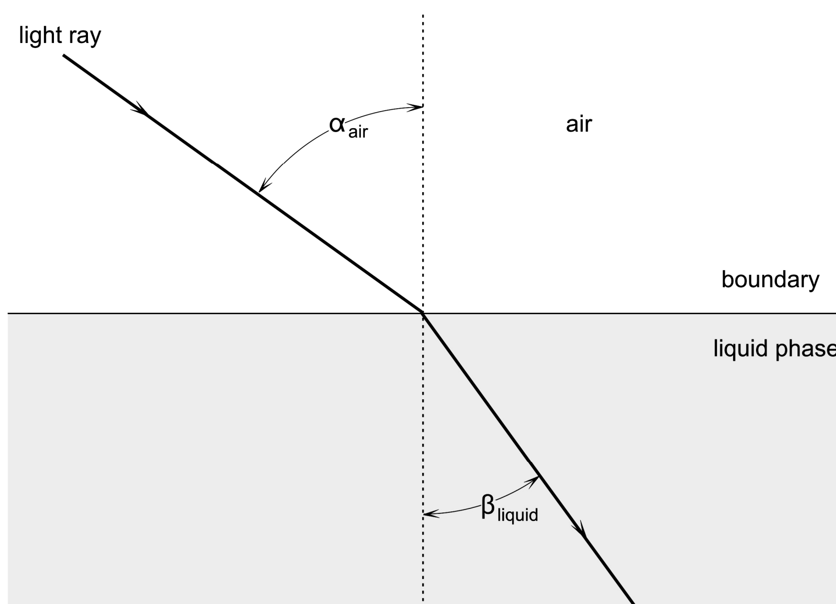


Fig. 2.2.4. Refraction of light ray after crossing the border of two different centres.

The measurement of the refraction index is possible using a refractrometer, i.e. Abbe refractometer. This apparatus contains two high quality glass prisms, between them the liquid form of the analyzed compound in the form of a thin film is brought in. Through these three media a light ray runs and parts the field of view into two areas – the light and the dark one. The border of the division of these fields is estimated by a boundary angle – the β angle of the light refraction. It is necessary to do the measurement in a precise temperature of the environment. The typical temperature is 20 °C.

2.2.5. Solubility of Organic Compounds

The solubility tests of organic compounds are a very helpful step in the identification process. There are two main categories of solubility: when a chemical reaction is the driving force of this process and the solubility where simple miscibility is the only mechanism involved.

Table. 2.2.1. The solubility classes of organic compounds determined in water, acids, bases and ether

Solubility class	Soluble in:	Not soluble in:	Types of organic compounds
S_1	water (litmus is unchanged) diethyl ether		Monofunctional alcohols, aldehydes, ketones, esters, nitriles, and amides with five carbon atoms or fewer.
S_A	water (litmus is red) diethyl ether		Monofunctional carboxylic acids with five carbon atoms or fewer, arylsulfonic acids.
S_B	water (litmus is blue) diethyl ether		Monofunctional amines with six carbon atoms or fewer.
S_2	water	diethyl ether	Salts of organic acids, also sodium salts, amine hydrochlorides, aminoacids, polyfunctional compounds with hydrophilic functional groups: carbohydrates, polyhydroxy compounds, polybasic acids etc.
A_1	5% NaOH 5% $NaHCO_3$	water	Strong organic acids: carboxylic acids with more than six carbon atoms, phenols with electron-withdrawing groups in *ortho* and/or *para* position(s); 1,3-diketones.
A_2	5% NaOH	water 5% $NaHCO_3$	Weak organic acids: phenols, enols, oximes, imides, sulfonamides, thiophenols, all with more than five carbon atoms; 1,3-diketones; nitro compounds with α-hydrogens.
B	5% HCl	water 5% NaOH	Aliphatic amines with eight or more carbon atoms; anilines (only one phenyl group attached to nitrogen); some ethers.
MN		water 5% NaOH	Miscellaneous neutral compounds containing nitrogen or sulfur and having more than five carbon atoms.
N	96% H_2SO_4	water 5% NaOH 5% HCl	Alcohols, aldehydes, ketones, esters with one functional group and more than five, but fewer than nine carbon atoms, ethers, epoxides, alkenes, alkynes, some aromatic compounds (especially those with activating groups).
I		water 5% NaOH 5% HCl 96% H_2SO_4	Saturated hydrocarbons, haloalkanes, aryl halides, other deactivated aromatic compounds, diaryl ethers.

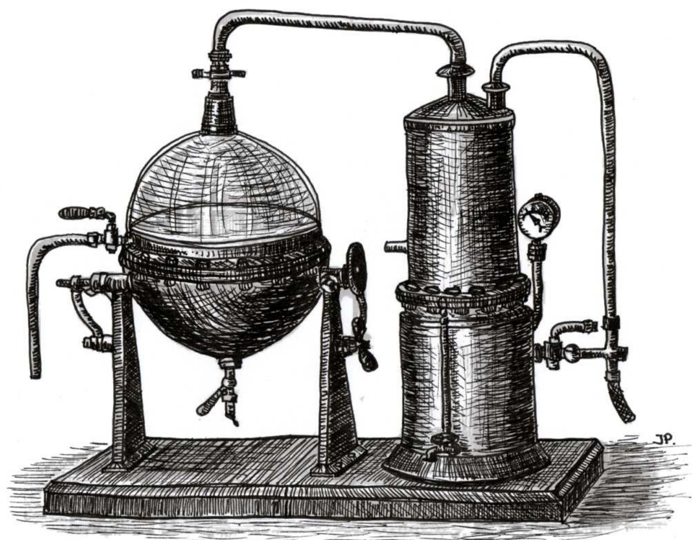
By solubilization it is possible to obtain three kinds of infomation. The presence of a functional group is usually indicated. Solubility in different solvents could give more specific information about the functional group. Finally, certain deductions about molecular size and composition may sometimes be made. The above table describes the main classes of solubility of organic compounds.
It is necessary to emphasize that some compounds with long aliphatic chains, with many condensed aromatic rests/residues?, or contain some attachments with big molecular size would give ambiguous results in the solubility tests.

2.3. Elemental Analysis

Organic compounds are mainly built from atoms of carbon, hydrogen and oxygen. However they also contain other elements such as nitrogen, sulfur, fluorine, chlorine, bromine and iodine, which are detected, by means of chemical tests, usually straightforward ones. Elemental analysis is routinely applied in the confirmation of unknown compounds. Firstly, the unknown sample must be checked for purity by thin-layer chromatography and/or gas chromatography after it has been recrystallized or distilled. The unknown can be dried to remove residual solvents.
Usually 5 mg of a sample is enough for carbon and hydrogen analysis, and another 5 mg to analyze atoms of sulfur, halogen or deuterium. Generally oxygen atoms are not analyzed in elemental analysis, and the percentage of them is normally obtained by the difference in the percentages of the other atoms.
The empirical formula can be determined by combustion, if the molecular formula is unknown prior to the analysis. Two methods are used in order to determine the amounts of carbon, hydrogen and nitrogen atoms in an analyzed sample. In the first method the sample is combusted in pure oxygen. The products are passed in a stream of helium gas over suitable reagents to completely oxidize these products: carbon dioxide from carbon atoms, water from hydrogen, nitrogen oxides from nitrogen atoms, and sulfur dioxide from sulfur atoms. The resulting mixture is swept over three thermal conductivity detectors with the adsorption traps. Another technique is based on the separation of the combustion products from an oxidation/reduction reactor, on a gas chromatograph connected to a thermal conductivity detector. To determine the amounts of halogens present in the sample, it is combusted in a Söniger micro combustion flask. The products of combustion are titrated with mercuric nitrate.

2.4. Spectrometric Techniques

The organic compounds can be quickly analyzed by spectrometric methods such as nuclear magnetic resonance (NMR), infrared spectrometry (IR) or mass spectrometry. The main advantages are: small amount of the sample, short time of analysis and very precise result. NMR and IR methods are crucial to the structure determination. In NMR spectrometry, the relationship of carbon atoms and hydrogen atoms to each other is determined mostly using ^{1}H NMR, ^{13}C NMR but also two-dimensional techniques like COSY, NOESY, ROESY methods. IR spectrometry helps in the identification of functional groups present in the analyzed structure. Mass spectrometry is less important. The main condition is that the analyzed sample must be in a gaseous state. The compound may be ionized in several ways and it is detected to give mass spectrum – characteristic for the analyzed compound, with the main information about the molecular mass.



3

Separation Techniques

The identification process of some substances in a mixture involves separating them at first and then characterizing each of them. There are a number of separation techniques frequently used by organic chemists. Before choosing the separation method, preliminary tests should be carried out. Two things are most important. The mixture should survive the separation procedure and its components should be stable under the conditions of this process. The used separation method should be the easiest and most efficient one. The most important separation techniques:

- extraction procedures,
- precipitation, crystallization,
- adsorption, absorption,
- distillation, evaporation,
- filtration, also dialysis,
- centrifugation,
- electrophoresis,
- chromatographic techniques such as: thin layer chromatography (TLC), liquid chromatography (LC), ion-exchange chromatography (IEC), high performance liquid chromatography (HPLC), gas chromatography (GC).

3.1. Distillation

The distillation technique is a useful method of separation of some components, based on differences in their volatilities in a boiling liquid mixture. There are many distillation methods, depending of the character of the mixture. The most popular device used in

distillation consists of a flask in which the source material is usually heated, Cleisen adapter with a thermometer, a condenser in which the heated vapour is cooled back to the liquid state, i.e. Liebig condenser, and a receiver in which the concentrated or purified liquid, called the distillate, is collected.
There are different types of distillation processes.

The **simple distillation** (Fig. 3.1.1. A) is based on cooling and condensation all hot vapours immediately, without some special conditions. Therefore, the distillate is usually not very pure, and is contaminated with other compounds of the separated mixture. This technique is useful only to separate liquids whose boiling points differ greatly, or to separate liquids from nonvolatile solids. The simple distillation is also used to separate and preliminarily purify the components of the mixture.
Short-path distillation (Fig. 3.1.1. B) is a distillation technique similar to the simple distillation, sometimes under reduced pressure, but it involves the distillate travelling a short distance, usually without the need for a condenser, often only a few centimetres. This technique is often used for unstable compounds at high temperatures or to purify small amounts of a compound.

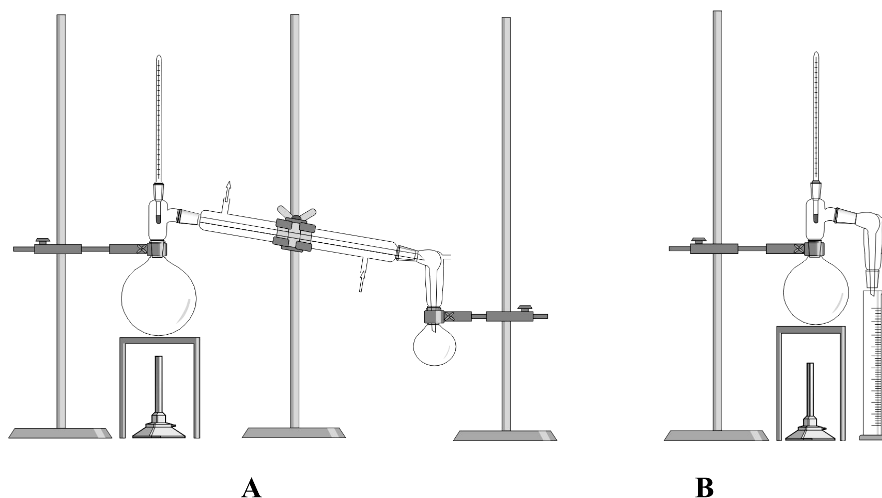


Fig. 3.1.1. The distillation techniques: A – the simple distillation device, B – the short-path distillation scheme.

For distilling the heat-sensitive compounds the **steam distillation** (Fig. 3.1.2. A) is the proper method, to purify them by co-distilling them with water. This process involves bubbling steam through a heated mixture. Water is added to the heating mixture continuously in a steam form, or alternatively – in small portions, drop wise using a glass dropper. Some of the target compounds are able to vaporize, in accordance with their partial pressure. The vapour mixture is cooled and condensed, usually yielding a layer of oil and a layer of water. The steam distillation is a useful process of isolating the essential oils from the plant materials in perfumery production, water distillates have many applications in aromatherapy, food processing and cosmetics. Some modification of this process is the **distillation under gas stream** (Fig. 3.1.2.B).

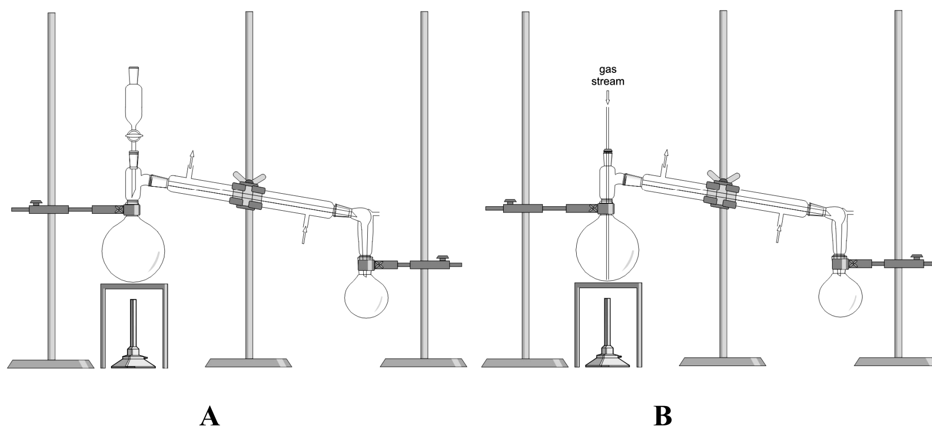


Fig. 3.1.2. The distillation devices: A – the steam distillation scheme, B – the distillation under gas stream.

Distillation of some compounds with very high boiling points, and relatively low flashpoints is dangerous. To be able to boil such compounds safely, it is often better to reduce the pressure at which such compounds are boiled at much lower temperature, lower than their ignition temperature. This technique is referred to as **vacuum distillation** or **distillation under the reduced pressure** (Fig. 3.1.3.), and it is commonly used in the laboratory during the process of evaporation of solvents on a rotary evaporator.

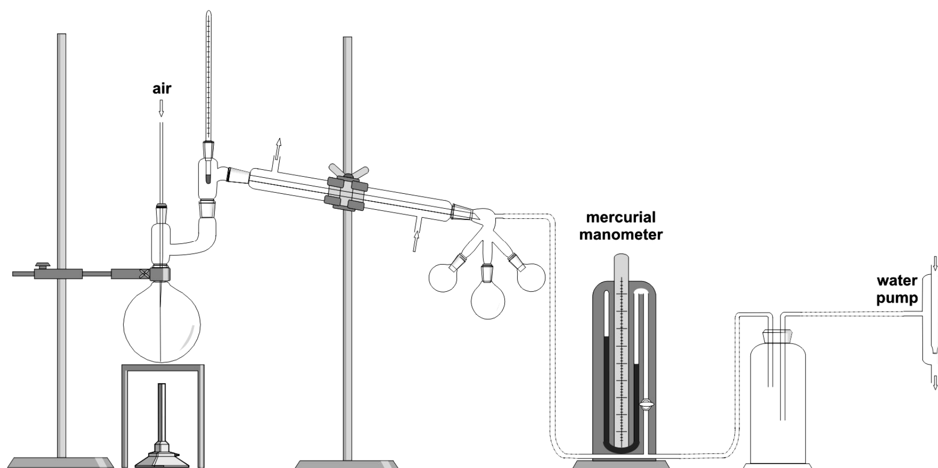


Fig. 3.1.3. The device used for the distillation under the reduced pressure.
The boiling mixture is a little bit bubbled by air to prevent overheating the boiling mixture.

The **fractional distillation** (Fig. 3.1.4. A) is used in order to separate the components well by repeated vaporization-condensation cycles within a packed fractionating column. This separation, by successive distillations, is also called **rectification**, and requires a special type of the condenser, usually cooled by air – the fractionating column. As it rises, it cools, condensing on the condenser walls and the surfaces of the packing material or on the spikes if there is the glass column of Vigreaux. The condensate continues to be heated by the rising hot

vapours, and it vaporizes once more. Each vaporization – condensation cycle, called a theoretical plate, yields purer distillate containing more volatile components. More theoretical plates lead to better separation.
Interactions between the components of the boiling mixture can sometimes result in a constant-boiling **azeotrope** which behaves as if it were a pure compound. It boils at a single temperature instead of a range and the solution contains the given components in the same proportion as the vapour – simple distillation does not change the purity of the distillate, so it does not affect the separation. For example, ethyl alcohol and water form an azeotrope of 95.63 % at 78.2 °C. There exists some technique to break the azeotrope to give a pure distillate, and it is known as the **azeotropic distillation** (Fig. 3.1.4. B). The azeotrope is easily broken in a distillation set-up by using a liquid-liquid separator, i.e. a decanter to separate the two liquid layers that are condensed overhead. Only one of the two liquid layers is refluxed to the distillation set-up.

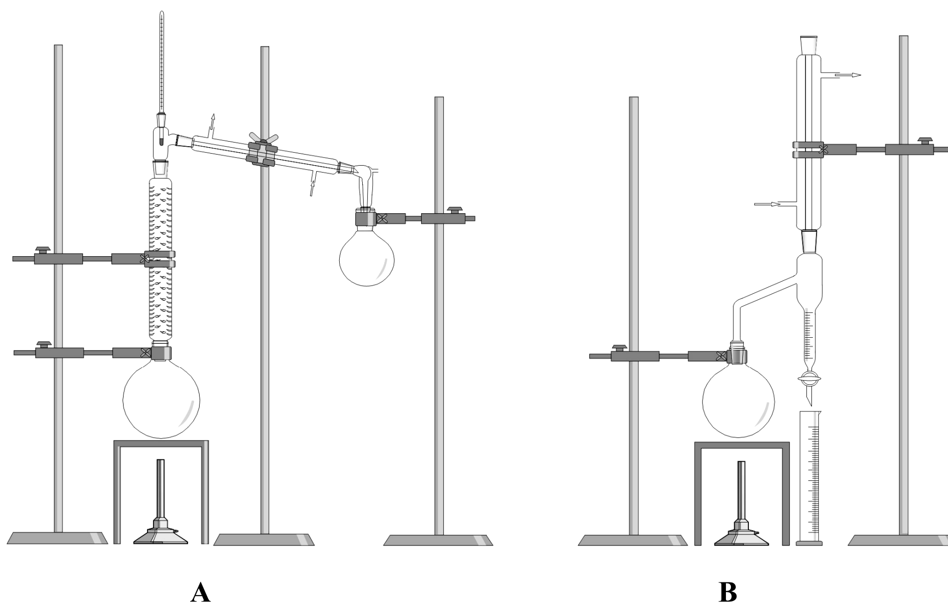


Fig. 3.1.4. The distillation schemes: A – the fractional distillation using Vigreaux column, B – the azeotropic distillation.

3.2. Sublimation

Sublimation is the transition of a substance from the solid phase to the gas phase without passing through an intermediate liquid phase. This is an endothermic process that occurs at high temperature, and the reduced pressure is advantageous. The reverse process to sublimation is **deposition**. Sublimation is a useful technique to purify solid state compounds. Typically a contaminated solid is placed into a sublimation apparatus and heated under vacuum. Under this reduced pressure the solid volatilizes and condenses as a purified solid state compound on a cooled surface, i.e. a cold finger, leaving a non-volatile residue of impurities behind. Once heating ceases and the vacuum is removed, the purified compound

may be collected from the cooling surface. In this process of purification it is necessary to heat the contaminated solid at the temperature lower than the melting points of the compounds of the mixture, not to melt them before sublimating the pure compound.

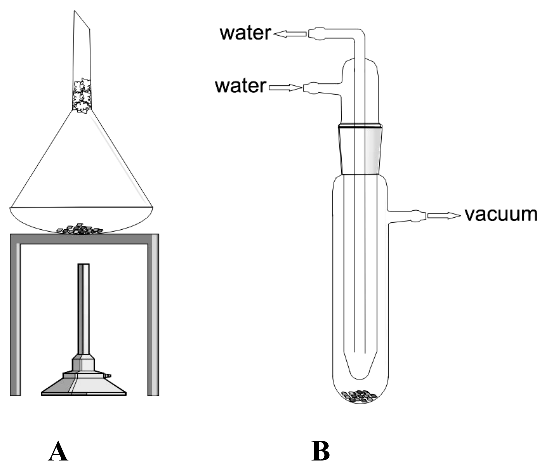


Fig. 3.2.1. The sublimation and deposition processes used for purification of a substance:
A – sublimation from a glass dish covered by a glass funnel and deposition of a pure substance in a narrow stem,
B – vacuum sublimation in the cold finger, cooled by circulated water.

3.3. Extraction

Extraction is the process of passage of a substance from a solid or liquid mixture to a liquid solvent. The solvent is not or only partially miscible with the solid or the liquid mixture containing the substance. By intensive contact the extracted compound transfers from the solid or liquid mixture into the solvent (extract). After mixing, these two phases are separated which happens either by gravity or centrifugal forces. To get the extracted compound in a pure form a further separation process is necessary (rectification or re-extraction). Depending on the phases the following types of extraction exist:

- solid – liquid extraction,
- liquid – liquid extraction,
- gas – liquid extraction, called absorption.

The solvent used to extraction process should have a few important features:

- selectivity – only the expected substance has to be extracted,
- capacity – to reduce the volume of the solvent used to the extraction,
- miscibility – with the second phase it should be the lowest,
- difference in density – the best is the highest difference between the two phases which have to be separated,
- optimal surface tension – not to form the emulsions with the second phase, difficult to separate,
- non-reactive with the separated substance and the second phase,
- chemically and thermally stable,
- flame temperature – should be 25 °C higher than the operating temperature,

– with low viscosity – leads to low pressure drop, good heat and mass transfer.

3.3.1. Liquid – liquid extraction

Liquid – liquid extraction, also known as **partitioning**, is a method of separation compounds based on their relative solubility in two different immiscible liquid phases, usually water or water solution, and an organic solvent. The substance is extracted from one liquid phase into another liquid phase. The most popular liquid-liquid extraction is provided using a separatory funnel. This type of a process is commonly performed after a chemical reaction to purify the product(s) of the reaction from the rest of the non-reacted compounds and from some other substrates like solvents and semi-products.

Acid-base extraction is a procedure using sequential liquid-liquid extractions to purify acids and bases from mixtures, based on their chemical properties. This type of extraction is very helpful, especially in the isolation of natural products from crude extracts.
If one or more of the compounds in the mixture to be separated is acidic or basic, the solubility of these acidic and basic components can be manipulated by applying simple acid-base reactions. Using such a manipulation, an acidic or basic compound that may be soluble in an organic solvent and water insoluble can be changed to be insoluble in this organic solvent and water soluble, by carrying out an acid-base reaction. The main disadvantage of this method is no possibility to separate chemically similar acids or bases. The fundamental theory behind this technique is that salts tend to be water-soluble, while neutral molecules tend not to.

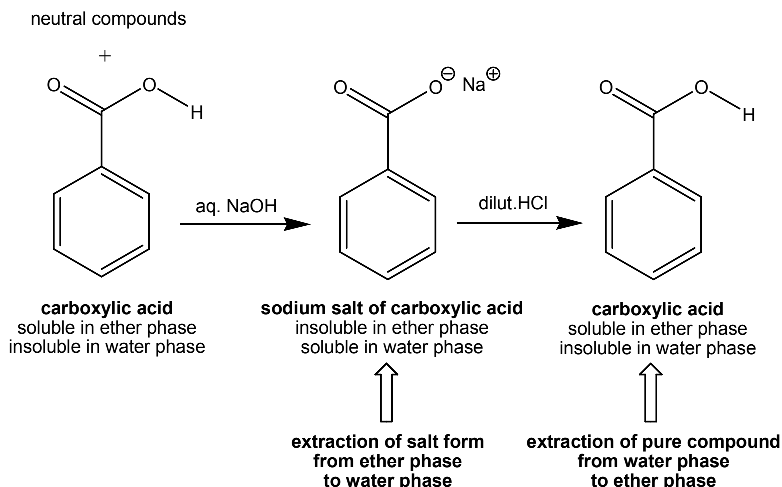


Fig. 3.3.1. The scheme of extraction process of carboxylic acid from the mixture of neutral compounds, based on changing its solubility – the example of acid-base extraction.

An example of the application of such solubility manipulation of carboxylic acid in its extraction process is shown in Figure 3.3.1. Usually, in this type of extraction a mixture of some compounds dissolved in a suitable organic solvent is mixed with an aqueous solution with the proper pH value, to be able to separate the compound of interest into its required form. After shaking and allowing for non-mixed phases separation, the one containing the

compound of interest is collected. The procedure is then repeated with this phase at the opposite pH value. The order of the steps is not important and the process can be repeated to increase the separation yield. However, it is often convenient to receive the separated compound dissolved in the organic phase after the last step, when it is easy to evaporate from the solvent, to finally receive the compound in the solid state form.
The acid-base extraction procedure is also used to separate very weak acids from stronger acids and very weak bases from stronger bases, as long as the difference of their pK_a (or pK_b) constants is large enough. The example are very weak acids with phenolic rests, and with $pK_a \approx 10$, which can be separated from stronger acids like benzoic acid, with $pK_a \approx 4 – 5$. Similar situation is in the case of very weak bases like caffeine or *p*-nitroaniline with $pK_b \approx 13 – 14$, which can be separated from stronger bases like dimethyltryptamine, with $pK_b \approx 3 – 4$. Usually the pH value is adjusted to this and it is roughly between pK_a (or pK_b) constants of the separated compounds. Weak acids, like citric acid, H_3PO_4, or diluted H_2SO_4 are used for moderately acidic pH values, whereas HCl or more concentrated H_2SO_4 is used for strongly acidic pH values. Similarly, weak bases like NH_4OH or $NaHCO_3$ are used for moderately basic pH values, while stronger bases like K_2CO_3 or NaOH are used to receive strongly alkaline conditions.

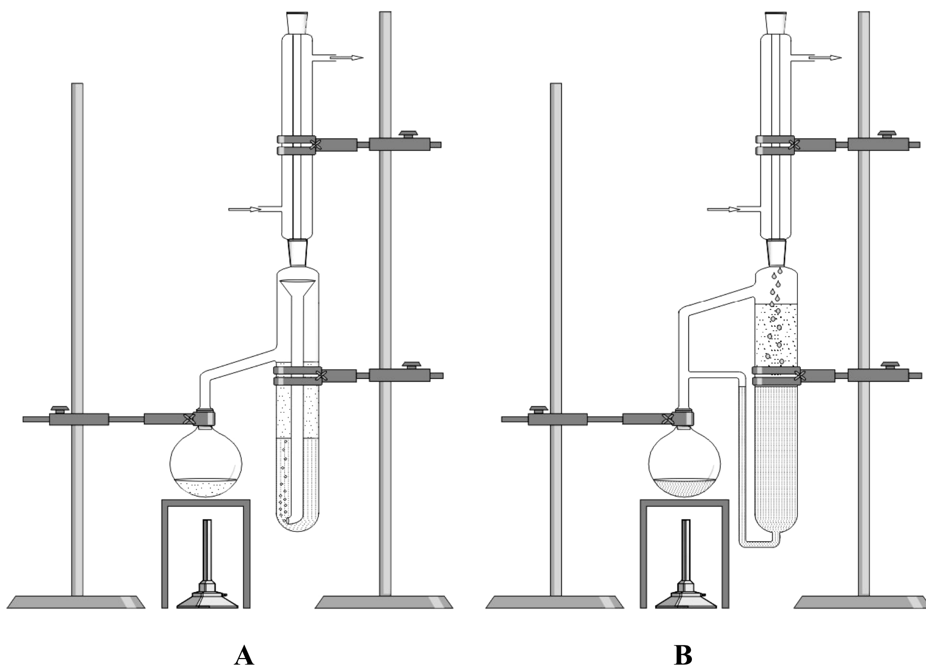


Fig. 3.3.1. The continuous extraction process devices: A – the extraction of the lighter solvent by the heavier one, B – the extraction of the heavier solvent by the lighter one.

Depending of the weight of the used solvents there are two types of the laboratory extractors which make it possible to provide **continuous extraction**. When the compound is extracted from the heavier solution to the lighter one, the lighter solvent is heated all the time to evaporate it, then its vapour is condensed on the walls of a cooler and its liquid form is

dropped into some kind of a long funnel down, on the bottom of the extractor, down the heavier solution, to be able to go through this solution and extract the substance from this phase (Fig. 3.3.1. A).
When the situation is reversed, and the isolated compound is extracted from the lighter solution to the heavier one, the heavier solvent is heated all the time to evaporate it and its vapour is condensed on the walls of a cooler to be able to drop into the extractor. Because of gravity, these drops go down naturally through the lighter solvent, and are able to contact with the extracted compound, to extract it. In the bottom of the extractor there is an overflow glass pipe. When too much of the heavier solvent is evaporated from the heated flask and condensed into the extractor, it is turned back to the heated flask (Fig. 3.3.1. B).

3.3.2. Solid – liquid extraction

Solid – liquid extraction is the process where a soluble compound of a solid matter is extracted by a solvent. The extraction matter has to be prepared in this way that the extract can be solved by the solvent in short time. The extracted material is not a homogeneous substance and it is usually porous. At the beginning the solvent diffuses into the pores and dissolves the expected compound. At the end of the extraction process still a certain amount of solution (consisting of solvent and extracted substance) is retained in the solid particles because of adhesive forces. This is the reason why the complete extraction is practically not possible.
The most popular extractor used to solid – liquid extraction process in the organic chemistry laboratory is Soxhlet apparatus. Originally, it was used in a semi-continuous method applied to the extraction of lipids from foods by repeated washing (percolation) with an organic solvent, usually hexane or petroleum ether, under reflux. In this extraction method the sample is ground into small particles and placed in a porous cellulose thimble. The thimble is placed in an extraction chamber, which is suspended above a flask containing the solvent and below a condenser. The flask is heated and the solvent evaporates and moves up into the condenser where it is converted into a liquid that trickles into the extraction chamber containing the sample. The extraction chamber is designed in such a way that when the solvent surrounding the sample exceeds a certain level it overflows by a siphon side arm back down into the boiling flask. This cycle may be repeated many times, for hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the expected compound, which is to be extracted, is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled.
After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble, and is usually discarded.

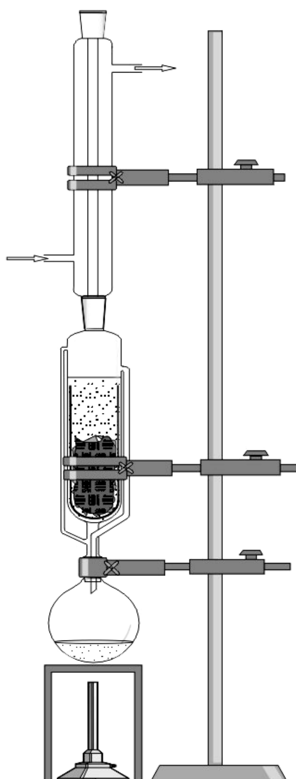


Fig. 3.3.2. The continuous extraction process from a solid state phase by a solvent – Soxhlet apparatus.

3.4. Chromatography techniques

Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase, finally to be separated.

The mobile phase may be liquid or gas, whereas the stationary phase could be composed of various types of materials. The chromatography techniques are usually divided into two main groups: gas chromatography (GC), which is used in the case of relatively volatile and thermally stable organic compounds, and liquid chromatography (LC), encompassing many different separation techniques, based on the same general rules.

From the separation scale point of view, chromatography may be analytical, semi-preparative or preparative. The purpose of preparative chromatography is to separate the components of a mixture for further use.

3.4.1. Thin–Layer Chromatography

Thin layer chromatography (TLC) is the rapid and easy chromatography technique, very often used to separate mixtures. In TLC the stationary phase is spread over a sheet of glass, plastic, or aluminium foil. Calcium oxide or an organic polymer serves to bind the adsorbent to the sheet, coated usually with silica gel, aluminium oxide, or cellulose. It is also possible to use a special kind of chromatography paper, i.e. Whatman type, as a stationary phase and then this process of separation is called paper chromatography (PC).
After the sample is applied on the plate, above the bottom of the slide, this slide is placed in a container with a shallow layer of a solvent or a solvent mixture, known as the mobile phase. The mobile phase with the separated mixture is drawn up on? the plate by capillary action. The distance to which the mobile phase moves the compounds up the chromatogram plate is dependent on the ability of these substances to adhere to this adsorbent system, as well as many other factors. This type of chromatography can be used to:

- monitor the progress of a reaction,
- identify the compounds present in a given mixture,
- determine the purity of an analyzed substance.

TLC plates are usually commercially available, with standard particle size ranges to improve reproducibility. They are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulfate and water. The thickness of the adsorbent layer is typically around 0.1 – 0.25 mm for analytical purposes, and around 0.5 – 2.0 mm for preparative TLC. If the UV light is used as a detection method, then the adsorbent may contain a fluorescent indicator, typically for λ_{max}= 254 nm.

To run a TLC, a small spot of solution containing the sample in the concentration of 1 – 10 % in the solvent should be applied to a plate using a capillary tube, about 1.5 centimetres from the bottom edge. The spot of the mixture should be small. The solvent should be allowed to completely evaporate off, otherwise a very poor or no separation will be achieved. If a non-volatile solvent was used to apply the sample, the plate needs to be dried in a vacuum chamber. In the separation chamber a folded piece of filter paper should be placed along the side. This filter paper should be saturated with the eluent and touch the bottom of the chamber. A small amount of the mobile phase should be poured into the separation chamber to a depth of less than 1 centimetre. The container must be closed with a cover glass or any other lid, and left for a few minutes to let the solvent vapours ascend the filter paper and saturate the air in the chamber. Then the TLC plate may be placed, spotted side down, into the chamber so that the spot of the sample does not touch the surface of the eluent or the filter paper, and the lid must be closed all the time. During the separation process the solvent will move up the plate by capillary action, until its front is about 1 centimetre from the top of the plate, not higher than the top of the filter paper in the chamber. Then the plate should be removed and dried from the solvent. The continuation of the elution would give a misleading result.
Different compounds in the sample mixture travel on the slide surface at different rates due to the differences in their attraction to the stationary phase, and because of differences in the solubility in the solvent. TLC technique is best used with compounds that are coloured, or visible under UV light. Alternatively, the plate may be placed in an enclosed chamber with a chemical developer. Several methods exist to visualize the colourless spots:

- iodine vapours are a general unspecific colour reagent, where nearly all compounds, except alkanes and aliphatic halides, form iodine charge-transfer complexes, and this process is reversible – the plate should be put into the chamber with iodine crystals for a few minutes and the dark brown spots should be quickly marked,

- specific colour reagents sprayed onto the plate, i.e. sulfuric acid, to cause the colourless compounds to darken by charring (the destructive technique), or spraying by *p*-anisaldehyde and baking the plate to dryness until the dark spots develop.

The most common method of reporting the results of TLC analysis is the determination of the retention factor R_f for each detected spot. This value is calculated by dividing the distance covered by the product by the total distance covered by the solvent (the solvent front):

$$R_f = \frac{distance\ spot\ of\ interest\ has\ moved\ from\ origin\ (b)}{distance\ solvent\ front\ has\ moved\ from\ origin\ (a)}$$

Alternatively, the results may be reported as R_x factor, where:

$$R_x = \frac{distance\ spot\ of\ interest\ has\ moved\ from\ the\ origin}{distance\ spot\ of\ reference\ compound\ has\ moved\ from\ the\ origin}$$

The R_f value depends on the used solvent, the type of TLC plate and it is not the physical constant.

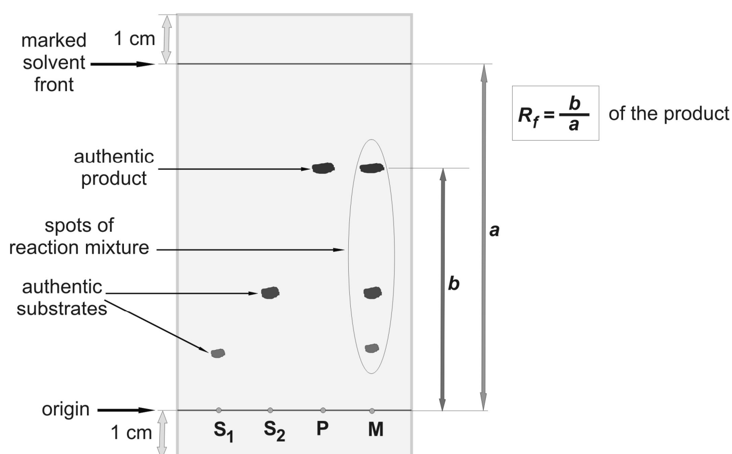


Fig. 3.4.1. Thin–layer chromatogram with separated reaction mixture in the presence of the authentic substrates and the authentic product, to identify the spots well.

This type of separation of compounds is based on the competition of the solute and the mobile phase for binding places on the stationary phase. For example, if normal phase silica gel is used as the stationary phase it can be considered polar. Given two compounds which differ in polarity, the more polar compound has a stronger interaction with the silica and is therefore more capable to dispel the mobile phase from the binding places. As a consequence, the less polar compound moves higher up the plate (resulting in a higher R_f value). If the mobile phase is changed to a more polar solvent or mixture of solvents, it is more capable of dispelling solutes from the silica binding places, and all compounds on the TLC plate will move higher up the plate. Changing the polarity of the mobile phase will normally not result in reversed order of running of the compounds on the TLC plate. An eluotropic series can be used as a

guide in selecting a mobile phase. If a reversed order of running of the compounds is desired, a non-polar stationary phase should be used, such as C_{18}–functionalized silica.

TLC can also be used on a semi-preparative scale to separate mixtures of up to a few hundred milligrams. The mixture is not "spotted" on the TLC plate as dots, but rather is applied to the plate as a thin even layer horizontally to and just above the solvent level. When developed with solvent, the compounds separate in horizontal bands rather than horizontally separated spots. Each band is scraped off the backing material. The backing material is then extracted with a suitable solvent and filtered to give the isolated material upon removal of the solvent.

3.4.2. Column Chromatography

Column chromatography is a method used to purify individual compounds from mixtures of compounds usually in the semi-preparative or preparative scale, to receive the final substances in micrograms up to kilograms. The classical preparative chromatography column, is a glass tube with a diameter from 5 mm to 50 mm and a height of 50 cm to 1 m. Such kind of the separation process usually takes a time commitment of many hours, with the classical gravity flow columns. In flash chromatography, which is a type of column chromatography, to reduce the time of separation, air or nitrogen pressure is applied to the top of the column, to push the solvent through the column filler at a much faster rate. The knowledge of preliminary TLC characterizing the separated mixture will be very useful before column chromatography.
In the column chromatography technique the mixture components are retained by the stationary phase and are separate from each other while they are running at different retention time through the column with the **eluent**. During the separation process the eluent containing the compounds – **eluate**, is collected in a series of fractions. The composition of the flowing eluent may be monitored currently using different techniques, i.e. UV absorption, fluorescence, refractive index, or each fraction may be analyzed by analytical methods, after collection. Coloured compounds can be seen through the glass wall of the column as moving bands.
Because the column chromatography has a constant flow of eluted solution passing through the detector at varying concentrations, the detector must plot the concentration of the eluted sample over a course of time. This plot of sample concentration versus time is called a **chromatogram**. The ultimate goal of chromatography is to separate different components from the mixture. The resolution expresses the extent of separation between the components from the mixture. The higher the resolution of the chromatogram, the better the extent of separation of the samples the column gives. The resolution can be calculated from the chromatogram. The separate curves in the diagram represent different sample elution concentration profiles over time based on their affinity to the column filler. To calculate resolution, the retention time and curve width are required.

A variety of chromatography columns are available commercially. In plane chromatography the column has a small piece of Teflon tube with a pinch clamp attached to its bottom to regulate flow rate. A more comfortable version is a stopcock, recommended by Teflon, and a filter disk of glass sinter above the stopcock. Typically, column chromatography is set up with a peristaltic pump flowing the eluent and the sample mixture through the top of the column. The eluate passes through the column where a fraction collector at the end of the column setup collects the eluted samples. The mobile phase should be chosen to separate effectively the different compounds of the mixture. The eluent is optimized in small scale pretests, often using TLC with the same type of the stationary phase.

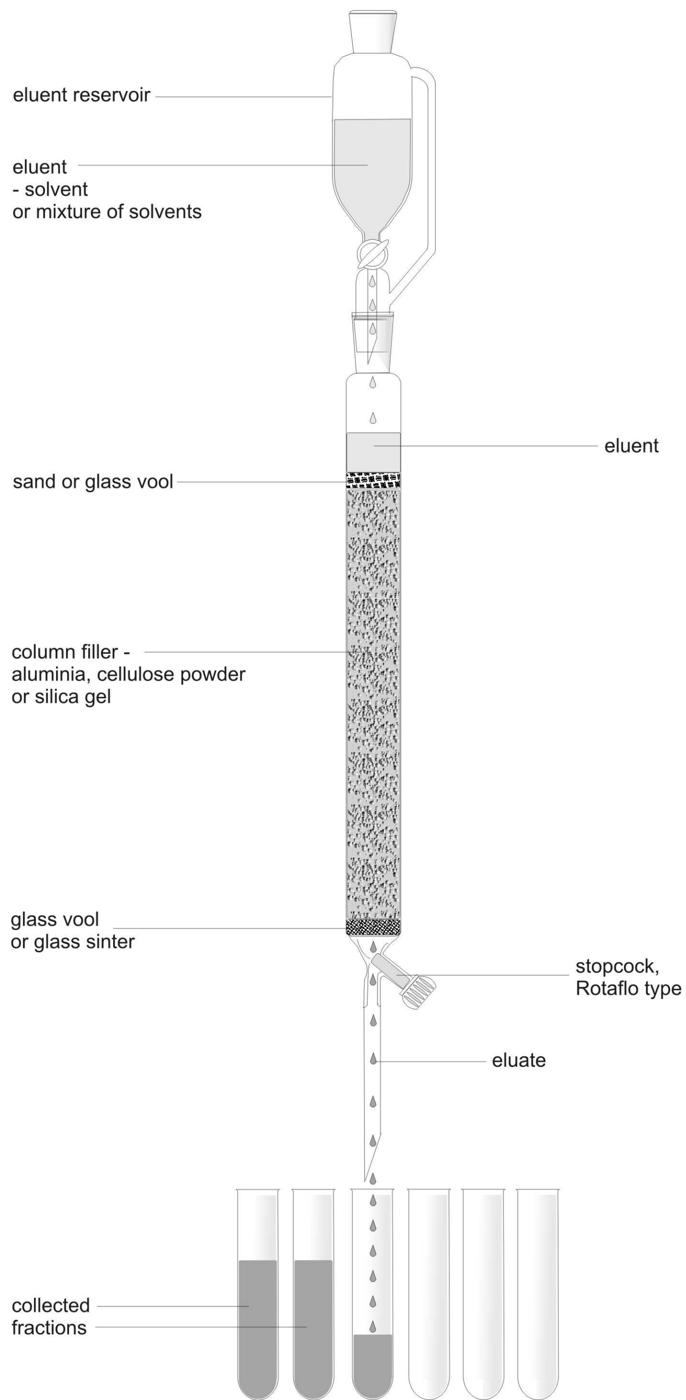


Fig. 3.4.2. The packed chromatography column.

The most common stationary phases used in column chromatography are silica gel, alumina and cellulose powder. It is also possible to use ion-exchangers, used in ion exchange chromatography, C_8– and C_{18}–coated silica typical in reversed-phase chromatography (RP), and many other fillers used in affinity chromatography or expanded bed adsorption (EBA). The particle size of the stationary phase is generally finer in flash column chromatography than in gravity column chromatography, i.e. one of the most widely used silica gel grades in the former technique is mesh 230 – 400 (40 – 63 μm), while the latter technique typically requires mesh 70 – 230 (63 – 200 μm) silica gel.

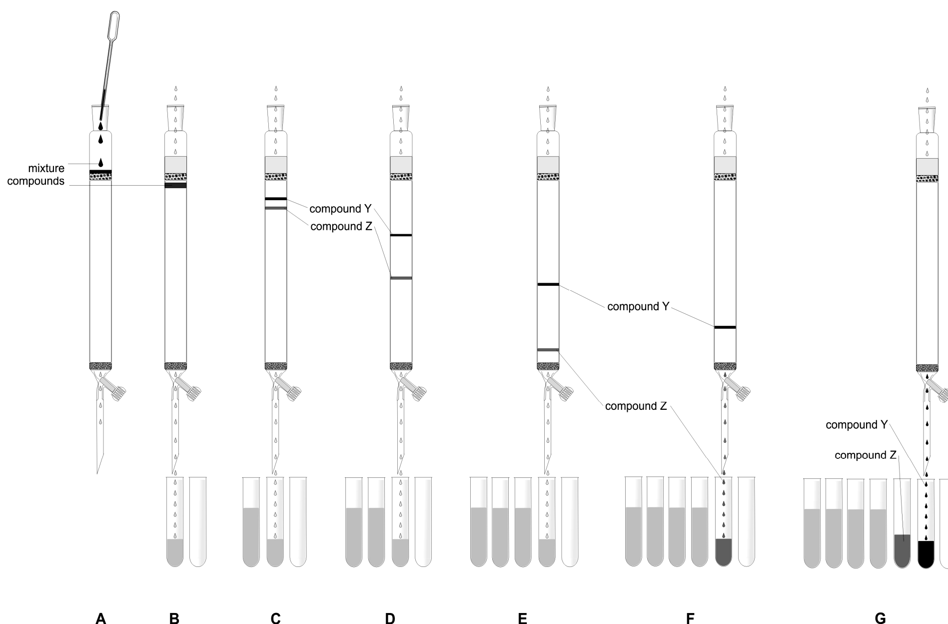


Fig. 3.4.3. The steps of the process of separation of a mixture compounds in the column chromatography process.
A – imposition of a mixture on the top of the column filler, **B**, **C**, **D** and **E** – the phases of separation process, **F** – collection of one of the compounds (compound Z) eluted through the column faster, **G** – collection of the next compound (compound Y) eluted through the column slower. All the time during the separation process the column filler is eluting with eluent, and the fractions are being collected.

Liquid chromatography may be divided into a few methods, depending on the type of the column preparation:

- dry column chromatography, where the column is first filled with dry stationary phase powder, followed by the addition of mobile phase, which is flushed through the column until it is completely wet, and from this point is never allowed to run dry; mixtures are placed on the dry column in a minimal amount of the eluent;
- wet column chromatography, called also a slurry method – it is prepared with the wet stationary phase powder suspended in the eluent, carefully poured into the column; care must be taken to avoid air bubbles, the separated mixture is pipetted on top of the stationary phase; this layer is usually topped with a small layer of sand, or with cotton or glass wool, to protect the shape of the organic layer from the velocity of newly added eluent;

- micro–scale column chromatography, uses either a Pasteur pipette or a 50-mL titration burette as a column; these types of columns can be used on the same day they are prepared and in the same manner as the larger-scale columns.

3.4.3. High Performance Liquid Chromatography

High-performance liquid chromatography, sometimes called **high–pressure liquid chromatography** (HPLC) is a chromatographic technique that attacks problems associated with traditional column chromatography. In this technique a particle size of the stationary phase is much smaller, and it is in the order of a few micrometers. This small size of the particles produces a problem such as potentially slow flow rates, so pressures well above atmospheric pressure are necessary to push the mobile phase through the tightly packed column.

HPLC typically utilizes different types of stationary phases, a pump that moves the mobile phase(s) and the analyzed mixture through the column, and a detector that provides a characteristic retention time for the separated compounds. The pulseless and able to generate reproducible flow rates piston pump(s) provides the higher pressure required to proper the mobile phase and analyte(s) through the column. This allows for a better separation on the columns of shorter length when compared to ordinary column chromatography, and much shorter time of separation. Other advantages are: reusable columns, automatic and continuous solvent addition, reproducible programmed gradients of eluents as well as automatic and continuous monitoring of the eluted substances. Less than 1 mg of a sample is commonly analyzed. The preparative–scale instruments are able to separate between several milligrams and a few grams of a sample, but the main disadvantage is that large amounts of eluent(s) are used.

A further refinement of HPLC is to vary the mobile phase composition during the analysis. Usually **gradient elution** is applied. The gradient depends on how hydrophobic the analyzed sample is. In the gradient elution it is possible to separate the mixture of some compounds as a function of their affinity. This partitioning process is similar to that which occurs during a liquid–liquid extraction but is not continuoued step–wise. The choice of eluents and gradient program depends on the nature of the column and the components of the separated mixture. Another important component in choosing the type of eluent is the influence of the pH value since this can change the hydrophobicity of the separated compounds. For this reason most methods use a buffering agent to control and stabilize the pH value. The buffers neutralize the charge on any residuals on the stationary phase and act as ion pairing agents to neutralize charge on the separated compounds. There is some important information concerning the mixture composition needed in the process of establishing the method. It is important to know the number of compounds in the analyzed mixture and their chemical structures, especially the presence of functional groups, their molecular weights, pK_a values of the compounds, their solubility and concentration in the mixture. A series of tests are often performed on the sample together with a number of trial runs in order to find the HPLC method which gives the best peak separation.

From the column filler nature point of view there are a few types of HPLC, but it is possible to classify them into two main groups: **elution chromatography** and **displacement chromatography**. In elution mode, the separated substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks is desired in order to achieve maximum

purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. However, for two substances to travel at different speeds, and thereby be resolved, there must be substantial differences in some interaction between the separated molecules and the chromatography filler. In the case of displacement chromatography a molecule with a high affinity for the chromatography gel will compete effectively for binding sites, and thus displace all molecules with lesser affinities, which is the basic principle of this method. Displacement chromatography has advantages over elution chromatography as the components are resolved into consecutive zones of pure substances rather than some peaks. Because the process takes advantage of the nonlinearity of the isotherms, a larger column feed can be separated on a given column with the purified components recovered at significantly higher concentrations.

Partition chromatography is the first kind of **elution chromatography** that chemists developed. **Normal–phase HPLC** (**NP–HPLC**), or adsorption chromatography, is the method in which the separation process is based on adsorption to a stationary surface. In this technique a polar stationary phase and a non–polar, non–aqueous mobile phase are used, NP–HPLC works effectively for separating mixture compounds readily soluble in non–polar solvents. Very polar solvents used in the eluent mixture may deactivate the stationary phase by creating a stationary bound water layer on the stationary phase surface. Adsorption strengths increase with the increased polarity of the separated substances, and the interaction between the polar compounds and the polar stationary phase extends also the elution time. The interaction strength depends not only on the functional groups of the separated molecules, but also on steric factors, which allows this method to resolve separate structural isomers.

Partition chromatography is also known as **hydrophilic interaction chromatography** (**HILIC**) in HPLC, where the compounds of the analyzed sample are separated based on their differences in polarity. In HILIC a bonded polar stationary phase and a non–polar, water miscible, mobile phase are most often used. Partition HPLC has been used historically on non–bonded silica or alumina supports, each works effectively by relative polar differences. However, HILIC has the advantage of separating a mixture of acidic, basic and neutral substances in a single chromatogram. The polar compounds diffuse into a stationary water layer associated with the polar stationary phase and are thus retained. Retention strengths increase with the increased polarity of a separated molecule, and the interaction between this polar compound and the polar stationary phase increases the elution time. The interaction strength depends on the functional groups in the molecule structure which promote partitioning but can also include electrostatic interaction and hydrogen donor capability. Application of more polar eluents in the mobile phase decreases the retention time of the separated compounds, whereas more hydrophobic eluents tend to increase retention times.

Reversed phase HPLC (**RP–HPLC** or **RPC**) is based on the usage of a non–polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica treated with RMe_2SiCl, where R is a straight chain of alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. In this type of HPLC retention time is longer for non–polar molecules, while polar molecules elute through the column more readily. The retention time of some analyzed compound can be increased by adding more water to the mobile phase, thereby making the affinity of the hydrophobic substance for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. The decreasing retention time is possible by adding more organic solvent to the eluent mixture. Gradient elution uses this effect by automatically reducing the polarity and the surface tension of the aqueous mobile phase during the course of the analysis.

In this type of HPLC the analyzed compounds with a larger hydrophobic surface area, containing lots of non–polar bonds like C–H and C–C, or S–S, results in a longer retention time, because they do not interact with the water structure. On the other hand, polar groups, such as –OH, $–NH_2$, $–COO^-$ or $–NH_3^+$ reduce retention time as they are well integrated into water. Branched chain compounds elute more rapidly than their corresponding linear isomers, because their overall surface area is decreased. Similarly organic compounds with single C–C bonds elute later than those with a C=C or C≡C bond, as they are shorter than a single bond.

Size–exclusion chromatography (**SEC**), also known as **gel permeation chromatography** (**GPC**) is used to separate compounds on the basis of their size. SEC is mainly used for the analysis of large molecules such as proteins, polysaccharides and other polymers. SEC works by trapping the smaller molecules in the pores of the particles of the column filler. The larger molecules simply pass by the pores as they are too large to enter the pores. Therefore larger molecules flow through the column quicker than smaller molecules.

Ion–exchange chromatography (**IEC**) is one of the **displacement chromatography** methods. In IEC, retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded. Types of ion exchangers include:
- polystyrene resins,
- cellulose and dextran ion exchangers,
- controlled–pore glass or porous silica gels.

In general, ion exchangers favour the binding of ions of higher charge and smaller radius. An increase in counter-ion concentration reduces the retention time. Separation in IEC depends upon the reversible adsorption of charged separated substances to immobilized ion–exchange groups of opposite charge.

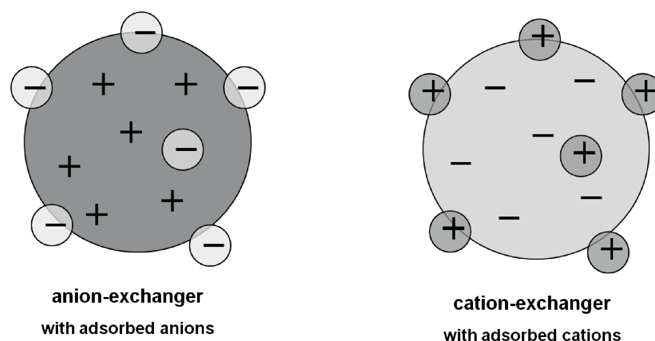


Fig. 3.4.4. Two kinds of ion-exchangers: anion exchanger with adsorbed counter-anions, and cation-exchanger with adsorbed counter-cations.

Most of such kind of experiments are performed in five main stages. The first step is equilibration in which the ion-exchanger is brought to a starting state, so that in terms of pH and ionic strength to it is able to bind the desired compounds. The groups of the exchanger are associated with counter-ions, which are usually simple anions or cations like Cl^- or Na^+, respectively. The second stage is the separated mixture application and adsorption on the resin. The desired mixture components carrying the appropriate charge displace counter-ions and bind reversibly to the column filler. The unbound components of the separated mixture are washed out from the exchanger bed using starting eluent, usually a buffer. During the third step of the separation process, the adsorbed substances are removed from the column by

changing the elution conditions unfavourably for ionic bonding of these molecules. This normally involves increasing the ionic strength of eluent, i.e. salt concentration in the eluent gradient or pH value changing. The most weakly bound substances are eluted first. The last two stages of the process are the removal of the compounds not eluted from the column under the previous experimental conditions and re–equilibration at the starting conditions for the next purification.

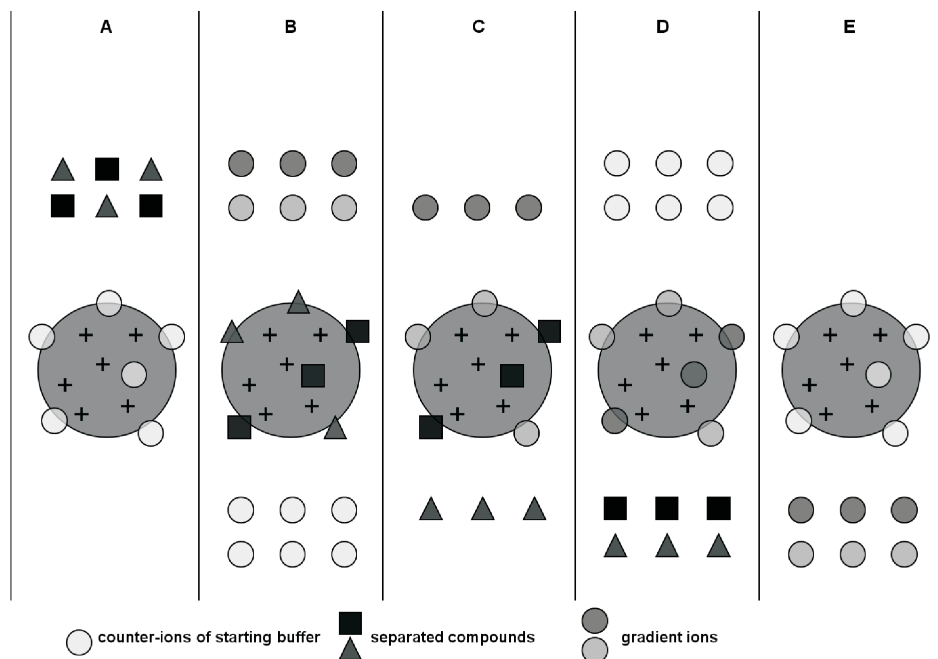


Fig. 3.4.5. Anion-exchange chromatography process with salt gradient elution. **A** - the starting conditions, where counter-anions of the starting buffer are adsorbed on the column resin, **B** – adsorption of the separated compounds on the resin, **C** – beginning of the desorption of the separated compounds by anions of the gradient salt solution, **D** – end of the desorption process, **E** – regeneration of the resin.

The separation process occurs when the separated substances have different degree of interaction with the ion-exchanger due to differences in their charges, charge densities and distribution of charge on their surfaces. These interactions can be controlled by varying parameters such as ionic strength and pH value.

Bioaffinity chromatography is a separation process which relies on the property of biologically active ligands mounted in a gel matrix to form stable, specific, and reversible complexes involved common molecular forces such as the Van der Waals interaction, electrostatic interaction, dipole–dipole interaction, hydrophobic interaction, and hydrogen bonds. This is a method based on a highly specific biological interactions such as that between antigen and antibody, enzyme and substrate, or receptor and ligand. Affinity chromatography combines the size fractionation capability of gel permeation chromatography with the ability to design a chromatography that reversibly binds to a known subset of molecules.

Detection type and operation affect the relative response of sample components and potential interferences in three interrelated ways: sensitivity, selectivity, and baseline noise. The most commonly used detection method in HPLC is **UV–Vis detection** using either a variable-wavelength (spectrophotometry) or a **diode-array detection** (**DAD**). The alternative types of detection are used primarily when the analyzed compounds have little or no UV–Vis absorbance or their concentrations are too low for this type of detection. In UV detectors the light source is typically a deuterium lamp, which provides acceptable light intensity in the range 190 – 400 nm, whereas the measurements at visible wavelengths require a high-energy tungsten-halide lamp with light intensity in the range 400 – 700 nm. Light from the lamp passes through a transmitting flow cell connected to the column and impinges on a diode that measures the light intensity (I), as well as this it is directed to a reference diode for measurement of the original light intensity (I_o). Good analytical results may be obtained only by careful selection of the wavelength used for detection. Such choice requires a knowledge of the UV–Vis spectra of the individual sample components. If the analyzed standards are available, their spectra can be measured prior HPLC method development. Alternatively, a DAD permits the acquisition of UV spectra for all analyzed mixture components during method development. Diode–array detectors allow simultaneous collection of chromatograms at different wavelengths during a single analysis. A chromatogram at any desired wavelength can be displayed, usually between 190 and 400 nm. DADs therefore provide more information on sample composition that is provided by a single wavelength measurement.
The oldest and the most widely used detector is the **refractive index** (**RI**) **detector**. Refractive index is a physical property of all compounds, so in theory every substance may be detected using this kind of method. However, because mobile phase components, including solvents and usually some additives, also show significant refractive index response, gradient elution using RI detectors is impractical. Moreover, other factors, like temperature of the analysis, dissolved in the mobile phase gases, lack of sensitivity for the trace analysis, limit the usage of the RI detector. Another type of universal detector is **evaporative light-scattering** (**ELS**) **detector**. The eluted substances from the HPLC column are nebulized and evaporated as it passes through the drift tube, and particles of the analyzed compounds are detected as they pass through the light scattering cell. Therefore, the usage of such detector is restricted to non-volatile substances and volatile mobile phases. However, because of the ability to use ELS detectors with gradient elution, they are being used more frequently in these methods. Each of these two detectors has a similar sensitivity, allowing the analysis of substances present in the range of 0.1 μg/mL and higher, but they are usually two orders of magnitude poorer than UV detection.
Detection based on **fluorescence** (**FL**) of the analyzed compounds is a very sensitive and selective technique, typically there are? three orders of magnitude more sensitive than UV detection methods. The main advantage of this technique is its ability to discriminate the analyzed substances from interference or background peaks. Since the analyzed compounds possess natural fluorophoric groups, derivatization with a reagent that possesses a fluorophore must precede the use of this detector, i.e. carboxylic acid rest, hydroxyl group, rest of aldehyde, amine, ketone, thiol, phenol or peptide bond. In this technique light from the lamp passes through an excitation filter, which provides essentially monochromatic light of the desired wavelength for the excitation of analyzed compounds. This exciting light passes through the eluate in the flow cell, causing the analyzed substances to fluoresce (emit) at a higher wavelength than that used for excitation. A further complication in the usage of FL detectors is that the signal and optimum wavelengths for excitation and emission may be strongly dependent on separation conditions like temperature, eluent polarity and viscosity,

pH value. It means that the final result may require a compromise between good separation and good detection.
Another group of detectors commonly used in HPLC analysis are **electrochemical detectors** (**EC**), which can be classified according to their operation: **direct–current amperometric** (**DCA**) **detectors** or **conductivity detectors** mostly used for ion-exchange chromatography.
Mass spectrometry as the detection method in HPLC, called **liquid chromatography – mass spectrometry** (**LC-MS**) technique, is becoming popular, because of sensitivity, no need of derivatization of the analyzed samples and the possibility of the identification of the unknown compounds. There are many types of mass analyzers in MS, including magnetic electrostatic sectors, quadrupole, ion trap (ITP), time–of–flight (TOF) and Fourier transform ion cyclotron resonance (ICR) mass analyzers. The most commonly utilized one, interfaced with HPLC, is the triple quadrupole. There are also instruments which employ an electrospray ion source (ESI) connected with an octopole (dual quadrupole) ion filter prior to ITP mass analyzer. A quadrupole MS is constructed with four symmetrically arranged parallel rods. Diagonally opposed rods are electrically connected together to a radiofrequency (RF) and direct current (DC) voltage generator. After defragmentation ions of the analyzed compounds, are extracted into the quadrupole region drift toward the detector and are influenced by the combined DC and oscillating RF fields. By ramping the alternating–current and DC fields, which are such that values corresponding to the peaks within the stability diagram are maintained, ions of successive *m/z* are permitted to pass through the quadrupoles and impinge on the detector. In this way the mass spectrum is generated.

3.4.4. Gas Chromatography

Gas chromatography (**GC**), also known as **gas-liquid partition chromatography** (**GLPC**), as all other chromatographic processes, is based on the distribution of the mixture compounds between two phases, where the stationary phase is liquid and the mobile phase is carrier gas. The carrier gas is usually an inert gas such as helium or an unreactive gas such as nitrogen. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a column. The compounds being analyzed must be volatile and have to interact with the stationary phase.

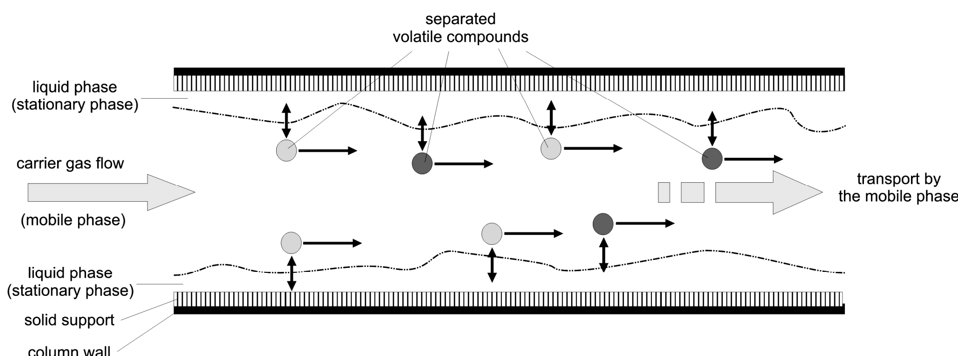


Fig. 3.4.6. The scheme of gas chromatography process. The separated substances interact differentially with the liquid stationary phase, depending on their molecular structure and affinity to this phase.

The different analyzed compounds will interact differentially with the stationary phase, depending on their molecular structure. Their distribution is described as the distribution

coefficient K_D, defined as the ratio of the separated substance weight in equal volumes of the stationary and mobile phases:

$$K_D = \frac{compound\ weight\ per\ unit\ volume\ stationary\ phase}{compound\ weight\ per\ unit\ volume\ mobile\ phase} = \frac{C_S}{C_M}$$

K_D is a true equilibrium constant, it depends only on the analyzed substance, the liquid phase, and the temperature. It is not dependent on the column type. The transport of the separated compounds through the column occurs only in the continuously moving gas phase with the same rate. Compounds which interact with the liquid phase to a greater extent remain on the column for a longer time and retention time is therefore larger.

Gas chromatography is in principle similar to column chromatography as well as other techniques such as HPLC, TLC, but has several notable differences. The column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, whereas column chromatography usually has no such temperature control. Moreover, the concentration of a separated compound in the gas phase is solely a function of the vapour pressure of the gas. Gas chromatography is similar to fractional distillation, since both processes separate the components of a mixture primarily based on boiling point (or vapour pressure) differences. However, fractional distillation is typically used to separate components of a mixture on a large scale, whereas GC can be used on a much smaller scale.

A **gas chromatograph** uses a flow of gas through a very narrow column. As the chemicals exit the end of the column, they are detected and identified electronically. A known volume of gaseous or liquid analyte is injected into the entrance of the column – the injection port, usually using a microsyringe, manually or using autoinjector or autosampler, where it is vaporized and carried into the column. Typically a capillary column is 15 to 30 m long, coated on the inside with a thin film (0.2 μm) of high boiling liquid (the stationary phase). A detector is used to monitor the outlet stream from the column. Thus, the time at which each component reaches the outlet and the amount of that component can be determined. Generally, substances are identified by the order in which they elute from the column and by the retention time of the analyte in the column.
The column is thermostated. A good separation will occur in a reasonable amount of time. It is often necessary to maintain the column at a wide variety of temperatures, from ambient to 360 °C. The control of temperature is one of the easiest and the most effective ways to influence the separation process. The injection port should be hot enough to vaporize the sample rapidly, but must be low enough not to decompose the analyzed mixture. The general rule is to have the injection temperature about 50 °C higher than the boiling point of the analyzed sample. The column temperature should be high enough to pass the analyzed compounds through it at a reasonable speed. It should not be higher than the boiling point of the separated sample, but considerably below this boiling point – it must be kept above the "dew point", but not above its boiling temperature. Reducing the temperature produces the greatest level of separation but can result in very long elution times. In some cases temperature is ramped either continuously or in steps to provide the desired separation. This is referred to as a **temperature program**. Electronic pressure control can also be used to modify flow rate during the analysis, aiding in faster run times while keeping acceptable levels of separation.

Two types of columns are used in GC:

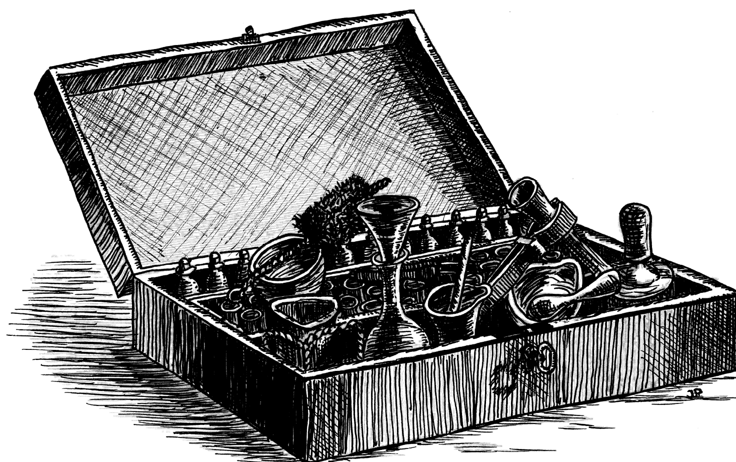
- packed columns – are 1.5 – 10 m in length and have an internal diameter of 2 – 4 mm; the column is usually made of stainless steel or glass and contains a packing of finely divided, inert, solid support material that is coated with a liquid or solid stationary phase; the nature of the coating material determines what type of materials will be most strongly adsorbed;
- capillary columns – have a very small internal diameter, on the order of a few tenths of millimetres, and lengths between 25 – 60 meters are common; the inner column walls are coated with the active materials (WCOT columns), where others are quasi solid filled with many parallel micropores (PLOT columns); most capillary columns are made of fused-silica (FSOT columns) with a polyimide outer coating, they are flexible, so a very long column can be wound into a small coil.

A number of detectors are used in gas chromatography. The most common ones are the **flame ionization detector** (**FID**) and the **thermal conductivity detector** (**TCD**). Both are sensitive to a wide range of components, and both work over a wide range of concentrations. While TCD is essentially universal and can be used to detect any component, other than the carrier gas, its thermal conductivity at the detection temperature must be different than the analyzed compounds, FID is sensitive primarily to hydrocarbons, and more sensitive to them than TCD. However, FID cannot detect water. Both detectors are also quite robust. Since TCD is non–destructive, it can be operated in series before FID, which is destructive, thus providing complementary detection of the same analytes. Other detectors are sensitive only to specific types of substances, or work well only in narrower ranges of concentrations.

Some gas chromatographs are connected to a **mass spectrometer** (**MS**), which acts as the detector. The combination is known as **GC–MS**. The MS principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments, and the measurement of the ratio of mass to charge (***m/z***) of the charged particles. It is used for determining the elemental composition of the molecules separated in GC. MS instruments consist of three modules: an **ion source**, which can convert gas phase sample molecules into ions, a **mass analyzer**, which sorts the ions by their masses by applying electromagnetic fields, and a **detector** measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present.
The technique has both qualitative and quantitative uses. These include identifying unknown compounds, determining the isotopic composition of elements in a molecule, and determining the structure of a compound by observing its fragmentation. There are many types of mass analyzers, using either static or dynamic fields, and magnetic or electric fields, but all operate according to the above differential equation. Two of them are popularly used as the detectors in GC: **quadrupole** mass filter and quadrupole **ion trap**.
Quadrupole mass analyzers use oscillating electrical fields to selectively stabilize or destabilize the paths of ions passing through a radio frequency (RF) quadrupole field created between 4 parallel rods. Only the ions in a certain range of *m/z* ratio are passed through the system at any time, but changes to the potentials on the rods allow a wide range of *m/z* values to be swept rapidly, either continuously or in a succession of discrete hops. A quadrupole mass analyzer acts as a mass–selective filter and is closely related to the quadrupole ion trap, particularly the linear quadrupole ion trap except that it is designed to pass the untrapped ions rather than collect the trapped ones, and for that reason it is referred to as a transmission quadrupole.
The **quadrupole ion trap** works on the same physical principles as the quadrupole mass analyzer, but the ions are trapped and sequentially ejected. Ions are trapped in a mainly

quadrupole RF field, in a space defined by a ring electrode between two endcap electrodes. The sample is ionized either internally, e.g. with an electron or laser beam, or externally, in which case the ions are often introduced through an aperture in an endcap electrode.
A **linear quadrupole ion trap** is similar to a quadrupole ion trap, but it traps ions in a two dimensional quadrupole field, instead of a three-dimensional quadrupole field as in a **3D quadrupole ion trap**. This kind of ion trap can be visualized as a linear quadrupole curved around and connected at the ends or as a cross section of a 3D ion trap rotated on edge to form the toroid, donut shaped trap. The trap can store large volumes of ions by distributing them throughout the ring-like trap structure. This toroidal shaped trap is a configuration that allows the increased miniaturization of an ion trap mass analyzer. Additionally all ions are stored in the same trapping field and ejected together simplifying detection that can be complicated with array configurations due to variations in detector alignment and machining of the arrays.

Some GC−MS are connected to an NMR spectrometer which is a backup detector. This combination is known as **GC−MS−NMR**. Some GC−MS−NMR are connected to an infrared spectrophotometer which acts as a backup detector. This combination is known as **GC−MS−NMR−IR**. In most of the GC−MS systems match MS spectra to library spectra. The biggest MS library spectra dedicated to GC−MS systems is NIST library, which is universal for the instruments of different producers.



4

The Chemical Characterization of Functional Groups

The organic compounds are classified into several groups, according to their characteristic structures, especially to their characteristic functional substituents. After the purification of an unknown substance, and physical and its spectral identification, there is an important need to complete the characterization using some specific chemical tests for functional groups present in the identified structure. In this chapter there are descriptions of the most popular procedures of reactions of the organic compounds in microscale – some "wet" tests commonly used as the methods of functional groups and compound identification. It is possible to find out more about such tests in the book "The systematic identification of organic compounds" by authors Shriner R. L., Hermann K. F., Morrill T. C., Curtin D. Y., Fuson R. C.

4.1. Hydrocarbons

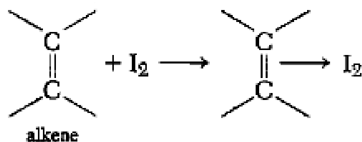
4.1.1. Alkanes

Alkanes are not usually characterized chemically because they are quite inert to the most of reactions. Since chemists usually rely heavily on physical and spectral characterization, it is possible to use the lack of reaction to conclude that the identified compound is not in a more reactive class. This is consistent with the fact that alkanes fall into the solubility class I.

4.1.2. Alkenes and Alkynes

Iodine Test for Unsaturated Hydrocarbons

The C=C bond characteristic for alkenes (olefins) may be detected very easily by chemical tests. A very popular test is based on the reaction with iodine.



Iodine in methylene chloride solution

A couple of crystals of iodine should be added to 100 mL of methylene chloride. The solution should be tightly closed by a stopper.

Experiment

To 0.25 mL or 0.25 g of the unknown sample 0.5 mL of iodine in methylene chloride solution (a few crystals of iodine dissolved in 100 mL of methylene chloride) should be added and mixed.

Discussion

Aromatic hydrocarbons, saturated, fluorinated and chlorinated hydrocarbons do not react. However, unsaturated hydrocarbons like alkenes produce a light–tan solid, while retaining the purple colour of the iodine solution.

Bromine Test

Addition of bromine across the carbon–carbon double or triple bond occurs in the bromine test with dissipation of the brown-red bromine colour. If the bromine colour is discharged and hydrogen bromine is evolved, then substitution has occurred on the unknown. Hydrogen bromine gas may be detected by placing moistened blue litmus paper across the mouth of the test tube and noting whether it turns red, indicating the presence of an acidic gas. Some alkenes bearing electron-withdrawing groups often undergo a slow or negligible reaction.

$$\mathrm{>C{=}C<} + \underset{\text{(red-brown)}}{Br_2} \longrightarrow \mathrm{-C(Br)-C(Br)-}$$

alkene (red-brown) 1,2-dibromoalkane (colorless)

$$\mathrm{-C{\equiv}C-} \xrightarrow[\text{(brown)}]{Br_2} \mathrm{BrC{=}CBr} \xrightarrow[\text{(brown)}]{Br_2} \mathrm{-CBr_2-CBr_2-}$$

alkyne 1,2-dibromoalkene (colorless) 1,1,2,2-tetrabromoalkane (colorless)

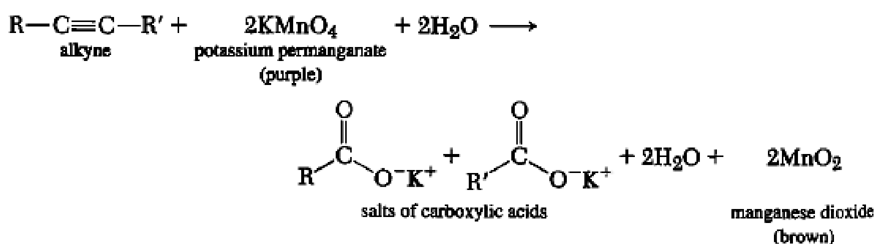
Experiment
0.1 g or 0.2 mL of the unknown compound should be added to 2 mL of methylene chloride, and then 5% solution of bromine in methylene chloride, drop wise, shaken until the bromine colour persists. The methylene chloride–bromide solution has a shelf life of about a year.

Discussion
This test is widely used to indicate the presence of an olefinic or acetylenic linkages. It should be employed in conjunction with the potassium permanganate test. Discharge of the bromine colour is characteristic for many compounds. In this category there are enols, many phenols, and enolizable compounds. Ketones, like other carbonyl compounds, may exhibit an induction period because the liberated hydrobromic acid acts as a catalyst for the enolization step of the bromination. Simple esters do not give this test. Ethyl acetoacetate decolorizes the solution immediately, whereas ethyl malonate may require as much as a minute. A number of active methylene compounds will readily give a positive test at 70 °C, and benzyl cyanide may require several minutes at this temperature.

Potassium Permanganate Test
Alkenes as well as alkynes are oxidizing to 1,2-diols with potassium permanganate, with reduction of the manganese from +7, which is purple, to +4 which is brown. The disappearance of the purple colour and the formation of a brown suspension, which is manganese (II) oxide, at the bottom of the test tube is a positive indication for carbon–carbon double or triple bond.

$$3\ \mathrm{>C{=}C<} + 2KMnO_4 + 4H_2O \longrightarrow 3\ \mathrm{-C(OH)-C(OH)-} + 2KOH + 2MnO_2(s)$$

alkene (purple) 1,2-diol manganese dioxide (brown)



Experiment

Typically, 0.1 g or 0.2 mL of the analyzed compound should be dissolved in 2 mL of water or methanol, and then 2% aqueous potassium permanganate solution should be added drop by drop and shaken until the purple colour of the permanganate persists. If the colour of the mixture is not changed in 0.5 – 1 minute, the tube with the compounds should be allowed to stand for 5 minutes, with occasional vigorous shaking.

Discussion

A slight reaction may be due to the presence of impurities. In cold dilute solutions, the chief product of the reaction on an olefin is glycol. If the reaction mixture is heated, further oxidation takes place, leading ultimately to cleavage of the carbon chain.

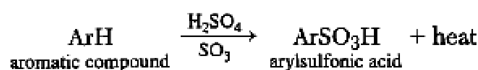
The speed of the reaction depends on the solubility of the identified compound, and if it is very insoluble it is necessary to powder the compound and shake the mixture very vigorously for several minutes or to dissolve the substance in a solvent unaffected by permanganate.

This test, though superior to the bromine test for unsaturated compounds, offers certain complications in return. All easily oxidizable substances give this test. Carbonyl compounds that decolorize bromine solutions generally give a negative result. Acetone is a good example of this, therefore it can be used as a solvent in potassium permanganate test. Aldehydes give a positive result in this test, however, many of them do not decolorize the bromine solution. Formic acid and its esters also reduce permanganate. Alcohols form another important class of substances that decolorize the permanganate solution, but not the bromine one. Pure alcohols do not give the test readily, however, they often contain some impurities that are easily oxidized. Phenols and arylamines also reduce permanganate solution and undergo oxidation to quinones.

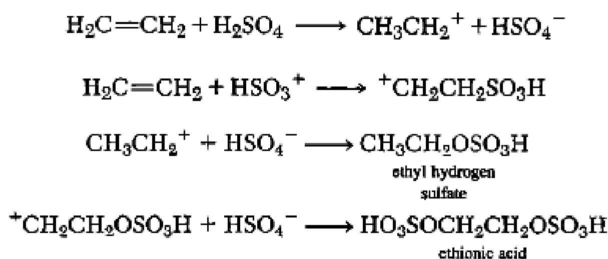
4.1.3. Aromatic Hydrocarbons

Fuming Sulfuric Acid Test

Fuming sulfuric acid converts aromatic compounds to arylsulfonic acids. The aromatic compound dissolves completely with the evaluation of heat.



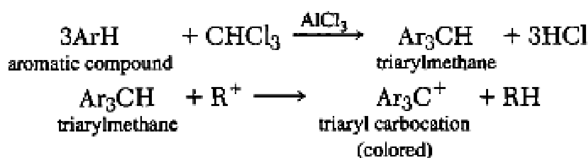
Concentrated sulfuric acid is a remarkable solvent in two respects. It is dielectric constant appears to be much greater than that of many other compounds for which this property has been measured. Thus, forces of attraction between dissolved ions are so small in dilute solution that activity coefficients may be taken as unity. The other unusual property is that, in addition to the authoprotolysis like that found in hydroxylic solvents such as water, there is a self-dissociation resulting initially in the formation of sulfur trioxide and water.
Concentrated sulfuric acid converts ethylene to ethyl hydrogen sulfate, but sulfuric acid containing added sulfur trioxide (fuming sulfuric acid) yields ethionic acid. The reason for the difference between concentrated sulfuric acid and fuming sulfuric acid is understood if it is realized that concentrated sulfuric acid contains sulfonating species such as SO_3 or HSO_3^+ in small concentration and therefore the sulfonating reagent fails to compete with proton addition.



Caution: *This reagent must be used with relatively inert compounds only, such as those that do not dissolve in the solubility tests with concentrated sulfuric acid. Substances for which preliminary tests indicate highly activating groups, like –OH, -NH_2, etc., may be decomposed violently by fuming sulfuric acid. This test must be done in a fume hood.*

Experiment
The amount of 0.5 mL of 20% fuming sulfuric acid should be put into a dry, clean test tube and then 0.25 mL or 0.25 g of the unknown compound should be added. The mixture should be shaken vigorously and allowed to stand for a few minutes. A positive test for the presence of aromatic ring is a complete dissolution of unknown, evolution of heat, and minimal charring.

Chloroform and Aluminium Chloride Test
Aromatic compounds are able to react with chloroform and aluminium chloride to produce triaryl carbocations in a variety of colour derivatives, dependent upon the functional groups on the aryl ring. The triaryl carbocations are in the solution as Ar_3C^+ $Al_3Cl_4^-$ salts and are responsible for the observed colours.



Experiment
The amount of 2 mL of chloroform is added to a test tube with 0.1 mL or 0.1 g of the analyzed substance, then it should be mixed thoroughly and the test tube should be inclined so as to moisten the wall. Then 0.5 – 1.0 g of anhydrous aluminium chloride is added? so that some of the powder strikes the side of the test tube. The colour of the powder on the side, as well as the solution should be noted.

Discussion
The colours of the resulting aromatic products of the reaction with chloroform and aluminum chloride are quite characteristic. Aliphatic compounds, which are insoluble in sulfuric acid, give no colour or are only very light yellow. Typically, benzene and its homologs give orange to red colour, as well as aryl halides, naphthalene becomes blue, biphenyl and phenanthrene – purple, and anthracene gives a green product. Aromatic esters, ketones, amines, and other oxygen– or nitrogen–containing compounds may also give blue or green colours. This test should be used in conjunction with other tests to confirm the presence or absence of an aromatic ring in the analyzed structure.

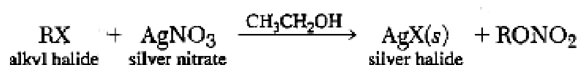
4.2. Halides

Silver Nitrate Test

The silver nitrate reaction proceeds by a carbocation (S_N1) process, thus there is the following reactivity order:

$AgNO_3$/ethanol test: $R_3CX > R_2CHX > RCH_2X$.

The halide is displaced from the alkyl halide to form an insoluble salt – a silver halide precipitate:

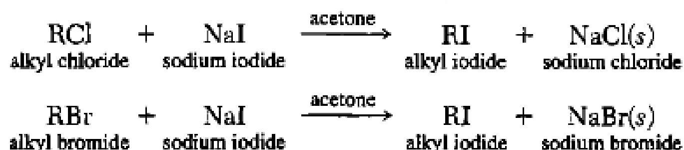


Experiment
One drop or a couple of crystals of the unknown compound should be added to 2 mL of the 2% ethanolic silver nitrate solution. If no reaction is observed after 5 minutes of standing at room temperature, the mixture should be heated to boil, to be able to observe some precipitate and to note its colour. Then there is a need to add two drops of 5% nitric acid, to observe if the precipitate is dissolving. Silver halides are insoluble in dilute nitric acid, instead of silver salts of organic acids.

Discussion
The identity of the halogen can sometimes be determined from the colour of the silver nitrate halide. Silver chloride is white, silver bromide is pale yellow, whereas silver iodide has yellow colour. The most reactive halides are those that are ionic. Among organic compounds, the amine salts of the halogen acids constitute the most common examples.

Sodium Iodide in Acetone Test

The sodium iodide reaction with halides goes by a direct displacement (S_N2). The sodium iodide test can be used to test for the presence of bromine or chlorine, where sodium halides precipitate from the solution.



This test is used to classify aliphatic chlorides and bromides as primary, secondary or tertiary, in the following reactivity order:

$$\text{NaI/acetone test:} \quad R_3CX < R_2CHX < RCH_2X.$$

Sodium iodine in acetone reagent

15 g of sodium iodide should be diluted in 100 mL of acetone. The solution, colourless at first, becomes a pale lemon yellow. It should be kept in a dark bottle and discarded as soon as a definite red-brown colour develops.

Experiment

Two drops of the analyzed compound should be added to 1 mL of the sodium iodide in acetone reagent. If the compound is solid, 0.1 g of it should be dissolved in the smallest possible volume of acetone, and then this solution should be added to the reagent. The reacting mixture in the test tube should be shaken and allowed to stand at room temperature for 3 minutes. It should be noted whether a precipitate is formed and also whether the solution turns reddish brown, because of the liberation of free iodine. If no change occurs at room temperature, the test tube should be placed in a water bath at 50°C. Excessive heating causes loss of acetone and precipitation of sodium iodide, which can lead to false-positive results. At the end of the sixth minute, the mixture should be cooled to room temperature.

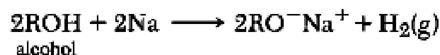
Discussion

With sodium iodide, primary bromides give a precipitate of sodium bromide within 3 minutes at 25°C whereas the chlorides give no precipitate and must be heated to 50°C in order to effect the reaction. Tertiary chlorides will react if the test solutions are allowed to stand for a day or two.

4.3. Compounds with Oxygen Atom

4.3.1. Alcohols, Phenols

Sodium Detection of Active Hydrogen
Sodium metal undergoes reaction with hydroxyl groups of many alcohols to liberate hydrogen gas and form the salt of the alcohol. The rate is highly variable and depends upon the alcohol structure.

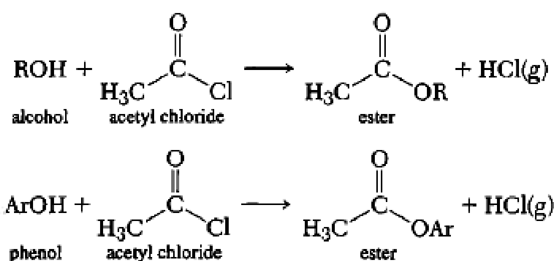


Experiment
Caution: *The test must be performed in the fume hood.* Small thin slices of freshly cut sodium should be added to 0.25 mL or 0.25 g of the analyzed sample, until no more will dissolve. Evolution of hydrogen gas indicates the presence of an acidic hydrogen, such as a hydroxyl group in alcohol. The solution should be cooled and observed. Then an equal volume of ether should be added. Another positive test is the formation of the solid salt. Prior to testing, the sample should be dried with calcium sulfate. Any residual water will undergo reaction with sodium. This test may be applied to solid compounds or very viscous liquids by dissolving them in an inert solvent such as anhydrous ligroin or toluene.

Discussion
The order of reactivity of alcohols with sodium is unknown to decrease with the increasing size of the alkyl portion of molecule. This test is subject to many limitations, and the results should be interpreted with caution. This test is most useful with alcohols of intermediate molecular weight, such as those containing from three to eight carbon atoms. Lower alcohols are difficult to obtain in anhydrous condition.
Metallic sodium is thus a useful reagent for detecting the types of reactive hydrogen compounds that are not sufficiently active to produce hydrogen ions in an ionizing solvent. It is obviously unnecessary and dangerous to try the action of sodium on compounds known to be acids. Structural effects upon acidity are complex. It is well known that liquid samples of alcohols follow this order of reactivity:

In the gas phase, the reverse order applies. It has been suggested that larger alkyl groups stabilize the alkoxide ion by polarization, while bulky groups about/around? the oxygen destabilize solvation.

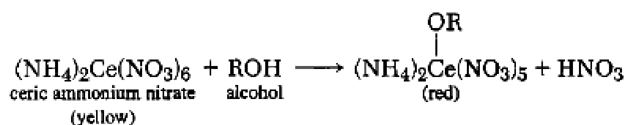
Detection of Active Hydrogen with Acetyl Chloride
Another method of detecting such active hydrogen is by adding acetyl chloride to an alcohol or a phenol to form the ester, which is less dense than the aqueous layer.



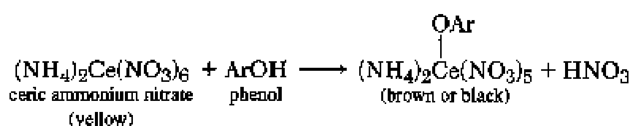
Experiment
Caution: *The test must be performed in the fume hood.* To the amount of 0.2 mL or 0.2 g of the analyzed compound, 0.2 mL of acetyl chloride should be added drop by drop. Evolution of heat and hydrogen chloride gas is a positive test. To destroy any unreacted acetyl chloride, the mixture should be allowed to stand for a minute or two, and then it should be poured cautiously into 1 mL of water.

Discussion
Alcohols and phenols react with acetyl chloride to form esters, which is indicated by the formation of a top layer in the flask. Tertiary alcohols form primarily the alkyl chloride, due to the reaction of the liberated hydrogen chloride on another molecule of the alcohol.

Ceric Ammonium Nitrate Test
The method of testing for the presence of the alcoholic hydrogen involves ceric ammonium nitrate. The yellow ceric ammonium nitrate forms a red organometallic compound with alcohols. The ceric ammonium nitrate reagent forms red complexes with primary secondary and tertiary alcohols of up to l0 carbons. Also, all types of glycols, polyols, carbohydrates, hydroxy acids, hydroxy aldehydes, and hydroxy ketones give red solutions.



Phenols give a brown colour or precipitate.



Ceric ammonium nitrate reagent
To 40 mL of distilled water 1.3 mL of concentrated nitric acid should be added. Thereafter 10.96 g of yellow ceric ammonium nitrate should be dissolved in the dilute nitric acid solution. This solution should be then diluted to 50 mL with water. The test is carried out at room temperature (20 – 25 °C). Hot solutions (50 – 100 °C) of Ce (IV) oxidize many types of organic compounds. This reagent is usable for about a month.

Experiments

For water-soluble compounds:

Four to five drops of a liquid sample or 0.1-0.2 g of a solid analyzed compound should be added to 1 mL of the ceric ammonium nitrate reagent. The mixture should be shaken well and it should be noted whether the yellow colour of the reagent will change to red.

Alcohols react with the reagent to form a red alkoxy cerium (IV) compound. If a red colour develops, the solution should be watched carefully and the time of becoming the mixture to colourless should be noted. If no change is noted in 15 min, the test tube should be allowed to stand several hours or overnight. It should be also noted whether bubbles of carbon dioxide are liberated.

For water-insoluble compounds:

Before the experiment, dioxane should be checked with ceric nitrate solution, to be sure that it does not give a positive test. Dioxane sold as "histological grade" is usually pure enough so that it may be used, and it does not give a red complex. Commercial dioxane sometimes contains glycols or antioxidants as preservatives and must be purified.

2 mL of dioxane should be added to 1 mL of the ceric ammonium nitrate reagent. If a red colour develops or if the solution becomes colourless, dioxane must be purified. If the mixture remains yellow or is only light orange-yellow, it may be used to test water-insoluble compounds. Then 6 mL of the solution should be divided in half, reserving 3 mL for observation as a control. To the other 3 mL of the dioxane containing reagent four to five drops of a liquid analyzed compound or 0.1-0.2 g of its solid form should be added. Then it should be mixed thoroughly and the same observations as in the test for water-soluble compounds should be made.

Discussion

The red cerium (IV) compounds has been shown to be the intermediate for the oxidation of alcohols by Ce (IV) solutions. Hence, a second phase of this test involves the disappearance of the red colour due to oxidation of the coordinated alcohol and reduction of the coloured Ce (IV) complex to the colourless Ce (III) complex. Thus a positive test includes the successive formation, and then the disappearance of the red colour, assuming the oxidation step occurs within a reasonable time.

A red colour is produced by aqueous 40% formaldehyde (formalin). This is due to methanol present in the solution. Acetaldehyde frequently gives a red colour due to the presence of 3-hydroxybutanal, acetaldol. Alternatively, these aldehydes may hydrate in aqueous solution to form gem diols, $RCH(OH)_2$, which may be the species that are oxidized.

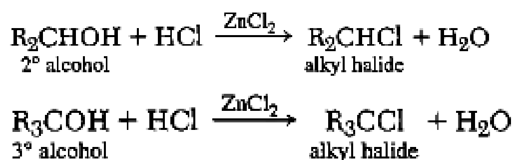
Negative tests are indicated by the absence of the red complex with retention of the yellow colour of the reagent. All pure aldehydes, ketones, saturated and unsaturated acids, ethers, esters, and dibasic and tribasic acids produce a negative test. The dibasic acids, oxalic and malonic ones, do not give a red colour but do reduce the yellow Ce (IV) to colourless Ce (III) solutions.

Alcohols containing halogens give positive tests to form red complexes. Very insoluble alcohols of high molecular weight such as l-hexadecanol, triphenylmethanol, or benzipinacol fail to react even in the dioxane solutions and do not give a red colour. Long-chain alcohols, C_{12} through C_{18}, will give a positive test when added to an acetonitrile solution of ammonium hexanitratocerate at the boiling point, 82 °C.

Lucas Test

Substrates that easily give rise to cationic character at the carbon bearing the hydroxyl group undergo the Lucas test readily. Therefore, only secondary (2’) and tertiary (3’) alcohols form

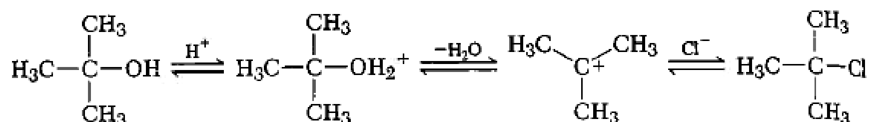
the alkyl halide, which appears as a second liquid layer, tertiary alcohols being the most reactive. Primary (1') alcohols undergo reaction with the zinc chloride and hydrochloric acid either negligibly slowly or not at all.



Lucas reagent
To prepare Lucas reagent 13.6 g (0.1 mole) of anhydrous zinc chloride should be dissolved in 10.5 g (0.1 mole) of concentrated hydrochloric acid, with cooling.

Experiment
The amount of 2 mL of the Lucas reagent should be added to 0.2 mL or 0.2 g of the sample in a test tube, at 26 – 27°C. The tube should be stopped and shaken very well, then allowed to stand. The time required for the formation of the alkyl chloride should be noted, it appears as an insoluble layer or emulsion. Alternatively 1.2 mL of concentrated hydrochloric acid should be added to 0.2 mL or 0.2 g of the alcohol in a test tube. The mixture should be shaken well, and allowed to stand. The mixture should be observed carefully during the first 2 minutes.

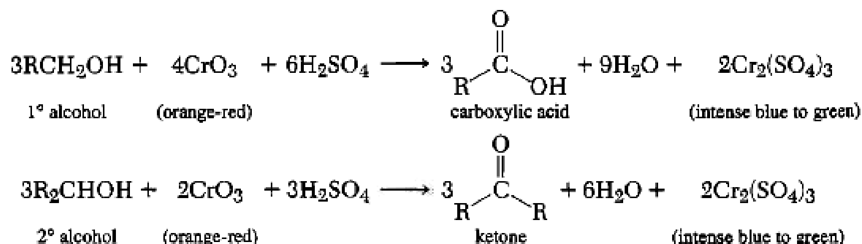
Discussion
The mechanism of the Lucas test is an S_N1-type process is as follows:



Since the Lucas test depends on the appearance of the alkyl chloride as a second liquid phase, it is normally applicable only to alcohols that are soluble in the reagent. This limits the test in general to nonfunctional alcohols lower than hexyl and certain polyfunctional molecules.
Tertiary alcohols react with concentrated hydrochloric acid so rapidly that the alkyl halide is visible within a few minutes at room temperature, first as a milky suspension and then as an oily layer. The acidity of the medium is increased by the addition of anhydrous zinc chloride, which is a strong Lewis acid, and, as a result, the reaction rate is increased. The high reactivity of tertiary alcohols is a consequence of the relatively great stability of the intermediate carbocation. Allyl alcohol, although a primary alcohol, yields a carbocation that is relatively stable because its charge is distributed equally on the two terminal carbon atoms. As a result, allyl alcohol reacts rapidly with the Lucas reagent and the reaction is accompanied by the evolution of heat. Addition of ice water to the reaction results in the formation of the allyl chloride as a separate layer.
Secondary alcohols are intermediate in reactivity between primary and tertiary alcohols. Although they are not appreciably affected by concentrated hydrochloric acid alone, they react with it fairly rapidly in the presence of anhydrous zinc chloride. A cloudy appearance of the mixture is observed within 5 minutes, and in 10 minutes a distinct layer is usually visible.

Chromic Anhydride (Jones Oxidation) Test

The Jones oxidation, in conjunction with the sodium metal test and the Lucas test, may be used to differentiate among primary (1'), secondary (2'), and tertiary (3') alcohols. The Jones oxidation only detects the presence of a hydroxyl substituent that is at least one hydrogen on a carbon bearing. Thus, only primary and secondary alcohols are oxidized to the corresponding carboxylic acids and ketones. Tertiary alcohols are not oxidized under these conditions.



Jones reagent

A suspension of 25 g of chromic anhydride (CrO_3) in 25 mL of concentrated sulfuric acid should be poured slowly with stirring, into 75 mL of water. Then the deep orange-red solution should be cooled to room temperature before use. It is necessary to use a good grade of acetone. Some samples of acetone may become cloudy in appearance in 20 seconds, but this does not interfere, providing the test solution becomes yellow. If the acetone gives a positive test, it should be purified by adding a small amount of potassium permanganate, and the mixture should be distilled.

Experiment

One drop of the analyzed liquid or about 10 mg of a solid compound should be added to 1 mL of acetone in a small test tube. Then one drop of the Jones reagent should be added to the mixture, and the result should be noted within 2 seconds. A control test should be made, where instead of the reagent one drop of acetone should be added, and the result should be compared. A positive test for primary or secondary alcohols consists in the production of an opaque suspension with a green to blue colour. Tertiary alcohols give no visible reaction within 2 seconds, the solution remaining orange in colour. Disregard any changes after 2 seconds.

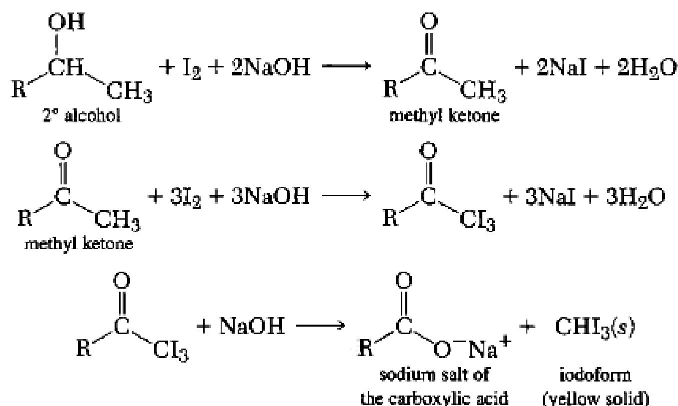
Discussion

This test is a rapid method for distinguishing primary and secondary alcohols from tertiary alcohols. Positive tests are given by primary and secondary alcohols without restriction as to molecular weight. Even cholesterol ($C_{27}H_{46}O$) gives a positive test. Aldehydes give a positive test but would be detected by other classification experiments. Aldehydes produce the green color in 5−15 seconds, with aliphatic aldehydes reacting more quickly than aromatic aldehydes. Ketones do not react. Olefins, acetylenes, amines, ethers, and ketones give negative tests within 2 seconds provided that they are not contaminated with small amounts of alcohols. Enols may give a positive test, and phenols produce a dark-coloured solution entirely unlike the characteristic green-blue colour of a positive test.

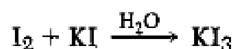
Iodoform Test

The iodoform test gives positive results for secondary alcohols in which a methyl group is attached to the carbon bearing of the hydroxyl group. This type of alcohol is oxidized to a

methyl ketone and under these basic conditions it forms a triiodo intermediate, which is then oxidized to the sodium salt of the acid and iodoform. Iodoform is a foul-smelling yellow precipitate.



Iodine-potassium solution
20.0 g of potassium iodide and 10.0 g of iodine should be added to 80.0 mL of water and stirred until the reaction is complete. The solution is deep brown due to the triiodide anion (I_3^-).



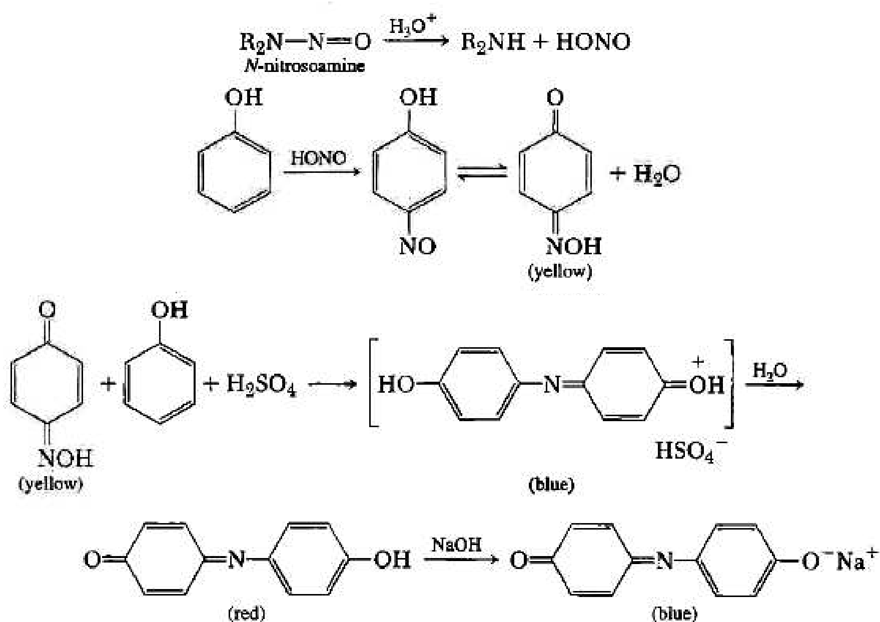
Experiment
Four drops of an analyzed compound in a liquid form or 0.1 g of a solid should be added into a test tube. Then 5 mL of dioxane should be added and shaken until all the sample have gone into solution, and thereafter 1 mL of l0 % NaOH solution is added?. The iodine-potassium iodide solution should be added slowly, with shaking, until a slight excess yields a definite dark colour of iodine. If less than 2 mL of the iodine solution is decolorized, there is a need to place the test tube in a water bath at a temperature of 60 °C. If the slight excess of iodine already present is decolorized, the addition of the iodine solution should be continued (the iodine solution must be kept at 60 °C), with shaking, until a slight excess of iodine solution again yields a definite dark colour. The addition of iodine should be continued until the dark colour is not discharged by 2 minutes of heating at 60 °C. The excess of iodine should be removed by the addition of a few drops of 10 % NaOH solution, with shaking. Thereafter the test tube should be filled with water and allowed to stand for 15 minutes. A positive test is indicated by the formation of a foul-smelling yellow precipitate (iodoform). The precipitate should be collected by filtration and dried to be able to measure the melting point. Iodoform (CHI_3) melts at 119 –121 °C and has a distinctive foul odour. If the iodoform is reddish, it is necessary to dissolve it in 3 – 4 mL of dioxane, and 1 mL of 10 % NaOH solution should be added, and shaken until only a light lemon colour remains. The precipitate should be diluted with water and filtered.

Discussion
The test will, be positive for any compound that reacts with the reagent to give a derivative containing one of the requisite groupings. Conversely, compounds that contain one of the requisite groupings will not give iodoform if that grouping is destroyed by the hydrolytic

action of the reagent before iodination is complete. The cleavage of trihalo ketones with base, exemplified by the second step of the iodoform test, is related to the reversal of the Claisen condensation. Bifunctional alcohols and ketones give positive iodoform tests. β-Keto esters do not produce iodoform by the test method, but their alkaline solutions do react with sodium hypoiodite. Acetoacetic acid is unstable, and its acidic aqueous solutions decompose to give CO_2 and acetone. Moreover, acetone will give a positive iodoform test. This behaviour is generally useful if a β-keto ester is one of the considered possibilities since these esters are hydrolyzed by boiling with 5 % sulfuric acid (acid-induced retro condensation).

Liebermann's Nitroso Reaction for Phenols

Liebermann's nitroso reaction is very helpful to indicate the presence of phenols. The final product of this reaction is the blue colour of the analyzed sample. This reaction is characteristic of phenols in which an ortho- or para- position is unsubstituted.



Experiment

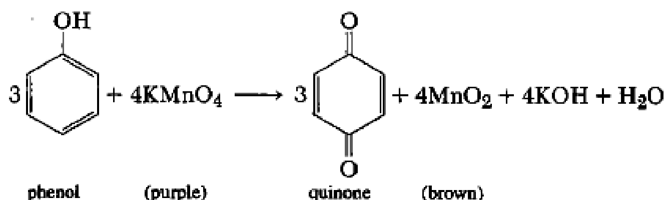
0.05 g of *N*-nitrosoamine, 0.05 g of the analyzed phenolic compound, and 2 mL of concentrated sulfuric acid should be mixed in a test tube, and then warmed gently for 20 seconds. The solution should be cooled slightly. A blue colour should develop, which changes to red when the solution is poured into 20 mL of ice water. Thereafter 10% sodium hydroxide solution should be added until the mixture is alkaline, and the blue colour is produced again.

To run a comparative test in order to check the colours, the following procedure may be followed. A crystal of sodium nitrite should be added to 2 mL of concentrated sulfuric acid, and the mixture should be shaken until dissolved. Then 0.1 g of phenolic compound should be added, and a blue colour will appear. The solution should be poured into 20 mL of ice water,

and the colour of the solution will change to red. Addition of 10% sodium hydroxide, until the mixture is alkaline, results in the return of the blue colour.

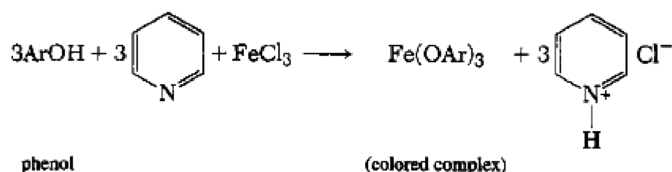
Discussion
The *N*-nitrosoamine liberates nitrous acid in the presence of sulfuric acid. The nitrous acid then undergoes a reaction with phenol to yield the yellow 4-nitrosophenol (quinone monoxime). The blue colour observed in this reaction is due to phenolindophenol formed from the reaction of the initially produced 4-nitrosophenol with excess phenol. The coupling reaction between certain diazonium salts and phenols has been shown to involve reaction between a diazonium ion and a phenoxide ion. If the solution is too acidic, the phenoxide ion is converted to phenol and thus the reaction is retarded. If the solution is too basic, the diazonium ion reacts with the hydroxide ion to give diazotate – ArN_2O^- which does not couple. The solution must, therefore, be properly buffered for a satisfactory coupling reaction.

Potassium Permanganate Test
Phenols reduce potassium permanganate solutions and undergo oxidation to quinones. An excess of the reagent yields a series of oxidation products, including maleic acid, oxalic acid, and carbon dioxide. The manganese is reduced from $^{+}7$, which gives a purple solution, to $^{+}4$, which is brown.



There is more information about the procedure of this experiment in chapter No 4.1.2.

Ferric Chloride-Pyridine Test
Phenols can be detected by treatment with ferric chloride. The procedure using pyridine solvent has resulted in accurate results in 90% of the phenolic substrates tested. Previous procedures using water or alcohol-water solvents had only a 50% success rate. The colour of the solution changes immediately to blue, violet, purple, green, or red-brown.



1% ferric chloride in chloroform
1 g of the black crystals of anhydrous ferric chloride should be added to 100 mL of pure chloroform. The mixture should be shaken occasionally for about an hour and allowed to

stand to permit the insoluble material to settle. The pale-yellow solution should be decanted into a screw-cap bottle fitted with a medicine dropper.

Experiment

Caution*: This test must be done in the fume hood.* 30 to 50 mg of the analyzed solid compound or four to five drops of its liquid form should be added to 2 mL or pure chloroform in a clean, dry test tube. The solution should be stirred. If the unknown compound does not seem to dissolve, even partially, an additional 2-3 mL of chloroform should be added and the mixture should be warmed gently, and then cooled to 25°C. Two drops of 1% solution of anhydrous ferric chloride in chloroform should be added followed by three drops of pyridine. The tube with the mixture should be shaken and the colour produced immediately should be noted. A positive test is shown by the production of a blue, violet, purple, green, or red-brown solution. Frequently the colours change in a few minutes.

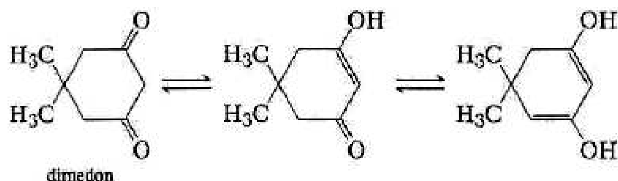
Discussion

This reagent is useful for detecting compounds containing a hydroxyl group directly attached to an aromatic nucleus. Treatment of chloroform solutions of phenols, naphthols, and their ring-substituted derivatives with a chloroform solution of anhydrous ferric chloride and pyridine produces characteristic blue, violet, purple, green, or red-brown complexes.

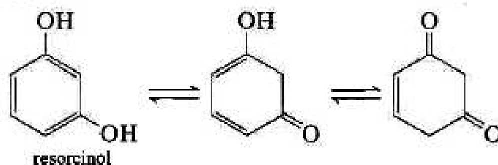
Alcohols, ethers, aldehydes, acids, ketones, hydrocarbons, and their halogen derivatives give negative results of colourless, pale-yellow, or tan solutions. This method is especially valuable for substituted phenols and naphthols that are very insoluble in water. Even 2,4,6-trichlorophenol, 2,4,6-tribromophenol, nonylphenol, phenolphthalein, and thymolphthalein give positive tests that sufficient chloroform (about 5 mL) is used to get them into solution.

Phenolic compounds that have failed to give positive tests are picric acid, 2,6-di-*tert*-butylphenol, phenolsulfonic acid, naphtholsulfonic acid, hydroquinone, *dl*-tyrosine, 4-hydroxylphenylglycine, and 4-hydroxybenzoic acid. The 4-hydroxybenzoic acid gives a distinct yellow colour, a negative result, whereas salicylic acid gives a violet colour, a positive test. The esters of 4-hydroxybenzoic acid produce purple colours and 4-hydroxybenzaldehyde a violet-purple colour.

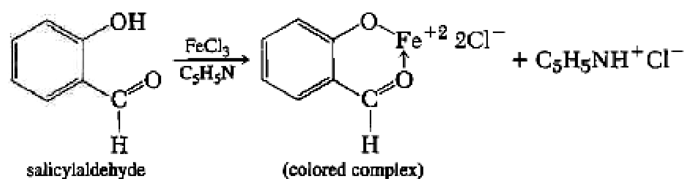
It interesting that 5,5-dimethyl-1,3-cyclohexandione (dimedon, methone) gives a beautiful purple colour.



Resorcinol gives a blue-violet colour. Note that several tautomeric forms of these compounds are similar in structure to tautomeric forms of phenols.



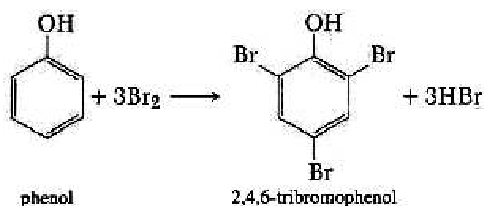
Salicylaldehyde forms a highly coloured complex with ferric chloride.



In aqueous or aqueous alcoholic solutions, some enols, oximes, and hydroxamic acids produce red-, brown-, or magenta-coloured complexes with aqueous ferric chloride. In aqueous solutions, aldehydes and ketones with α-hydrogens may tautomerize to the enol form, which will then give a violet-, red-, or tan-coloured complex with the ferric chloride.

Bromine Water Test

Since the aromatic nucleus of a phenol is substantially more reactive toward electrophilic aromatic substitution than benzene, bromination of phenols should be carried out under mild conditions. The discharge of the bromine colour is a positive test.



Experiment

0.1 g of the unknown compound should be dissolved in 10 mL of water. Then bromine water should be added drop by drop until the bromine colour is no longer discharged. The discharge of the bromine colour is a positive test. In some cases, a white precipitate (the brominated phenol) may also be formed.

Discussion

It has been shown that in the bromination of benzene and 2-nitroanisole with bromine water, the brominating agent operates by complex mechanisms. The advantage of bromine in water over bromine in carbon tetrachloride or methylene chloride is that the more polar solvent greatly increases the rate of bromination by the ionic mechanism. Of course, it is impossible to observe the evolution of hydrogen bromide with this solvent. An excess of bromine water converts tribromophenol to a yellow tetrabromo derivative, 2,4,4,6-tetrabromocyclohexadienone. The tetrabromo compound is readily converted to the tribromophenol by washing with 2% hydroiodic acid.

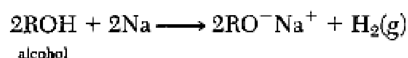
4.3.2. Carbohydrates

Monosaccharides, oligo- or polysaccharides are polyhydroxyaldehydes and ketones. These compounds are usually water-soluble solids that melt with decomposition. These

characteristics correctly point toward the presence of a large number of highly polar functional groups in these molecules.

Sodium Detection of Active Hydrogen

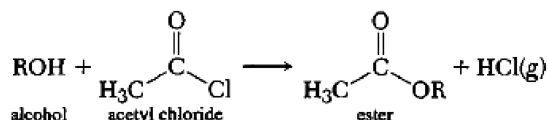
Sodium metal undergoes reaction with hydroxyl groups of many compounds, it is/forms?? a liberate hydrogen gas and forms the salt of the alcohol.



This experiment is described in chapter No 4.3.1., with reference to alcohols identification.

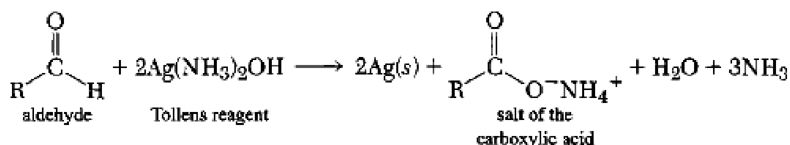
Detection of Active Hydrogen with Acetyl Chloride

The presence of the hydroxyl group in a carbohydrate structure can be detected with acetyl chloride. The reaction of the hydroxyl group with the acetyl chloride yields an ester, which appears as another liquid layer. This experiment is described in chapter no 4.3.1.



Tollens Test

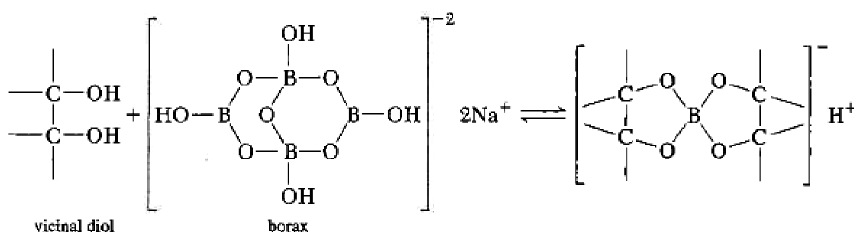
The Tollens test can be used to test for the presence of the aldehyde group in these carbohydrates which are aldoses. The aldehyde functional group is oxidized to the salt of the carboxylic acid with the silver ion being reduced to elemental silver which is deposited as a coating inside the reaction flask.



This reaction is described in chapter 4.3.3.

Borax Test

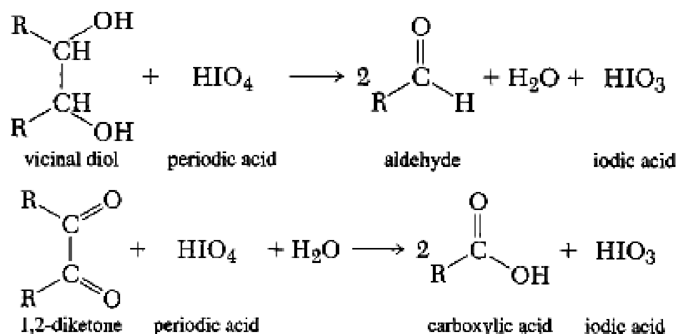
Vicinal diols can be detected with borax – sodium tetraborate decahydrate. Borax exists as $Na_2[B_4O_5(OH)_4] \times 8\ H_2O$. The resulting solution, with the addition of phenolphthalein indicator is colourless at room temperature, but yields a pink solution when warmed.



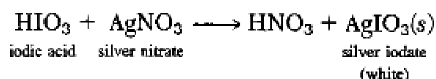
Experiment
In a test tube, a few drops of phenolphthalein should be added to 0.5 mL of a l % solution of borax. A pink solution is formed. A couple of drops or a few crystals of the unknown compound should be added to the mixture. If the pink colour begins to fade after the analyzed compound and the reagent have been mixed together, then it is necessary to continue adding small amounts of the substance until the pink colour lades completely. The test tube should be placed in a hot-water bath. If the pink colour reappears on warming, and dissipates again on cooling, then the unknown compound is a polyhydric alcohol.

Discussion
Carbohydrates and 1,2-diols give a positive test.

Periodic Acid Oxidation
α-Hydroxy ketones and α-hydroxy aldehydes are oxidized with periodic acid.



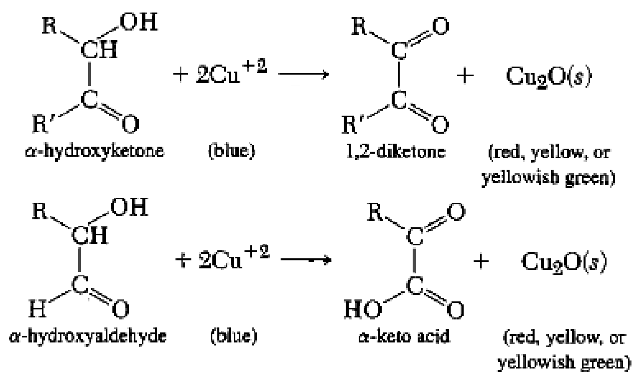
The iodic acid produced above is detected with 5% silver nitrate solution. An immediate precipitation of silver iodate occurs.



There is more information about this reaction in chapter 4.3.3.

Benedict's Solution Test

Benedict's solution will undergo reaction with reducing sugars such as α-hydroxy ketones and α-hydroxy aldehydes. The solution is initially blue from Cu^{+2} complex, but as the reaction proceeds, copper (I) oxide precipitates as a red, yellow, or yellowish-green solid.



Benedict's solution

A solution of 17.3 g of sodium citrate and 10.0 g of anhydrous sodium carbonate should be heated in 80.0 mL of water until the salts are dissolved. Then additional water should be added to bring the volume up to 85.0 mL. The solution of 1.73 g of hydrated copper sulfate in 10.0 mL of water should be poured slowly, with stirring, into the solution of the citrate and carbonate. The additional water should be added to bring the volume of the solution up to 100 mL.

Experiment

To a solution or suspension of 0.2 g of the compound in 5 mL of water, 5 mL of Benedict's solution should be added. Benedict's solution oxidizes a variety of compounds, with the corresponding reduction of Cu^{+2} to Cu^{+1}. The precipitation of the copper (I) oxide as a red, yellow, or yellowish-green solid is a positive test. If no precipitate is formed, the mixture should be heated to boiling and then cooled. It should be noted if any solid is formed. Two drops of concentrated hydrochloric acid should be added to a solution of 0.2 g of sucrose in 5 mL of water and the solution should be boiled for a minute. Thereafter the solution should be cooled, the acid shpuld be neutralized with dilute NaOH solution and the action of Benedict's solution should be tried again.

Discussion

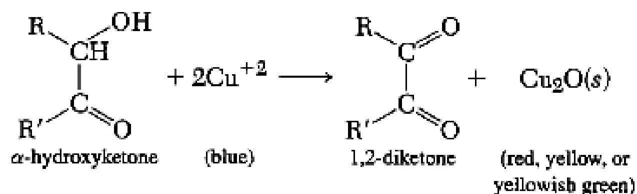
Benedict's solution, which contains the copper bound in the complex anion, functions as a selective oxidizing agent. It was introduced as a reagent for reducing sugars to replace Fehling's solution, which is very strongly alkaline. Benedict's reagent will detect 0.0l % of glucose in water. The colour of the precipitate may be red, yellow, or yellowish green, depending on the nature and amount of the reducing agent present.

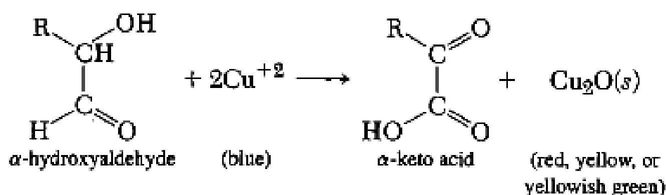
Benedict's reagent is reduced by α-hydroxy aldehydes, α-hydroxy ketones, and α-keto aldehydes. It does not oxidize simple aromatic aldehydes. Molecules containing only the alcohol functional group or only the keto group are not oxidized by Benedict's solution.

Hydrazine derivatives, as exemplified by phenylhydrazine and hydrazobenzene, are oxidized by this reagent. Other easily oxidizable systems, such as phenylhydroxylamine, aminophenol, and related photographic developers, also reduce Benedict's solution.

Fehling's Solution Test

Fehling's solution, similarly to Benedict's solution, is able to react with reducing sugars such as α-hydroxy ketones and α-hydroxy aldehydes. The solution is also initially blue from Cu^{+2} complex, but as the reaction proceeds, copper (I) oxide precipitates as a red, yellow, or yellowish-green solid.





Fehling's solution

2.5 mL of the following two solutions should be mixed immediately before use.

Fehling's solution no 1: 8.65 g of hydrated copper sulfate crystals should be dissolved in 100 mL of water and this solution should be diluted to 125 mL.

Fehling's solution no 2: 43.2 g of sodium potassium tartrate and 17.5 g of sodium hydroxide should be dissolved in 50 mL of water and then it should be diluted to 125 mL.

Experiment

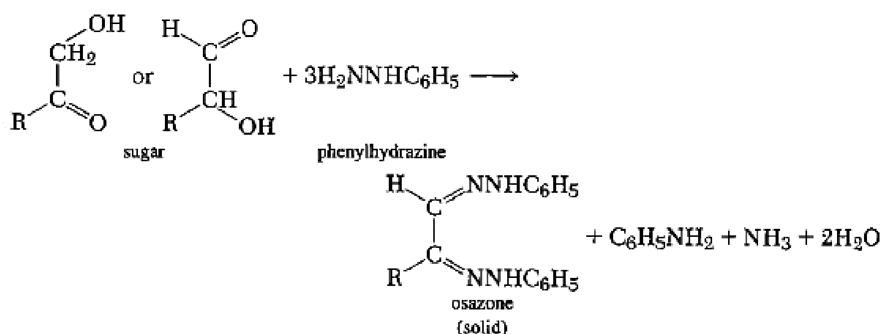
5 mL of Fehling's solution should be added to the solution of 0.2 g of the analyzed compound dissolved in 5 mL of water, the mixture should be heated to boiling, and thereafter it should be cooled. Fehling's solution oxidizes many compounds, and copper in the reagent is reduced from Cu^{+2} to Cu^{+1}. The precipitation of the copper (I) oxide as a red, yellow, or yellowish-green solid is a positive test.

Discussion

Benedict's and Fehling's solutions serve as a test for reducing sugars. Non-reducing sugars are hydrolyzed by heating with a small amount of l0 % HCl, then neutralized with 10% NaOH. The resulting solution will then give a positive test with Benedict's and Fehling's solutions.

Osazones Formation

The preparation of osazones from carbohydrates and phenylhydrazine along with the time required for the solid osazone to form can be used in distinguishing among the various sugars. Osazone formation involves hydrazone formation at C-l of an aldose or C-2 of a ketose, and oxidation of C-2 (or C-1) of an alcohol group to a ketone or an aldehyde.

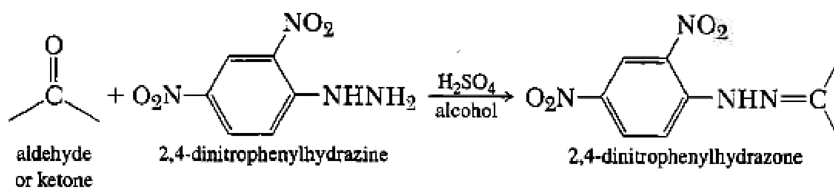


Experiment
0.2 g of the unknown sample should be put into a test tube, and then also 0.4 g of phenylhydrazine hydrochloride, 0.6 g of crystallized sodium acetate, and 4 mL of distilled water. The test tube should be put in a beaker of boiling water and the time that the test tube was immersed and, finally, the time of the precipitation should be noted. After 20 min, the test tube should be removed from the hot-water bath and cooled. A small amount of the liquid and solid should be put on a watch glass and tipped on the watch glass from side to side to spread out the crystals. Then some of the mother liquor should be absorbed with a piece of filtering paper, taking care not to crush or break up the clumps of crystals. The crystals should be examined under a low-power microscope (80 – 100 ×) and compared with photomicrographs.

Discussion
The time required for the formation of the osazone can be a valuable aid in distinguishing among various sugars. The following figures are the times required for the osazone to precipitate from the hot solution: fructose – 2 minutes, glucose – 4-5 minutes, xylose – 7 minutes, arabinose – 10 minutes, galactose – 15-19 minutes, raffinose – 60 minutes, and lactose osazone soluble in hot water, maltose osazone soluble in hot water and mannose – 0.5 minute, whereas sucrose – 30 min (owing to hydrolysis and formation of glucosazone).

4.3.3. Aldehydes, Ketones, Chinones

2,4-Dinitrophenylhydrazine Test
The reaction of aldehydes and ketones with 2,4-dinitrophenylhydrazine to form the 2,4-dinitrophenylhydrazone probably represents the most studied and most successful of all qualitative tests and derivatizing procedures. In addition, the general details of the reaction serve as a model for a number of other chemical reactions (osazone, semicarbazone, oxime, and other arylhydrazone preparations). The 2,4- dinitrophenylhydrazone precipitates from the solution.

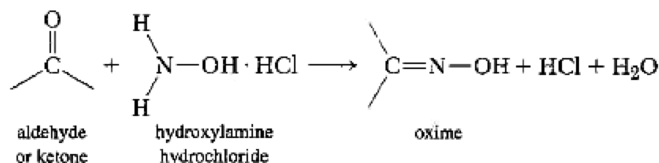


2,4-Dinitrophenylhydrazine reagent
3 g of 2,4-dinitrophenylhydrazine should be dissolved in 15 mL of concentrated sulfuric acid. This solution, with stirring, should be added to 20 mL of water and 70 mL of 95% ethanol. It should be mixed thoroughly and then filtered.

Experiment
A solution of one or two drops or about 50 mg of the analyzed compound should be dissolved in 2 mL of 95% ethanol and then added to 3 mL of 2,4-dinitrophenylhydrazine reagent. The mixture should be shaken vigorously and, if no precipitate forms immediately, it should be allowed to stand for 15 min. If needed, the precipitate can be recrystallized from ethanol.

Discussion
Most aldehydes and ketones yield dinitrophenylhydrazones that are insoluble solids. The precipitate may be oily at first and become crystalline on standing. A number of ketones, however, give dinitrophenylhydrazones that are oils. For example, 2-decanone, 6-undecanone, and similar substances fail to form solid dinitrophenylhydrazones. A further difficulty with the test is that certain allyl alcohol derivatives may be oxidized by the reagent to aldehydes or ketones, which then give a positive test. The colour of a 2,4-dinitrophenylhydrazone may give an indication as to the structure of the aldehyde or ketone from which it is derived. The dinitrophenylhydrazones of aldehydes and ketones in which the carbonyl group is not conjugated with another functional group are yellow. Conjugation with a carbon-carbon double bond or with a benzene ring shifts the absorption maximum toward the visible and is easily detected by an examination of the ultraviolet spectrum. However, this shift is also responsible for a change in colour from yellow to orange-red. In general, a yellow dinitrophenylhydrazone may be assumed to be unconjugated. However, an orange or red colour should be interpreted with caution, since it may be due to contamination by an impurity.

Hydroxylamine Hydrochloride Test
In the reaction of hydroxylamine hydrochloride with aldehydes, the formation of the oximes results in the liberation of hydrogen chloride, which can be detected by the change in colour from orange to red of a pH indicator.

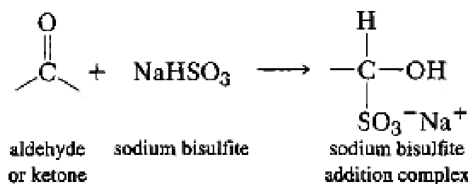


Bogen or Grammercy universal indicator – hydroxylamine hydrochloride reagent
0.3 mL of Bogen or Grammercy universal indicator should be added to a solution of 500 mg of hydroxylamine hydrochloride in 100 mL of 95% ethanol. Grammercy indicator is getting increasingly difficult to find. The colour of the solution should be adjusted to a bright-orange shade (pH 3.7-3.9) by adding 95% ethanolic sodium hydroxide or 95% ethanolic hydrochloric acid dropwise. The reagent is stable for several months.

Indicator solution
0.3 mL of either of the above solutions should be added to 100 mL of 95% ethanol to make a solution of the indicator.
Experiments
For neutral aldehydes or ketones:
A drop or a few crystals of the analyzed compound should be added to 1 mL of the Bogen or Grammercy indicator – hydroxylamine hydrochloride reagent. The colour change should be noted. If no pronounced change occurs at room temperature, the mixture should be heated to boiling. A change in colour from orange to red constitutes a positive test.

For acidic or basic aldehydes or ketones:
About 0.2 g of the identified compound should be added to 1 mL of the indicator solution. The colour of the mixture should be adjusted so that it matches 1 mL of the Bogen or Grammercy indicator – hydroxylamine hydrochloride reagent in a separate test tube of the same size. This is done by adding a few drops of 1% sodium hydroxide or 1% hydrochloric acid solution. Then the resulting solution should be added to 1 mL of the Bogen or Grammercy indicator – hydroxylamine hydrochloride reagent, and the result should be noted whether a red colour is produced.
Discussion
The change in colour of the indicator is due to the hydrochloric acid liberated in the reaction of the carbonyl compound with hydroxylamine hydrochloride, with the oxime not being sufficiently basic to form a hydrochloride. All aldehydes and most ketones give an immediate change in colour. Some higher-molecular-weight ketones such as benzophenone, benzil, benzoin, and camphor require heating. Sugars, quinones, and hindered ketones, such as 2-benzoylbenzoic acid, give a negative test.
Many aldehydes undergo auto-oxidation in the air and contain appreciable amounts of acids; hence the action of an aqueous solution or suspension on litmus must always be determined. If the solution is acidic, the second procedure must be used; this is also true of compounds whose solubility behaviour shows acids or bases. The reaction is reversible. Although in relatively weak acidic solutions the equilibrium may be made to lie far toward the right, strong acid removes semicarbazide by converting it to the conjugate acid and shifts the equilibrium toward the free ketone.

Sodium Bisulfite Addition Complex Reaction
The precipitation of a bisulfite addition complex is? indicative of a variety of carbonyl compounds reacting with sodium bisulfite. This reaction is greatly inhibited by the steric constraints about the carbonyl group.



Experiment
1 mL of ethanol should be added to 4 mL of a 40% aqueous sodium bisulfite solution to prepare an alcoholic solution of the sodium bisulfite. Any precipitated salt should be separated by decantation or filtration, before using the reagent. Then 1 mL of the reagent

should be placed in a test tube and 0.3 mL or 300 mg of the analyzed sample should be added. The test tube must be closed by a stopper and shaken vigorously. Aldehydes and ketones react with sodium bisulfite to form a solid. The formation of a solid is a positive test.

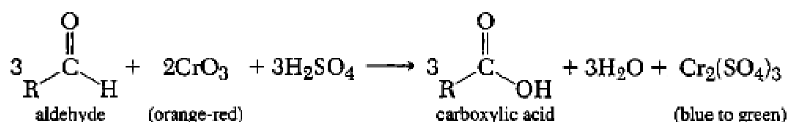
Discussion

The formation of bisulfite addition compounds, also known as α-hydroxyalkanesulfonates, is a general reaction of aldehydes. Most methyl ketones, low-molecular-weight cyclic ketones up to cyclooctanone, and certain other compounds having very active carbonyl groups behave similarly. Some methyl ketones, however, form the addition compounds slowly or not at all. Examples are aryl methyl ketones, pinacolone, and mesityl oxide, Cinnamaldehyde forms an addition compound containing two molecules of bisulfite.

The bisulfite addition compounds are in equilibrium with the carbonyl compound. These compounds are easily decomposed by either acids or alkalies to regenerate the original compounds, and thus they are stable only in neutral solutions. Compounds derived from low-molecular-weight carbonyl compounds are soluble in water. Another advantage of the bisulfite addition compounds is how easily they are purified. The nitrogen analogs of aldehydes, imines (or Schiff bases), also undergo reaction with sodium bisulfite. The product is identical with that formed by the action of a primary amine on the aldehyde bisulfite compound.

Chromic Anhydride (Jones Oxidation) Test

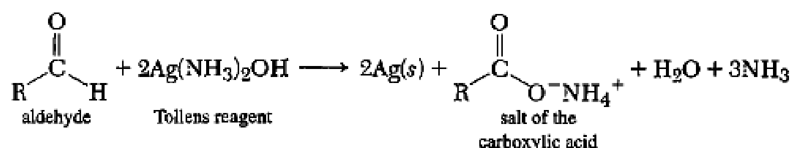
The Jones oxidation test may be used to the identification not only of alcohols, but also for detection of aldehydes, which oxidize to carboxylic acids. The chromium oxidizes from the 3rd oxidation state, which is an orange-red colour, to the 6th oxidation state, which is a deep-blue-green colour.



Aldehydes produce the green color in 5-15 seconds, with aliphatic aldehydes reacting more quickly than aromatic aldehydes, whereas ketones do not react. The procedure of this test is described in chapter 4.3.1.

Tollens Test

Aldehydes produce a silver mirror when mixed with Tollens reagent. As the aldehyde is oxidized to an acid, the silver is reduced from a +1 oxidation state to elemental silver and is deposited as a silver mirror or colloidal silver inside the reaction flask.



Tollens Reagent

A test tube should be cleaned with 10 % NaOH. 2 mL of a 5 % silver nitrate solution and a drop of 10 % sodium hydroxide should be mixed. Then 2% of ammonia solution should be added to this mixture, drop by drop, with constant shaking, until the precipitate of silver oxide just dissolves. In order to obtain a sensitive reagent, it is necessary to avoid a large excess of ammonia.

Caution: *This reagent should be prepared just before use and should not be stored, because the solution decomposes on standing and deposits a highly explosive precipitate.*

Experiment

One drop or a few crystals of the sample should be added to the freshly prepared Tollens reagent. A positive test is the formation of silver metal or colloidal silver. If no reaction takes place in the cold, the solution should be warmed slightly in a steam bath or in a hot water bath. However, excessive heating will cause the appearance of a false positive test by decomposition of the reagent.

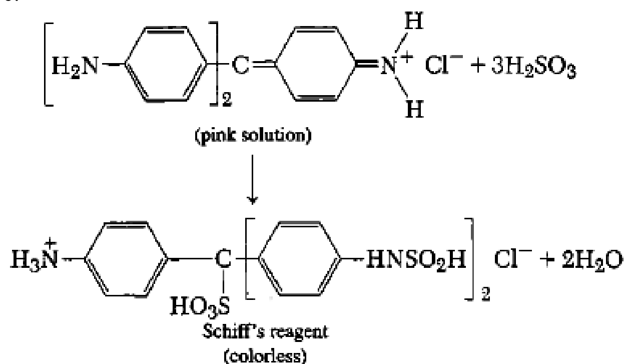
Discussion

It should be noted that diphenylamine and aromatic amines, as well as l-naphthols and certain other phenols, give a positive Tollens test. α-Alkoxy and α-dialkylamino ketones have been found to reduce ammoniacal silver nitrate. In addition, the stable hydrate of trifluoroacetaldehyde gives a positive test. This test often results in a smooth deposit of silver metal on the inner surface of the test tube; hence the name the "silver mirror" test. In some cases, however, the metal forms merely as a granular gray or black precipitate, especially if the glass is not scrupulously clean.

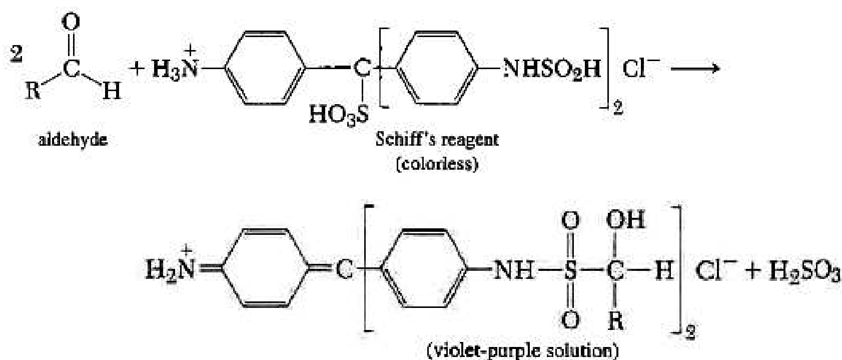
The reaction is autocatalyzed by the silver metal and often involves an induction period of a few minutes. False-negative tests are common with water insoluble aldehydes.

Fuchsin-Aldehyde Reagent (Schiff's Reagent) Test

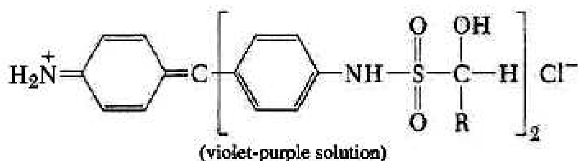
Schiff's reagent undergoes reaction with aldehydes to form a violet-purple solution. Fuchsin is a pink triphenylmethane dye that is converted to the colourless leucosulfonic acid by sulfurous acid. Apparently the reaction involves l,6-addition of sulfurous acid to the quinoid nucleus of the dye.



This violet-purple colour is different from the colour of the original fuchsin. It is not light pink, it has a blue cast bordering on violet or purple.



The leucosulfonic acid is unstable and loses sulfurous acid when treated with an aldehyde, resulting in a violet-purple quinoid dye.



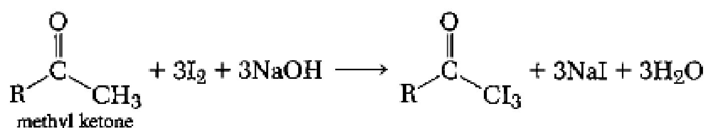
Schiff's reagent
0.05 g of certified basic fuchsin (4-rosaline hydrochloride) should be dissolved in 50 mL of distilled water. Then 2 mL of saturated sodium bisulfite solution should be added. After allowing the solution to sit for 1 h, 1 mL of concentrated hydrochloric acid must be added. It should be allowed to stand overnight. This reagent is practically colourless and very sensitive.

Experiment
2 mL of Schiff's reagent should be put into a test tube and two drops or a few crystals of the unknown compound should be added. The tube should be shaken gently, and the colour that will develop in 3-4 min should be observed. Aldehydes react with Schiff's reagent to form a complex which has a wine-purple colour.

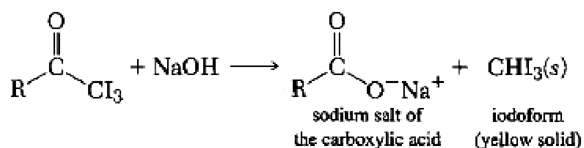
Discussion
Some ketones and unsaturated compounds react with sulfurous acid to regenerate the pink colour of the fuchsin. Therefore, the development of a light pink colour in the reagent is not a positive test for aldehydes. The fact that certain compounds cause the regeneration of the pink colour of the original fuchsin has been made the basis of a test. When a specially prepared reagent is used and the reaction time is 1 hour, aldoses produce a pink colour whereas ketoses and disaccharides, except maltose, do not. This modification of the Schiff test must be employed with caution, because many organic compounds produce a pink colour with the reagent when shaken in the air. Other compounds, such as α,β-unsaturated ketones, combine with sulfurous acid and thus reverse the first reaction given above.

Iodoform Test

The iodoform test will give positive results with methyl ketones. A positive test is indicated by? the precipitation of iodoform, a foul-smelling yellow solid.



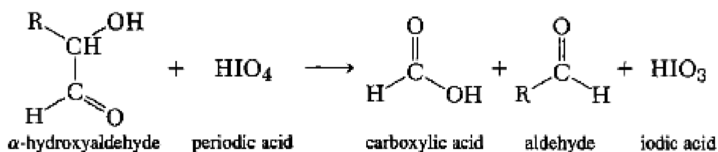
Cleavage produces the carboxylate salt and iodoform.

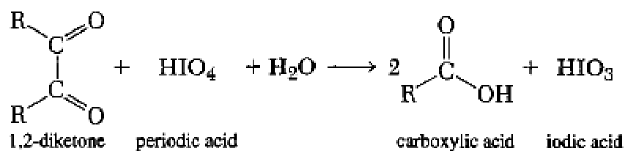


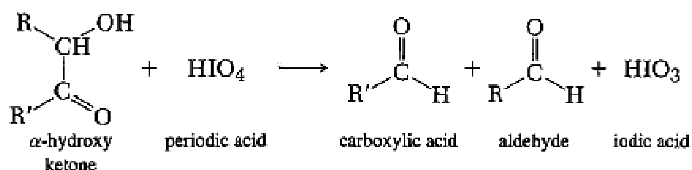
There is more information about this test in chapter 4.3.1.

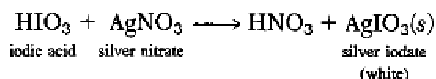
Periodic Acid Test

α-Hydroxy aldehydes, l,2-diketones as well as α-hydroxy ketones are oxidized with periodic acid, to give carboxylic acids, aldehydes and iodic acid. Iodic acid is detected with 5% silver nitrate solution. An immediate precipitation of silver iodate occurs.









Periodic acid reagent
0.5 g of *p*-periodic acid (H_5IO_6) should be dissolved in 100 mL of distilled water.

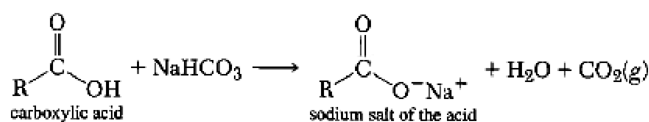
Experiment
One drop (no more) of concentrated nitric acid should be added to 2 mL of the periodic acid reagent in a small test tube and shaken thoroughly. Then one drop or a small crystal of the analyzed substance should be added. The mixture should be shaken for 10–15 seconds, and then one to two drops of 5% aqueous silver nitrate solution should be added. The instantaneous formation of a white precipitate (silver iodate) indicates that the analyzed organic compound has been oxidized by the periodate, which is thereby reduced to iodate. This constitutes a positive test. Failure to form a precipitate or the appearance of a brown precipitate that redissolves on shaking constitutes a negative test.

Discussion
Periodic acid has a very selective oxidizing action on l,2-glycols, α-hydroxy aldehydes, α-hydroxy ketones, 1,2-diketones, α-hydroxy acids, and α-amino alcohols. The rate of the reaction decreases in the above mentioned order. Under the conditions specified above, α-hydroxy acids sometimes give a negative test. β-Dicarbonyl compounds and other active methylene compounds also react.
It is important that the exact amounts of reagent and nitric acid must be used. The test depends on the fact that silver iodate is only slightly soluble in dilute nitric acid, whereas silver periodate is very soluble. If too much nitric acid is present, however, the silver iodate will fail to precipitate.
Olefins, secondary alcohols, 1,3-glycols, ketones, and aldehydes are not affected by periodic acid under the above conditions. The periodic acid test is best suited for water soluble compounds.

Benedict’s Solution Reaction and Fehling’s Solution Reaction
Benedict's solution and Fehling's solution will undergo reactions with aliphatic aldehydes but not with aromatic aldehydes. These reagents oxidize the aliphatic aldehyde to a carboxylic acid, and the copper in the reagent is reduced from +2 to +1. The copper (I) oxide precipitates as a red, yellow, or yellowish-green solid. These solutions will also undergo reactions with α-hydroxy ketones. There is more information about the procedures of these experiments in chapter 4.3.2.

4.3.4. Carboxylic Acids

Sodium Bicarbonate Test
Carboxylic acids should be primarily identified by spectroscopy and solubility tests. However, a few classification tests can be used to confirm the presence of the carboxylic group. Carboxylic acids react with a sodium bicarbonate solution to form the carboxylate anion and carbon dioxide gas.

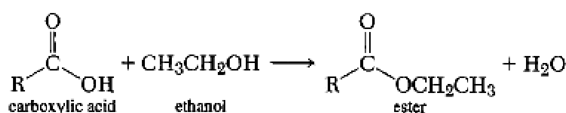


Experiment

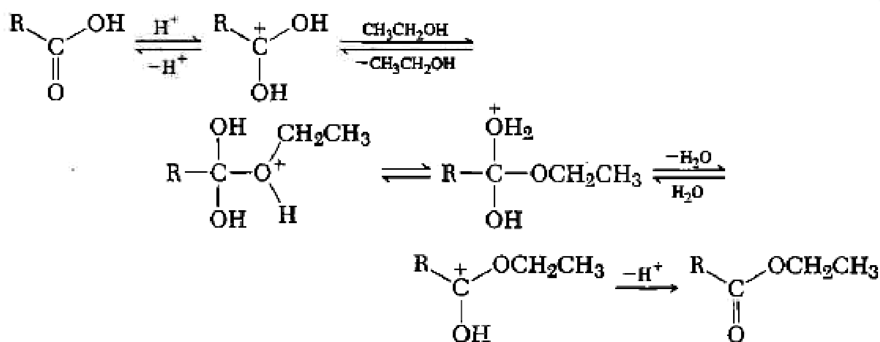
A few drops or a few crystals of the analyzed sample should be dissolved in 1 mL of methanol and slowly added to 1 mL of a saturated solution of sodium bicarbonate. Evolution of carbon dioxide gas is a positive test for the presence of the carboxylic acid.

Ester Formation Reaction

Another test for carboxylic acids involve the esterification of the acid. The ester forms another layer and has a sweet, fruity smell.



The mechanism of the esterification involves protonation as a method of enhancing the electrophilicity of the acid substrate toward nucleophilic attack by ethanol.



Experiment

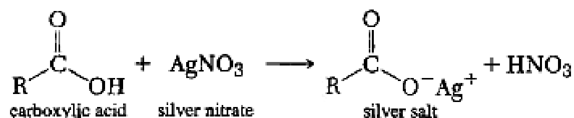
A mixture of 0.20 g of the identified compound should be warmed with 0.40 mL of absolute ethanol, and 0.20 mL of concentrated sulfuric acid over a steam bath or hot-water bath, for 2 minutes. The mixture should be poured slowly into an evaporating dish containing 2 mL of saturated sodium bicarbonate solution. A second layer should be formed. The mixture should be smelt carefully.

Discussion

The presence of a sweet, fruity smell in the product where no such smell existed in the original unknown, indicates that the original compound was a carboxylic acid and the acid was esterified. Large-molecular-weight carboxylic acids produce esters that are odourless.

Silver Nitrate Solution

Silver nitrate reacts with carboxylic acids to form silver salts of the carboxylic acid. These silver salts are soluble in dilute nitric acid, whereas silver halides are insoluble in nitric acid.

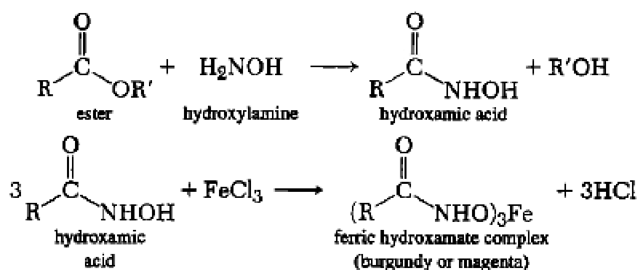


The procedure of this experiment is described in chapter 4.2.

4.3.5. Esters of Carboxylic Acids

Hydroxamic Acid Formation Reaction

Esters characteristically have a sweet fruity smell. Esters combine with hydroxylamine to yield an alcohol and hydroxamic acid. The solution is then treated with ferric chloride to produce the ferric hydroxamate complex, which has a characteristic burgundy or magenta colour.



Experiment

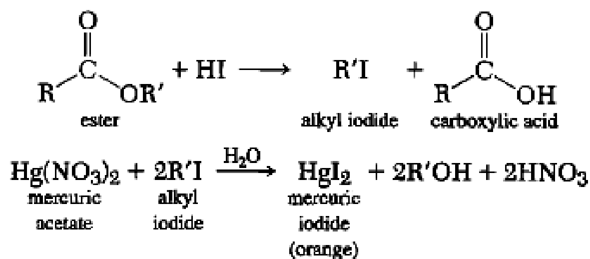
A mixture of one drop or about 40 mg of the analyzed compound, 1 mL of 0.5 M hydroxylamine hydrochloride in 95% ethanol, and 0.2 mL of 6 M sodium hydroxide should be heated to boiling. After the solution has cooled slightly, 2 mL of 1 M hydrochloric acid should be added cautiously. Anhydrides, acyl halides, and esters react with hydroxylamine to form the hydroxamic acid. If the solution is cloudy 2 mL of 95% ethanol should be added, to be able to observe the produced colour, one drop of 5% ferric chloride solution is added. If the colour caused by the drop of ferric chloride solution does not persist, there is a need to continue to add the ferric chloride solution drop wise until the observed colour permeates the entire test solution. A positive test will be a distinct burgundy or magenta colour of the ferric hydroxamate complex which is formed upon the reaction of the hydroxamic acid with the ferric chloride. The colour of this solution should be compared with the yellow one observed when the original compound is tested with ferric chloride in the presence of acid.

Discussion

All esters of carboxylic acids, including polyesters and lactones, give definite magenta colours of varying degrees of intensity. Acid chlorides, acid anhydrides, and trihalo

compounds such as trichloromethylbenzene and chloroform give positive magenta test results. Formic acid produces a red colour; with all other carboxylic acids the test is negative. Commercial lactic acid gives a positive test. Phthalic acid usually contains phthalic anhydride and thus gives a positive test.

Hydroiodic Acid Test (Zeisel's Alkoxyl Method)
Esters are cleaved by hydroiodic acid to form an alkyl iodide and a carboxylic acid. The allyl iodide is treated with mercuric nitrate to yield mercuric iodide which is orange in colour.



Gauze plugs
A solution of 0.10 g of lead acetate in 1.0 mL of water should be added to 6.0 mL of 1 M sodium hydroxide solution and should be stirred until the precipitate dissolves. Then a solution of 0.5 g of hydrated sodium thiosulfate in 1.0 mL of water should be added to this mixture, and next 0.5 mL of glycerol. This mixture should be finally diluted to 10.0 mL. Then 5 mL of this solution should be pipetted onto strips of double cheesecloth 2 by 45 cm. The strips of cloth should be dried and rolled to fit the test tube.

Mercuric nitrate solution
24.5 mL of a saturated solution of mercuric nitrate should be prepared, and thereafter 0.5 mL of concentrated nitric acid should be added into this solution.
Caution: *Mercury compounds are very toxic and require handling with special care.*

Experiment
Caution: *The test must be performed in the fume hood.* About 0.1 g or 0.1 mL of the analyzed compound should be placed in a 16-by-150-mm test tube. 1 mL of glacial acetic acid and 1 mL of 57% hydroiodic acid should be carefully added by means of a pipet. Then a boiling chip should be added and a gauze plug is inserted into the mouth of the test tube as described below. The gauze plug should be twisted so as to make a good fit and it should be pushed down so that it is 4 cm from the mouth of the test tube. A small piece of nonabsorbent cotton should be pushed down on the top of the plug by means of a glass rod so as to make a disk of cotton 2-3 mm thick. A piece of filter paper should be folded 2 by 10 cm longitudinally and moistened with a solution of mercuric nitrate, then it is placed on the cotton disk. The test tube should be immersed to a depth of 4-5 cm in an oil bath that is kept at 120-130 °C. When the reaction mixture boils, vapours rise through the porous plug which usually turns grey.
The volatile alkyl halide, rising through the plugs, reacts with the mercuric nitrate to produce a light-orange or vermilion colour due to the formation of the mercuric iodide. A positive test consists in the formation of an orange or vermilion colour on the test paper within a 10-minute heating period. A yellow colour constitutes a negative or doubtful test.

Discussion
This test is based on the classic Zeisel method for quantitatively estimating the percentage of methoxyl or ethoxyl groups. Functional groups containing methyl, ethyl, l-propyl, or 2-propyl radicals attached to oxygen are cleaved by the hydroiodic acid with the formation of a volatile alkyl halide. Alkoxy derivatives in which the group is butyl or larger are difficult to cleave, and the iodide is too high boiling to be volatilized. Some butoxy compounds give a positive test but the procedure is not reliable (the boiling point of butyl iodide is 131 °C). This class reaction is most useful for ethers, esters, and acetals in which the groups are methyl or ethyl. Methanol, ethanol, 1-propanol, 2-propanol, and even higher alcohols such as l-butanol and 3-methyl-1-butanol will also give a positive test. The test has been applied to numerous alkaloids and methylated sugars. The chief interference is caused by the presence of a sulfur-containing a functional group that liberates hydrogen sulfide when heated with hydroiodic acid.

4.3.6. Ethers

Ethers are only a little more polar and slightly more reactive than either saturated hydrocarbons or alkyl halides. The ether oxygen can be protonated by concentrated sulfuric acid.

Caution: *Ethers form extremely explosive peroxides upon standing, especially when exposed to air and/or light. Liquid ether that shows solid precipitates should not be handled at all.*

Peroxides can be detected by treating the ether with starch-iodide paper that has been moistened with dilute hydrochloric acid; peroxides will cause the paper to turn blue. Many laboratories have substituted *t*-butyl methyl ether for diethyl ether because of much greater peroxide danger for the latter. Pure ethers are more likely to be initially diagnosed by their failure to undergo reactions rather than by their ability to undergo chemical reactions.

Ferrox Test
The ferrox test is used to distinguish ethers from hydrocarbons. Ferric ammonium sulfate reacts with potassium thiocyanate to form iron hexathiocyanatoferrate, which reacts with oxygen-containing compounds to form a reddish-purple solution.

Experiment
A crystal of ferric ammonium sulfate and a crystal of potassiurn thiocyanate should be grinded together using a glass stirring rod. The iron hexathiocyanatoferrate that is formed sticks to the stirring rod. Thereafter 30 mg or three drops of the unknown compound should be mixed in a minimal amount of toluene in a test tube. The stirring rod with the iron

hexathiocyanatoferrate solid should be used to stir the analyzed compound. If the solid dissolves and a reddish-purple colour develops, the compound contains oxygen.

Discussion
Hydrocarbons, alkyl halides, diaryl ethers, and other high-molecular-weight ethers give a negative test. Most compounds in which the oxygen is in a carbonyl group that is in conjugation with an aromatic ring or a double bond produce a negative test. Such compounds including cinnamic acid, aromatic acids, aromatic esters, aromatic anhydrides, aromatic ketones and phenols also give a negative test. However, aromatic aldehydes give a positive test. Other compounds that produce a positive test are aliphatic acids, aliphatic aldehydes, aliphatic ketones, aliphatic esters, aliphatic anhydrides, and aliphatic ethers. This test should be used in conjunction with other tests. If the identified compound produces a positive test but yields a negative test for an aldehyde, a ketone, an ester, a carboxylic acid, or an anhydride, then the compound is an aliphatic or a low-molecular-weight ether.

Iodine Test for Ethers
Ethers can be detected by the iodine test, as the ether undergoes reaction with iodine, the colour of the solution changes from purple to tan.

$$\underset{\text{ether}}{R_2\ddot{O}:} + I_2 \longrightarrow R_2\ddot{O}: \longrightarrow I_2$$

The experiment is described in chapter 4.1.2.

Hydroiodic Acid Test (Zeisel's Alkoxyl Method)
Treatment of an ether with hydroiodic acid results in the cleavage of the ether. The alkyl iodide product is heated and its vapours undergo a reaction with mercuric nitrate to form mercuric iodide which is orange.

$$\underset{\text{ether}}{R'OR} + \underset{\text{hydroiodic acid}}{2HI} \longrightarrow \underset{\text{alkyl iodides}}{R'I + RI} + H_2O$$

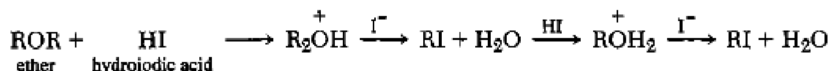
$$\underset{\text{ether}}{ArOR} + \underset{\text{hydroiodic acid}}{HI} \longrightarrow \underset{\text{alkyl iodide}}{RI} + \underset{\text{phenol}}{ArOH}$$

$$\underset{\text{mercuric acetate}}{Hg(NO_3)_2} + \underset{\text{alkyl iodide}}{2R'I} \xrightarrow{H_2O} \underset{\text{mercuric iodide (orange)}}{HgI_2} + 2R'OH + 2HNO_3$$

The procedure of this experiment is described in chapter 4.3.5.

Some ethers may require a more vigorous reagent for cleavage. It is sometimes advantageous to use 2 mL of hydroiodic acid, 0.l g of phenol and 1 mL of propanoic anhydride for each 0.1 g of the sample.

Hydroiodic acid is a strong acid and it protonates the ether. Iodide ion nucleophilically displaces the protonated alkoxy group giving alkyl iodide. This process is expected to predominate when the R groups are primary.

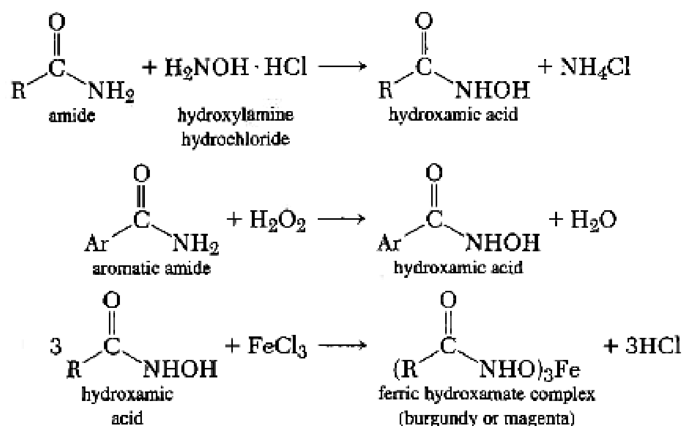


4.4. Compounds with Nitrogen Atom

4.4.1. Amides, Imides

Hydroxamic Acid Formation Reaction

Aliphatic amides react with hydroxylamine hydrochloride to form hydroxamic acid. Similarly, aromatic primary amides react with hydrogen peroxide to produce hydroxamic acid. The hydroxamic acid then reacts with ferric chloride to form the ferric hydroxamate, which has a characteristic magenta colour.



Experiment

One drop or 30 mg of the analyzed compound dissolved in a minimum amount of propylene glycol should be added to 2 mL of 1 M hydroxylamine hydrochloride solution in propylene glycol. Then 1 mL of 1 M potassium hydroxide should be added and the mixture should be boiled gently for 2 minutes. The mixture should be allowed to cool to room temperature, and then 0.5-1 mL of a solution of 5% alcoholic ferric chloride should be added. A red to violet colour constitutes a positive test. Yellow colours indicate negative tests, and brown colours or precipitates are indeterminate.

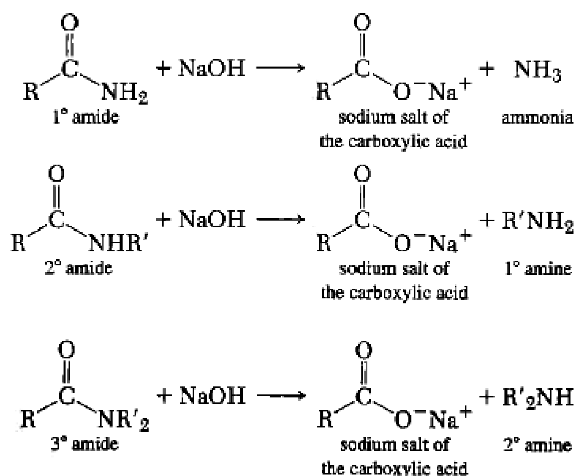
If the procedure gives a negative result, then 50 mg of the unknown compound should be dissolved in 5 mL of water. Thereafter 0.5 mL of 3% hydrogen peroxide and two drops of 5% ferric chloride solution should be added, and the solution should be heated to boiling. The hydrogen peroxide reacts with the aromatic amide to form the hydroxamic acid, which then reacts with the ferric chloride to form ferric hydroxamate complex. The characteristic magenta colour should develop if the compound is an aromatic primary amide.

Discussion

Most imides give positive tests, aliphatic amides and salicylamide give light magenta colours, and most nitriles give a negative test with this procedure. Benzanilide, diacetylbenzidine, and certain sterically hindered amides fail to give a positive test. However, aromatic primary amides will give a positive result only with hydrogen peroxide in the presence of ferric chloride.

Hydrolysis by Sodium Hydroxide

Amides can be hydrolyzed to yield the salt of the carboxylic acid and ammonia or amine. The presence of ammonia or a low-molecular-weight amine is detected with litmus paper.

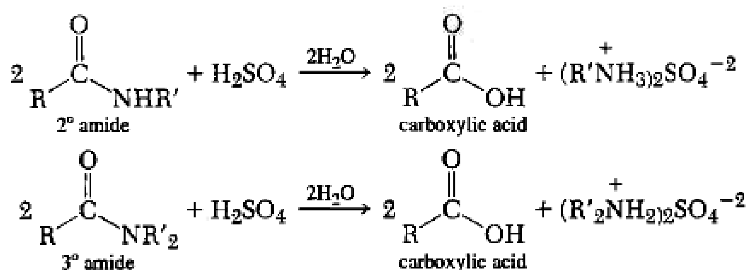


Experiment

0.2 g of the unknown compound should be added into a test tube containing 5 mL of 10 % sodium hydroxide solution. The mixture should be shaken well and it should be noted whether or not ammonia will be evolved. Then the solution should be heated to boiling to allow to observe and to note the odour. The action of the vapour should be tested on either pink moist litmus paper or filter paper moistened with a copper sulfate solution. If ammonia or amine is being evolved, the litmus paper turns blue. Ammonia, which is evolved only from primary amines, will turn the copper sulfate solution on the filter paper blue.

Discussion

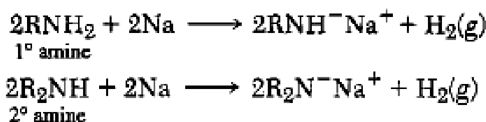
The ammonia or amine that is the product of this alkaline hydrolysis may be characterized by the Hinsberg's method – the reaction with benzenesulfonyl chloride (Chapter no 4.4.2). Many substituted amides are hydrolyzed more easily by heating under reflux with 20% sulfuric acid.



4.4.2. Amines

Sodium Detection of Active Hydrogen

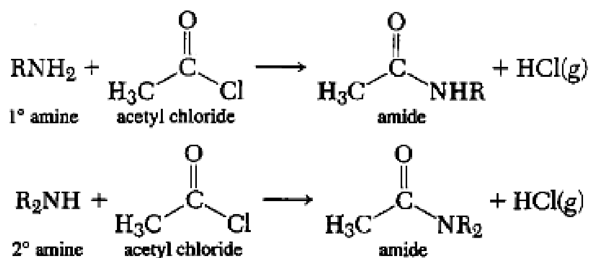
The active hydrogen on primary and secondary amines undergoes reaction with sodium to form the salt and to liberate hydrogen gas. Tertiary amines do not undergo reaction since they do not have an active hydrogen.



The procedure is described in detail in chapter No 4.3.1.

Detection of Active Hydrogen with Acetyl Chloride

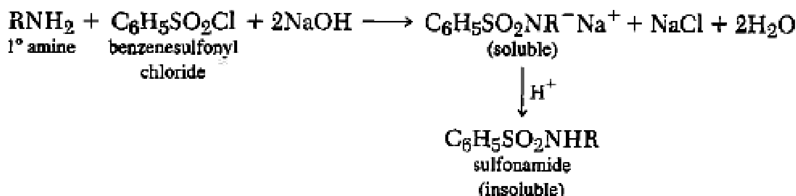
Primary and secondary amines react with acetyl chloride to produce amides which often precipitate from the solution. This reaction is usually accompanied by the evolution of heat. Tertiary amines do not react with acetyl chloride due to the lack of a hydrogen on the nitrogen.



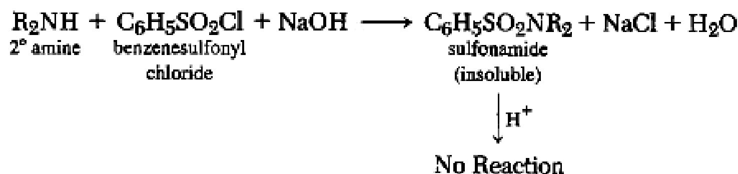
The experiment is described in chapter No 4.3.1.

Many substituted anilines, especially those with nitro groups in the ortho- and para- position relative to the amino group, do not react with acetyl chloride.

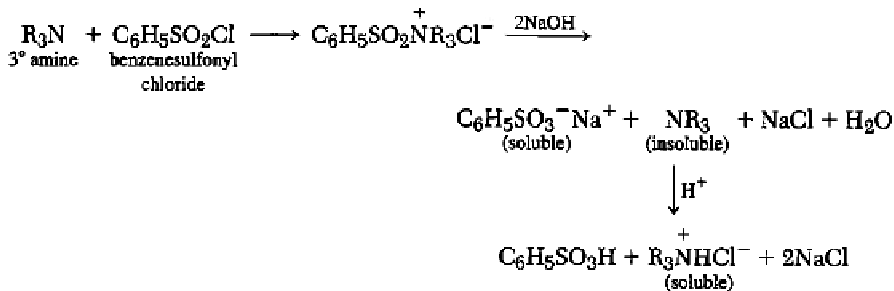
Benzenesulfonyl Chloride Test (Hinsberg's Method)
The Hinsberg test can be used to distinguish among primary, secondary, and tertiary amines. Benzenesulfonyl chloride undergoes reaction with primary amines in basic solution to form the sodium salts of the sulfonamide which are soluble in the reaction mixture. Acidification of the reaction mixture results in the precipitation of the sulfonamides.



Secondary amines, when treated with benzenesulfonyl chloride, yield the sulfonamides which precipitate from the solution. Acidification of the solution does not dissolve the sulfonamide.



Tertiary amines undergo reaction with benzenesulfonyl chloride to produce quaternary ammonium sulfonate salts which yield sodium sulfonates and insoluble tertiary amines in basic solution. Acidification of the reaction mixture results in the formation of sulfonic acids and soluble amine salts.



Experiment
0.3 mL or 300 mg of the analyzed substance should be put into a test tube, and then 5 mL of 10% sodium hydroxide solution and 0.4 mL of benzenesulfonyl chloride should be added. The test tube should be closed by a stopper and the mixture should be shaken very vigorously. The solution should be tested to make sure that it is alkaline. After all the benzenesulfonyl chloride has reacted, the solution should be cooled and the residue, if present, should be separated from the solution. The residue should be tested for solubility in l0% hydrochloric acid. If no residue remains, then the solution should be treated with l0% hydrochloric acid and observed whether a precipitate forms.

Discussion

If all of the original compound dissolves in the base, no residue remains, and acidification produces a precipitate, the original unknown is a primary amine. Primary amines react with benzenesulfonyl chloride in basic solution to form the sodium salt of the sulfonamide, which is normally soluble in basic solution, but the sulfonamide precipitates upon acidification.

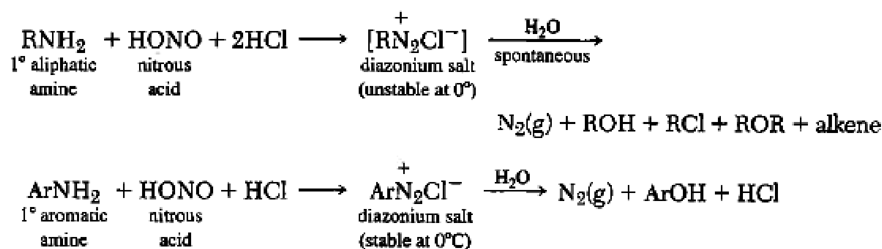
If a residue is formed and it is insoluble in acid, the original unknown is a secondary amine. Secondary amines undergo reaction with benzenesulfonyl chloride to precipitate the sulfonamide and acidification does not result in any change.

If a residue is present and is soluble in acid, it indicates that the residue is the unreacted tertiary amine. Tertiary amines undergo reaction with benzenesulfonyl chloride to produce quaternary ammonium sulfonate salts which yield sodium sulfonates and water-insoluble tertiary amines in basic solution. Acidification of the reaction mixture results in the formation of sulfonic acids and water-soluble amine salts. Any solid that is formed should be isolated and purified and its melting point should be compared against the original amine. If the amount of the solid is in sufficient quantity, it may be saved and used as a derivative for that unknown.

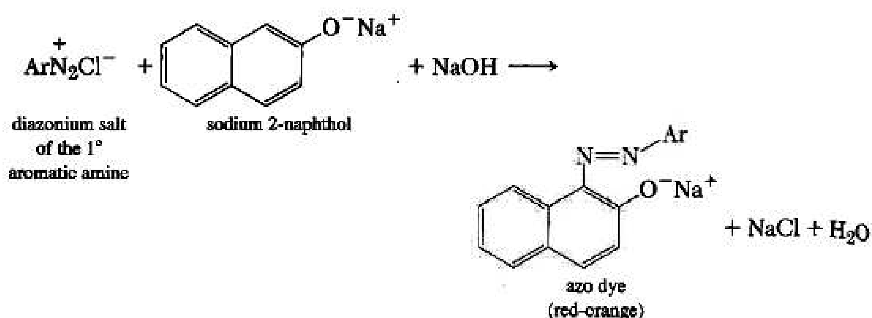
The sodium salts of certain sulfonamides of cyclohexyl through cvclodecylamine and certain high-molecular-weight amines are insoluble in 10% hydroxide solution. Usually, they are soluble in water. Certain primary amines may yield alkali-insoluble disulfonyl derivatives. These may be hydrolyzed by boiling for 30 min with 5% sodium ethoxide in absolute ethanol. If the solution heats up considerably, it should be cooled. Certain *N,N*-dialkylanilines produce a purple dye if the mixture becomes too hot. This may be prevented by carrying out the reaction at 15-20 °C.

Nitrous Acid Test

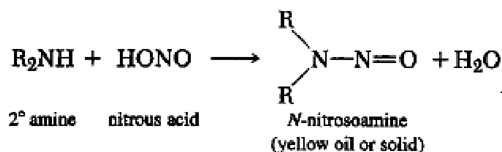
The reaction of amines with nitrous acid classifies the amines not only as primary secondary or tertiary but also as aliphatic or aromatic. Primary aromatic and aliphatic amines react with nitrous acid to form an intermediate diazonium salt. The aliphatic diazonium salts decompose spontaneously by rapid loss of nitrogen, particularly when the original amino group is attached to a secondary or tertiary carbon. Most aromatic diazonium salts are stable at 0 °C but lose nitrogen slowly on warming to room temperature.



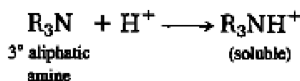
The diazonium salt of the primary aromatic amine reacts with sodium 2-naphthol to produce a red-orange azo dye.



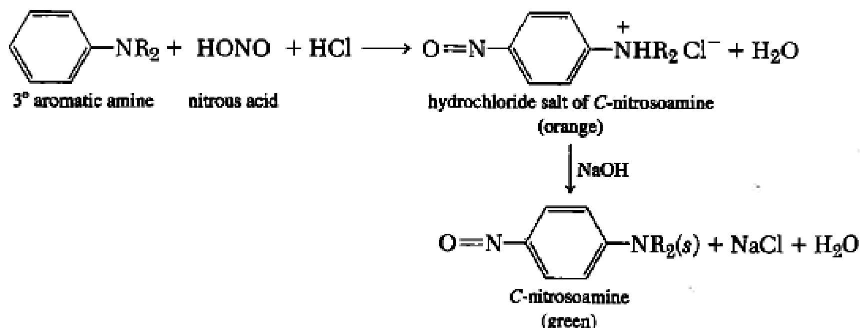
Secondary amines undergo a reaction with nitrous acid to form *N*-nitrosoamines which are usually yellow solids.



Tertiary aliphatic amines do not react with nitrous acid, but they form a soluble salt. The reaction mixture gives an immediate positive test on the starch-iodide paper for nitrous acid.



Tertiary aromatic amines react with nitrous acid to form the orange-coloured hydrochloride salt of the C-nitrosoamine. Treating the solution with base liberates the blue or green C-nitrosoamine.



Experiment
0.5 mL or 0.5 g of the identified sample should be dissolved in 1.5 mL of concentrated hydrochloric acid and next diluted with 2.5 mL of water, then the mixture should be cooled to

0°C in a beaker of ice. Thereafter 0.5 g of sodium nitrite should be dissolved in 2.5 mL of water and added drop wise, with shaking, to the cold solution of the amine hydrochloride. The addition should be continued until the mixture gives a positive test for nitrous acid. The test should be performed by placing a drop of the solution on starch-iodide paper. A blue colour indicates the presence of nitrous acid. If the test is positive, 2 mL of the solution should be moved to another test tube and warmed gently to make it possible to examine for the evolution of gas.

Discussion

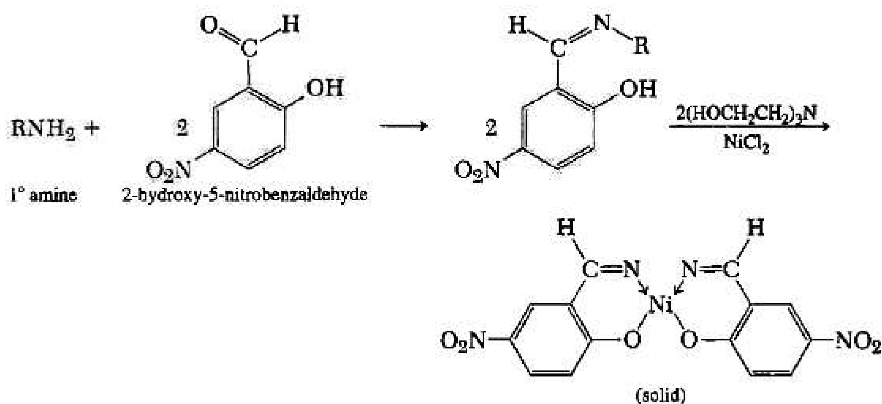
Reaction of primary amines. The observation of rapid bubbling or foaming as the aqueous sodium nitrite solution is added at 0 °C indicates the presence of a primary aliphatic amine. The evolution of gas upon warming indicates that the amine is a primary aromatic amine. Both aliphatic and aromatic primary amines react with nitrous acid to initially give the corresponding diazonium ions. The aliphatic diazonium compounds are so unstable that their existence has not been directly detected. Nitrogen gas, alcohol, olefin, and products of other displacement and carbocation reactions are formed. Aromatic diazonium salts, on the other hand, are generally stable in a solution at 0°C. When heated in aqueous solution, they quickly lose nitrogen to give the aryl cation Ar^+. This ion reacts rapidly with water to give phenol.
Caution: *If allowed to dry these compounds can be an explosion hazard, so the aryldiazonium ions should be used immediately upon preparation.*

Reaction with secondary amines. If a pale yellow oil or low-melting solid, which is the *N*-nitrosoamine, is formed with no evolution of gas, the original amine is a secondary amine. Both aliphatic and aromatic secondary amines react with nitrous acid to form *N*-nitroso compounds, commonly called nitrosoamines.
Caution: *Many of these compounds are carcinogenic and should be handled carefully.*

Reaction with tertiary amines. An immediate positive test for nitrous acid with no evolution of gas indicates the presence of a tertiary aliphatic amine. The tertiary aliphatic amine is simply protonated to form a soluble salt under these conditions and does not react with the nitrous acid. The reaction of a tertiary aromatic amine (an aniline) with nitrous acid produces a dark-orange solution or an orange crystalline solid which is the hydrochloride salt of the C-nitrosoamine. Treating 2 mL of the solution with 10% sodium hydroxide or sodium carbonate solution will produce the bright-green or -blue nitrosoamine base. The chemistry of the reaction of tertiary amines is quite complex. Under certain conditions, it may appear that tertiary amines undergo no reaction. This is actually true only at low pH, low temperature, and dilute conditions. The amine is simply protonated to form salts under these mild conditions. These salts can be recognized by their reaction with base to regenerate the original amine. Under higher temperatures, less acidic conditions, and other conditions, a variety of reactions occur when tertiary amines are treated with nitrous acid. For aliphatic amines *N*-nitrosoamines are formed. For aromatic amines, *N,N*-diaryl-4-nitrosoanilines are produced. *N*-Alkyl-*N*-nitrosoanilines and *N,N*-dialkyl-4-nitrosoanilines are produced from alkarylamines.

Test for Primary Aliphatic Amines

Primary aliphatic amines react quickly with 2-hydrory-5-nitrobenzaldehyde, followed by nickel chloride, to form a precipitate within several minutes.

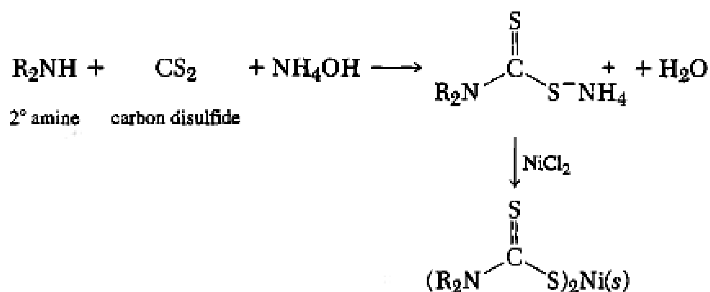


Nickel chloride and 2-hydroxy-5-nitrobenzaldehyde reagent
A solution of 0.5 g of 2-hydroxy-5-nitrobenzaldehyde dissolved in 25 mL of water should be added to 15 mL of triethanolamine. Then 0.5 g of nickel chloride hexahydrate dissolved in 10 mL of water should be added to this mixture and the final solution should be filled with water to 100 mL. If the triethanolamine contains ethanolamine, it may be necessary to add another 0.5 g of the aldehyde and remove the resulting precipitate by filtration.

Experiment
One or two drops or 50 mg of the compound to be tested should be added to 5 mL of water. If necessary, one or two drops of concentrated hydrochloric acid may be added to be able to dissolve the compound. Then 0.5 mL of this amine solution should be added to 3 mL of the nickel chloride and 2-hydroxy-5-nitrobenzaldehyde reagent. An immediate, copious precipitate is produced by primary aliphatic amines, whereas primary aromatic amines usually require 2-3 minutes to give a definite precipitate. The appearance of a slight turbidity is not a positive test. It indicates that traces of primary amines may be present as impurities.

Discussion
This test is so sensitive that care must be taken in interpreting it. Only a definite precipitate produced in considerable quantity indicates a primary amine. A slight turbidity is merely indicative of impurities. Care must be taken to use the amounts specified above since the addition of large amounts of solutions of secondary amines will also give a precipitate. Many commercial samples of secondary and tertiary amines contain traces of primary amines and produce a turbidity. The test is given by all primary amines capable of forming the Schiff base with 2-hydroxy-5-nitrobenzaldehyde. Hydroxylamine and hydrazines substituted on only one nitrogen atom give positive tests. Amides do not give a precipitate. The test is not applicable to amino acids.

Test for Secondary Aliphatic Amines
Secondary amines combine with carbon disulfide and ammonium hydroxide, followed by nickel chloride, to produce a solid product.

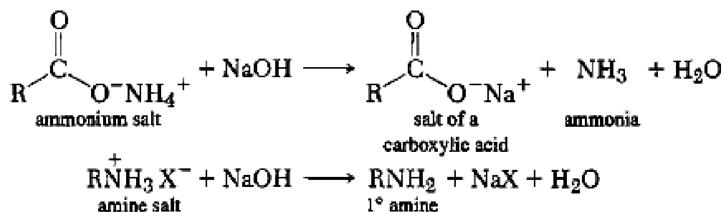


Nickel chloride in carbon disulfide reagent
An amount of carbon disulfide should be added to 0.5 g of nickel chloride hexahydrate in 100 mL of water so that after the mixture has been shaken, a globule of carbon disulfide is left on the bottom of the bottle. If stored in a tightly stoppered bottle, the reagent is stable for long periods of time. When the undissolved carbon disulfide evaporates, more of the substance must be added.

Experiment
An aqueous solution of the unknown compound should be prepared by adding one or two drops or 50 mg of the analyzed substance to 5 mL of water. If necessary one or two drops of concentrated hydrochloric acid may be added to dissolve the analyzed amine. Thereafter 0.5 - 1 mL of concentrated ammonium hydroxide should be added to 1 mL of the nickel chloride in carbon disulfide reagent in a test tube, followed by 0.5 - 1 mL of the amine solution. A definite precipitation indicates that the analyzed compound is a secondary amine. A slight turbidity is an indication of a trace of a secondary amine as an impurity.

Discussion
This test is given by all secondary amines but not by primary amines. *It is very sensitive, and many commercial samples of tertiary amines produce turbidity because of the presence of small amounts of secondary amines.* This is true for substituted pyridines, quinolines, and isoquinolines separated from coal-tar distillates.

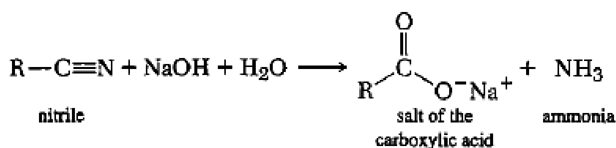
Sodium Hydroxide Treatment of Ammonium Salts and Amine Salts
Amine salts can be detected by treating the salt with sodium hydroxide to liberate the ammonia or amine.



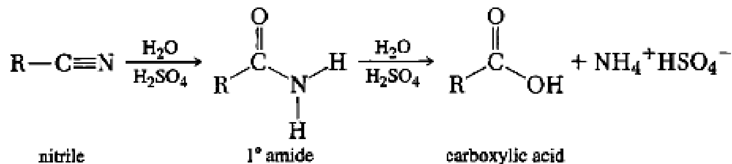
Experiment
0.2 – 0.4 g of the unknown compound should be added to 5 mL of 10% sodium hydroxide solution in a test tube, and the mixture should be shaken vigorously. The odour of ammonia or the formation of an oily layer of the amine should be noted. Moistened pink litmus paper placed in the vapour above the solution will turn blue if ammonia or a volatile amine is present.

4.4.3. Nitriles

Hydrolysis by Sodium Hydroxide
Nitriles can be hydrolyzed under basic conditions to yield the salt of the carboxylic acid and ammonia. The ammonia vapour is detected by litmus paper.

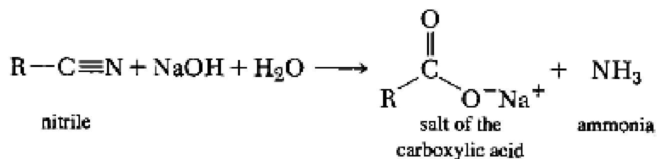


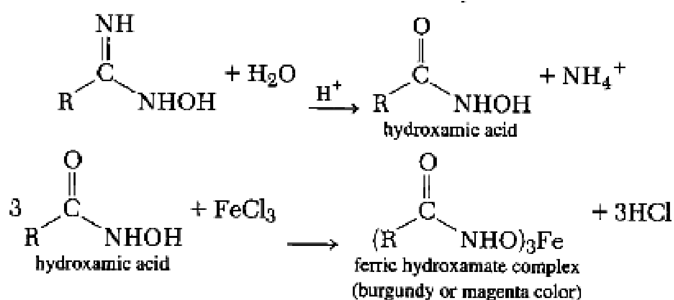
There is more information about this experiment in chapter 4.4.1.
Nitriles and ammonium salts will also give a positive test with the copper sulfate. Nitriles, particularly cyanohydrins, are frequently hydrolyzed by acids.



Treatment with concentrated hydrochloric acid, with heating, converts the nitriles to amides. The amides may be hydrolyzed further by diluting the mixture with water and heating for 0.5 - 2 h.

Hydroxamic Acid Formation Reaction
Nitriles, along with many other compounds, give a positive hydroxamic acid test. The hydroxamic acid is detected with ferric chloride to form the ferric hydroxamate complex, which has a burgundy or magenta colour.

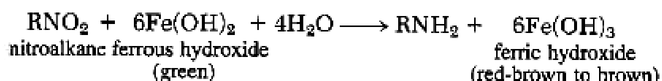




Experiment
One drop or 30 mg of the analyzed compound dissolved in a minimum amount of propylene glycol should be added to 2 mL of 1 M hydroxylamine hydrochloride solution in propylene glycol. Thereafter 1 mL of 1 M potassium hydroxide should be added and the mixture should be boiled gently for 2 minutes. The mixture should be allowed to cool to room temperature, and then 0.5 - 1 mL of a solution of 5% alcoholic ferric chloride should be added. A red to violet colour constitutes a positive test. Yellow colours indicate negative tests and brown colours or precipitates are indeterminate.

4.4.4. Nitro Compounds

Ferrous Hydroxide Reduction
The presence of a nitro group is detected in several different ways. In the ferrous hydroxide reduction, a positive test is noted by the change in colour from green to red-brown or brown due to the oxidation of iron from $^{+}2$ to $^{+}3$.



Ferrous sulfate reagent
5.0 g of ferrous ammonium sulfate crystals should be dissolved in 100 mL of recently boiled, distilled water. Then 0.4 ml, of concentrated sulfuric acid should be added, and next an iron nail to retard air oxidation.

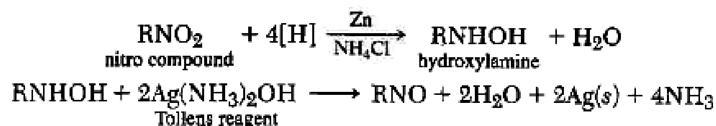
Alcoholic potassium hydroxide reagent
3 g of potassium hydroxide should be dissolved in 3 mL of distilled water and this solution should be diluted to 100 mL of 95% ethanol.

Experiment
10 mg of the compound should be dissolved in 1 mL of the ferrous sulfate reagent in a test tube and then 0.7 mL of the alcoholic potassium hydroxide reagent should be added. A glass tube should be inserted so that it reaches the bottom of the test tube and a stream of inert gas

should pass through the tube for about 30 seconds in order to remove air. The tube should be closed by a stopper quickly and then shaken. The colour of the precipitate should be noted after 1 minute. A positive test is the formation of the red-brown to brown precipitate of iron (III) hydroxide.

Discussion
The red-brown to brown precipitates of iron (III) hydroxide (ferric hydroxide) is formed by the oxidation of iron (II) hydroxide (ferrous hydroxide) by the nitro compound, which in turn is reduced to the primary amine. A negative test is indicated by a greenish precipitate. In some cases partial oxidation may cause darkening of the ferrous hydroxide.
Practically all nitro compounds give a positive test in 30 seconds. The speed with which the nitro compound is reduced depends on its solubility. 4-Nitrobenzoic acid, which is soluble in the alkaline reagent, gives a test almost immediately, whereas 2-nitronaphtalene must be shaken for 30 seconds. A positive test is also given by other compounds that oxidize ferrous hydroxide. Nitroso compounds, quinones, hydroxylamines, alkyl nitrates, and alkyl nitrites are in this group. Highly coloured compounds cannot be tested.

Zinc and Ammonium Chloride Reduction
From among the nitro compounds, only tertiary aliphatic nitro compounds and aromatic nitro compounds are reduced by zinc and ammonium chloride to the hydroxylamine. The hydroxylamine is then detected by the formation of metallic silver in the Tollens test.

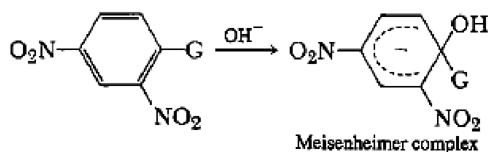


Experiment
0.2 mL or 0.2 g of the unknown compound should be dissolved in 4 mL of 50% ethanol, and then 0.2 g of ammonium chloride and 0.2 g of zinc dust should be added. The mixture should be shaken and heated to boiling. Thereafter it should be allowed to stand for 5 minutes, filtered, and the action of the filtrate should be tested on Tollens reagent (chapter no 4.3.3). A positive test with Tollens reagent is the formation of a black or grey precipitate or a silver mirror.

Discussion
This test depends on the reduction of the unknown compound to a hydrazine, a hydroxylamine, or an aminophenol. All these compounds are oxidized by Tollens reagent. This test cannot be applied if the original compound reduces Tollens reagent. Tertiary aliphatic compounds and aromatic nitro compounds give a positive test. Nitroso, azoxy and azo compounds are reduced with zinc and ammonium chloride, with the products being oxidized by the Tollens reagent.

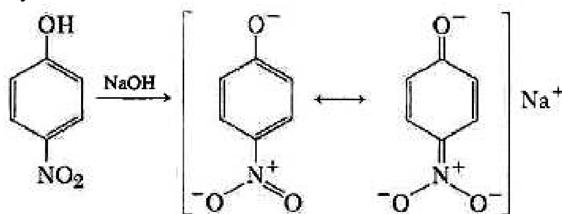
Treatment with Sodium Hydroxide
The number of nitro groups on an aromatic ring can be determined by the reaction of the unknown with sodium hydroxide. In the reaction with sodium hydroxide, the mononitro aromatic compounds yield no colour change, dinitro aromatic compounds produce a bluish-

purple colour, and trinitro aromatic compounds give a red colour. The colour of the solution is due to a Meisenheimer complex.



Experiment
2 mL of ethanol should be added to 5 mL of 20% sodium hydroxide solution and a drop or a crystal of the unknown compound. The mixture should be shaken vigorously. The colour of the solution should be noted. Alternatively, 0.1 g of the analyzed substance should be dissolved in 10 mL of acetone. Thereafter 2 - 3 mL of l0% sodium hydroxide solution should be added drop wise with shaking. The colour of the solution should be noted.

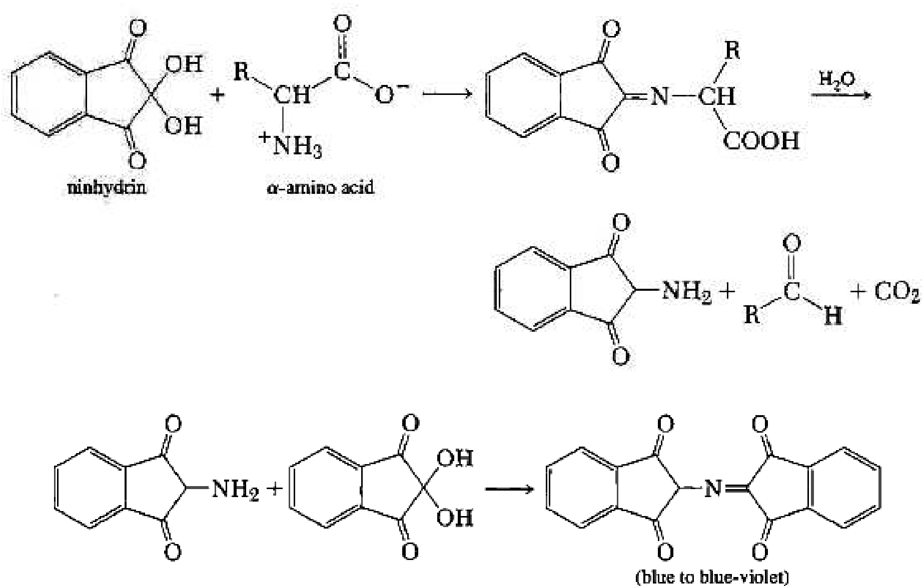
Discussion
Mononitro benzene compounds give no colour or a very light yellow one with these reagents. If two nitro groups on the same ring are present, a bluish-purple colour develops – the presence of three nitro groups produces a blood-red colour. The presence of an amino, substituted amino, or hydroxyl group in the molecules inhibits the formation of the characteristic red and purple colours. Polynitro compounds can form Meisenheimer complexes, which may lead to coloured solution.



Nitrophenols can form highly conjugated and stable phenoxide anions that may be a source of colour.

4.4.5. Amino Acids

Ninhydrin Test
The ninhydrin test is the chemical basis for the amino acid analyzer, it can be used to distinguish between different types of amino acids. α-Amino acids and β-amino acids react with ninhydrin to give a positive test which is blue-violet colour.



Experiment

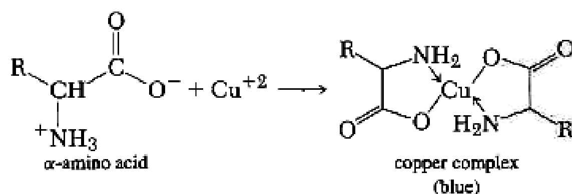
2 mg of the sample should be added to 1 mL of a solution of 0.2 g of ninhydrin (1,2,3-indanetrione monohydrate) in 50 mL of water. The mixture should be heated to boiling for 15-20 seconds. A blue to blue-violet colour is given by α-amino acids and constitutes a positive test. Other colorus (yellow orange or red) are negative.

Discussion

This reaction is important not only because it is a qualitative test, but also because it is the source of the absorbing material that can be measured quantitatively by an automatic amino acid analyzer. This colour reaction is also used to detect the presence and position of amino acids after paper chromatographic separation. Proline, hydroxyproline, and 2-, 3-, and 4-aminobenzoic acids fail to give a blue colour but produce a yellow colour instead. Ammonium salts give a positive test. Some amines, such as aniline, yield orange to red colours, which is a negative test.

Copper Complex Formation

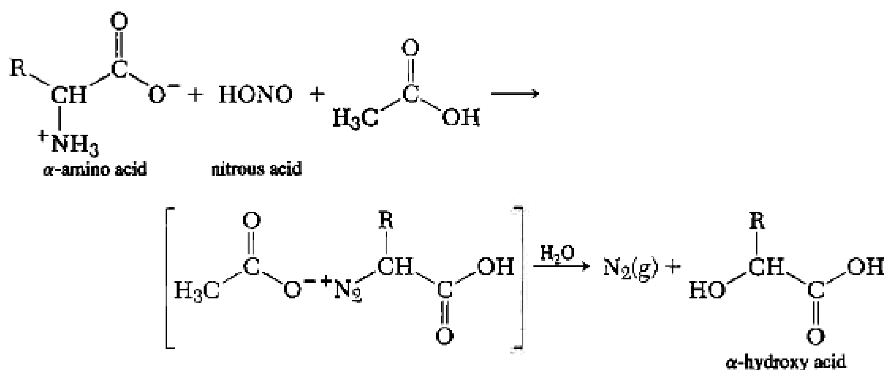
Copper complexes are formed from the reaction of α-amino acids with copper sulfate. A deep-blue colour is produced, indicating the presence of the copper complex.



Experiment
A small amount of the analyzed compound should be dissolved in 1 mL of water. Then two drops of 1 M copper (II) sulfate should be added. If a blue colour is not formed immediately, then the test tube should be heated in a hot-water bath for 5 minutes. A moderate- to deep-blue liquid or a dark-blue solid is a positive test.

Discussion
Some α-amino acids are not very soluble in cold water. However, these amino acids are soluble in hot water and will give a positive test when the solution is heated. Aliphatic amines yield a blue precipitate. Anilines give a brown or green colour but other aromatic amines produce a blue-purple colour.

Nitrous Acid Test
α-Amino acids combine with nitrous acid to produce an intermediate which decomposes to form nitrogen gas and an α-hydroxy acid.

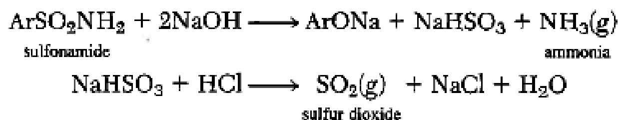


The procedure should be followed for amines, except substitute acetic acid for hydrochloric acid. A positive test is the evolution of nitrogen gas. The α-amino acid reacts with the nitrous acid to form an intermediate, which decomposes to nitrogen gas and an α-hydroxy acid.

4.5. Compounds with Sulfur Atom

The group of the organic compounds containing sulfur atoms encompass mostly sulfonamides, sulfonic acids and sulfonyl chlorides.

Sodium Hydroxide Fusion of Sulfonamides
The presence of sulfonamides can be detected by fusing with sodium hydroxide and testing for the evolution of amine or ammonia and of sulfur dioxide.



Sulfonic acids are structurally different from sulfuric acid only in the substitution of an organic group by one hydroxyl group and thus the high acid strength of sulfonic acids is not surprising. Sulfonic acids and their metal salts are usually soluble in water. ?

Nickel(II) hydroxide reagent

The nickel (II) hydroxide reagent should be prepared immediately before use by slowly adding 1 M sodium hydroxide to 0.20 g of nickel (II) chloride until no more solid precipitates. The precipitate should be washed with 10 mL portions of cold water until the washings are no longer basic. The nickel (II) hydroxide should be moistened with water and applied as a paste to a strip of filter paper.

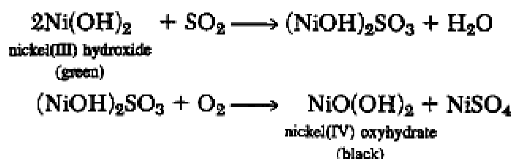
Experiment

In a test tube, 0.25 g of the analyzed compound should be fused with 1.5 g of powdered sodium hydroxide by heating with a bunsen burner. The escaping gas should be tested for the presence of ammonia or amines by placing pink moist litmus paper in the test tube very carefully to avoid touching the sides of the test tube with the paper. If ammonia or amine is being evolved, the litmus paper turns blue.

The test tube should be allowed to cool. Then just enough distilled water should be added to dissolve the sample. The solution should be acidified with 2 M hydrochloric acid. A filter paper that has been covered with a paste of nickel (II) hydroxide should be suspended over the test tube. Then it is necessary to warm the test tube gently to speed up the production of sulfur dioxide. If sulfur dioxide is present, the green nickel (II) hydroxide is oxidized to grey-black nickel (IV) oxyhydrate. A positive test for the presence of a sulfonamide is the evolution of both ammonia or amine and sulfur diode.

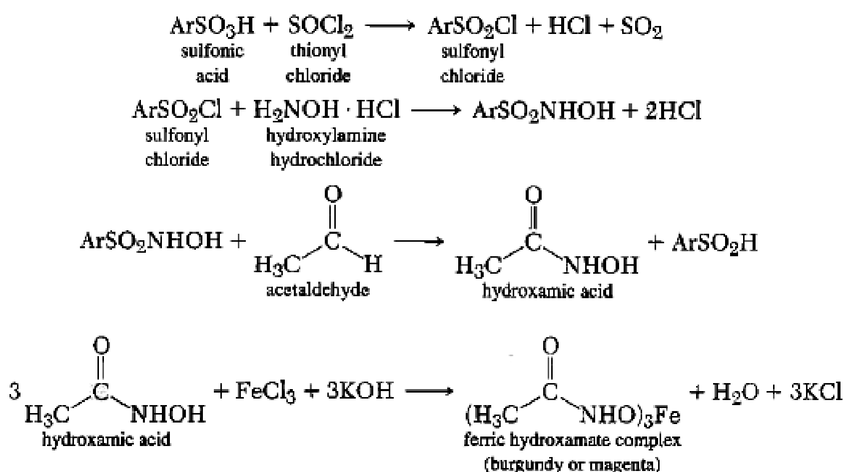
Discussion

The sulfur dioxide undergoes reaction with the green nickel (II) hydroxide to yield black nickel (IV) oxyhydrate.



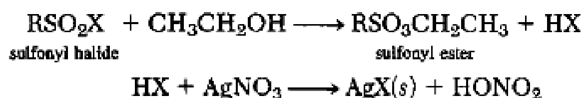
Hydroxamic Acid Formation Test

Sulfonyl chlorides and sulfonic acids can be detected through the hydroxamic acid test. The sulfonyl chloride is produced from the sulfonic acid and thionyl chloride. The sulfonyl chloride is treated with hydroxylamine, which undergoes reaction with acetaldehyde to form the hydroxamic acid. The hydroxamic acid undergoes reaction with ferric chloride to form the ferric hydroxamate complex, which is a burgundy or magenta colour.



Experiment
To prepare the sulfonyl chloride from the sulfonic acid, it is necessary to combine five drops of thionyl chloride and 100 mg of the sulfonic acid in a test tube and to heat the mixture in boiling water for 1 minutes. The test tube should be allowed to cool. 0.5 mL of a saturated solution of hydroxylamine hydrochloride in methanol should be added to the test tube. Then a drop of acetaldehyde should be added. The sulfonyl chloride undergoes the reaction with the hydroxylamine to form an intermediate, which, when treated with the acetaldehyde, forms the hydroxamate acid. Add dropwise a solution of 2 M potassium hydroxide in methanol until the solution is slightly basic when checked with pH paper. The solution should be heated to boiling and then allowed to cool. The mixture should be acidified by dropwise adding 0.5 M hydrochloric acid, until blue litmus paper turns red. Thereafter a drop of 5% ferric chloride solution should be added. Ferric chloride converts the hydroxamic acids to the ferric hydroxamate complex. The magenta colour of' the ferric hydroxamate complex is a positive result.
Sulfonyl chlorides can be treated directly with the hydroxylamine hydrochloride. The salts of sulfonic acids should be neutralized with hydrochloric acid, and then should be evaporated to dryness. The residue should be treated with thionyl chloride as described above.

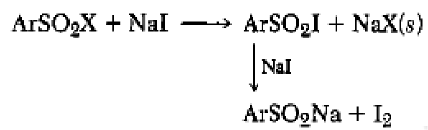
Silver Nitrate Reaction
The presence of the halogen in the sulfonyl halides can be detected by ethanolic silver nitrate solution. The sulfonyl halide is converted to the sulfonic ester and hydrogen halide. The halide ion reacts with the silver cation to form the insoluble silver halide.



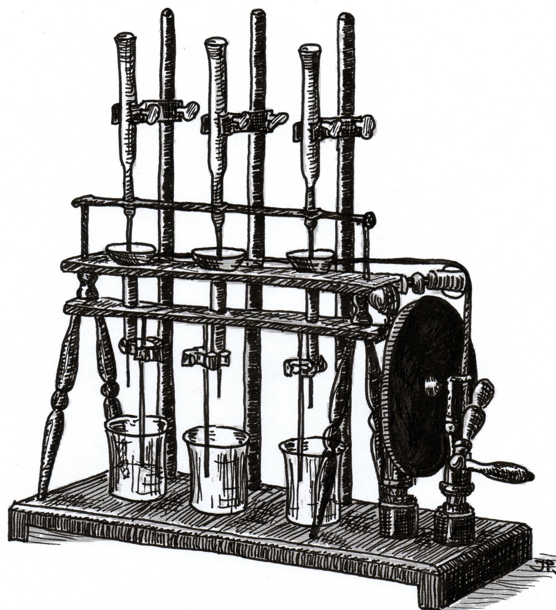
The experiment is described in detail in chapter no 4.2.

Sodium Iodide in Acetone Test

The halogen in sulfonyl bromides or chlorides can also be detected by sodium iodide in acetone, with the formation of the solid sodium chloride and the liberation of iodine.



The procedure is described in chapter no 4.2.



5

Synthetic Organic Drugs – Laboratory Exercises

There is more need to identify and quantify medicines than ever before. The drug analysis is based mainly on two elements.

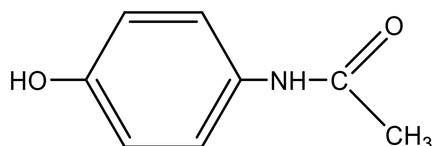
The first important step in the analysis of a medicine is usually to verify that it does contain the correct active ingredient(s). The first step in organic qualitative analysis has usually been a test-tube subject. These ”wet” tests are commonly used as methods of indication of functional groups, based on changes easy to observe, i.e. in specific colour tests, visual observations of physical state, smell, etc. Other identifying techniques presented in this chapter encompass the measurement of melting point for solid state products, the measurement of the refraction coefficient for liquids as well as analyses of IR, MS and NMR spectra.

Another important element in drug analysis is quantitative analysis. The content of the active ingredient(s) in a medicinal product is one of the most important quality parameters that assure its efficacy. Sometimes there is a need to isolate the biologically active compound from the medicinal product, depending on the form of the medicine, i.e. from ointment, suspension or from multiple-ingredient drugs. In the last case each active compound should be tested separately, using an appropriate analytical technique.

EXERCISE No 5.1.

PARACETAMOL

REAGENTS		EQUIPEMENT	
Paracetamol (1 tabl. 1f Panadol)	0.7 g	tubes	
distilled water		beaker	250 ml
1% $FeCl_3$	0.1 ml	heating mantel	
0.5% $K_2Cr_2O_7$	0.05 ml	pipette	5 ml
36% HCl	2 ml	measuring cylinder	50 ml
10% HCl	300 ml	conical flask	100 ml
95% ethanol	20 ml	burette	
ferroin	1 ml	mortar with pestle	
0.1 M ceric sulfate (IV)		balance	
ice			



Paracetamol or acetaminophen is a widely-used analgesic and antipyretic drug. Unlike aspirin, it is not a very effective anti-inflammatory agent. It is well tolerated, lacks many of the side-effects of aspirin, commonly used for the relief of fever, headaches, and other minor aches and pains. Paracetamol is also useful in the management of more severe pain, where it allows lower dosages of additional non-steroidal anti-inflammatory drugs (NSAIDs) to be used, thereby minimizing overall side-effects. Paracetamol is considered safe for human use at recommended doses. However, acute overdose can cause potentially fatal liver damage. The risk is heightened by the use of alcohol.
Another possibility is that paracetamol is able to block cycloxygenase as aspirin but this can take place in an inflammatory environment, where the concentration of peroxides is high and the oxidation state of paracetamol is high, which prevents its actions. This would mean that paracetamol has no direct effect at the site of inflammation but instead acts in the CNS to reduce temperature etc. where the environment is not oxidative.
Paracetamol consists of a benzene ring core, substituted by one hydroxyl group and the nitrogen atom of an amide group in the para (1.4) pattern. The amide group is acetamide (ethanamide). Paracetamol is a white crystal powder, easy to dissolve in ethanol, hard to dissolve in water and not dissolved in ether. Water solution of 20 mg of paracetamol has pH 5.5 – 6.5.

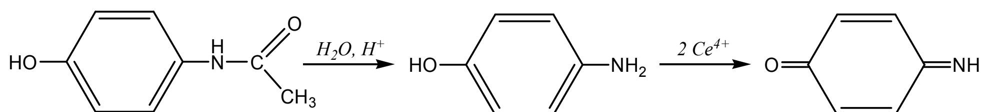
IDENTIFICATION ANALYSIS

1. Dissolve 0.1g of the substance (calculate the amount of tablet mass) in 10 ml of hot distilled water, next add 0.1 ml of freshly prepared 1% solution of $FeCl_3$. The solution of the resulting salt should be blue-violet.
2. Add 0.1g of the substance (calculate the amount of tablet mass) to 2 ml of concentrated HCl (36 %) and heat carefully for 2 minutes. Then add to the solution 10

ml of distilled water in small portions and cool it in ice bath. Then add 0.05 ml of 0.5 % solution of $K_2Cr_2O_7$. The colour of the solution should slowly change to violet.

QUANTITATIVE ANALYSIS

Quantity of paracetamol could be evaluated based on the cerometric method described in Polish Pharmacopoeia V.



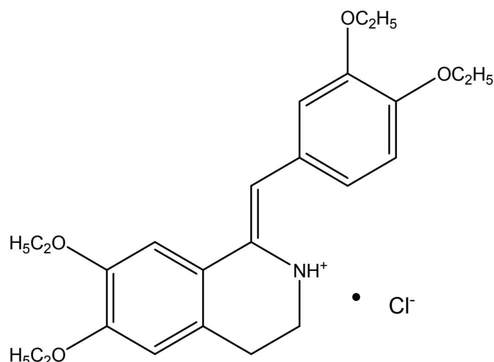
Dissolve 0.1 g of the substance (calculate the amount of tablet mass) in 4 ml of 95% ethanol, add 60 ml of 10 % HCl and 0.2 ml of ferroin solution. Prepare burette with 0.1 M solution of ceric (IV) sulphate (VI) and titrate the solution of paracetamol until the colour changes from red to green-blue, which takes only about 1 min. Make a titration process 3 times and a blind test once – the solution without paracetamol.
1 ml of 0.1M solution of ceric (IV) sulphate corresponds to 0.01280g of paracetamol.

EXERCISE No 5.2.

NO-SPA

(DROTAVERINE HYDROCHLORIDE)

REAGENTS		EQUIPEMENT	
NO-SPA (pills)	620 mg	tubes	
distilled water		beaker	250 ml
3% ferric (III) chloride	1 ml	heating mantel	
conc. sulphuric acid	10 ml	pipette	5 ml
conc. nitric acid	1 ml	measuring cylinder	50 ml
glacial acetic acid	20 ml	conical flask	100 ml
acetic acid anhydride	5 ml	burette	
mercuric (II) acetate	5 ml	mortar with pestle	
0.05 M chloric acid (VII)		balance	
crystal violet		reflux condenser	



6,7-diethoxi-1-[(3,4-diethoxiphenyl)-methylene]-1,2,3,4-tetrahydroisochinoline hydrochloride

Drotaverine (also known as **drotaverin**) is an antispasmodic drug, structurally related to papaverine. Drotaverine is a selective inhibitor of phosphodiesterase 4, and has no anticholinergic effects. It was found to be nearly 80% effective in treating renal colic.
Drotaverine hydrochloride is a green-yellow crystal powder, easily dissolved in methanol, ethanol, and almost not dissolved in water. Water solution of the substance (1 mg/ml) has pH 3.5 – 5.5.

IDENTIFICATION ANALYSIS

Mix 20 mg of drotaverine hydrochloride (calculate the amount of tablet mass) with 10 ml of concentrated sulfuric acid and then add 1 ml of 3% solution of ferric chloride (III) and heat the mixture for 3 minutes at 100 °C under reflux. In the presence of drotaverine hydrochloride the mixture should become green, because of its phenolic character. After cooling and adding 1 ml of cool solution of concentrated nitric acid to the mixture the colour should change to brown-red.

QUANTITATIVE ANALYSIS

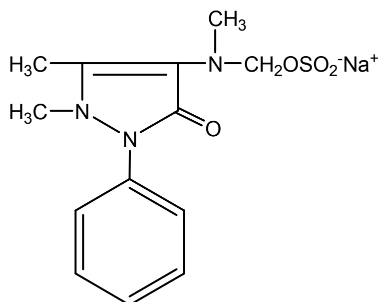
The amount of drotaverine hydrochloride is usually evaluated by the acidimetric method in non-water solution, after elimination of the chloride ions with mercuric (II) acetate. This method is in accordance with Polish Pharmacopoeia V.
Dissolve 0.2g of drotaverine hydrochloride (calculate the amount of tablet mass) in 20 ml of glacial acetic acid, and add 5 ml of acetic acid anhydride and 5 ml of mercuric (II) acetate solution. Add very small quantity of crystal violet to the mixture. Fill burette with 0.05M solution of chloric acid (VII) and titrate the solution until the colour will change to blue. Make a titration process 3 times, and a blind test once – the solution without drotaverine hydrochloride.
1 ml of 0.05M solution chloric acid (VII) corresponds to 21.70 mg of drotaverine hydrochloride.
Before the experiment calculate the amount of drotaverine hydrochloride in used pills.

EXERCISE No 5.3.

PYRALGINUM, ANALGIN

(METAMIZOLE SODIUM)

REAGENTS		EQUIPEMENT	
Metamizole sodium (pills)	1.4 g	tubes	
10% nitric acid	2 ml	beaker	250 ml
1% sodium nitrate (III)	0.2 ml	heating mantel	
5% silver nitrate (V)	0.4 ml	pipette	5 ml
10% hydrochloric acid	10 ml	measuring cylinder	50 ml
Schiff reagent	1 ml	conical flask	100 ml
0.02 M hydrochloric acid	5 ml	burette	
0.05 M solution of iodine		mortar with pestle	
solution of starch	5 ml	balance	



N-methyl-*N*-[1-phenyl-2,3-dimethyl-5-ketopirazolynyl(4)]-aminemethylsulfonate sodium

Metamizole sodium is a non-steroidal anti-inflammatory drug, commonly used in the past as a powerful painkiller and fever reducer. It is better known under the names **Dipyrone**, **Analgin**, and **Novalgin**. Metamizole was first synthesized by the German company Hoechst AG in 1920 and its mass production started in 1922. It remained freely available worldwide until the 1970s, when it was discovered that the drug carries a small risk of causing agranulocytosis – a very dangerous and potentially fatal condition. Recent studies estimate that the incidence rate of metamizole-induced agranulocytosis is between 0.2 and 2 cases per million person days of use, with approximately 7% of all cases fatal (provided that all patients have access to urgent medical care). In other words, one should expect 50 to 500 deaths annually due to metamizole in a country of 300 million, assuming that every citizen takes the drug once a month.

Metamizole was banned in Sweden in 1974, in the United States in 1977. More than 30 countries, including Japan, Australia, and most of the European Union, have followed suit. In these countries, metamizole is still occasionally used as a veterinary drug. Some pharmaceutical companies, notably Hoechst and Merck, continue to develop metamizole-containing drugs and market them in some countries. In the rest of the world (especially in Mexico, India, Brazil, Russia, Third World countries) metamizole is still freely available over-the-counter and remains one of the most popular analgesics.

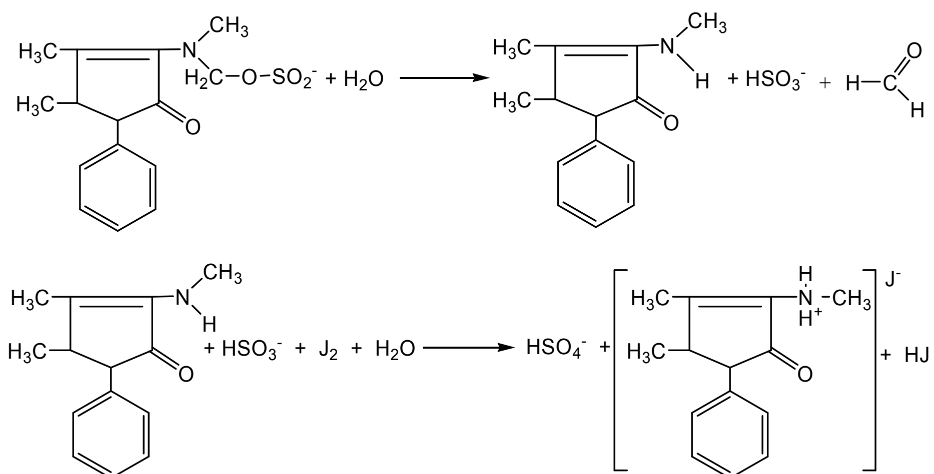
Metamizole sodium is a white crystal powder, easy dissolved in water, and almost not dissolved in ethanol and diethyl ether. Water solution of the substance (50 mg/ml) has pH 6.5-7.5.

IDENTIFICATION ANALYSIS

1. Dissolve 400 mg of metamizole sodium (calculate the amount of tablet mass) in 4 ml of water, add 2 ml of 10% nitric acid (V), and 0.2 ml of 1% solution of sodium nitrate (III). The colour of this solution changes to blue for only a while, and then it become colourless. Add 0.4 ml of 5% solution of silver nitrate (V) to the colourless mixture. The solution becomes cloudy and then blue. After a while the colour of the mixture changes from blue to yellow-green and metallic from silver precipitates.?
2. Dissolve 400 mg of metamizole sodium (calculate the amount of tablet mass) in 10 ml of 10% hydrochloric acid and heat to boiling. First sulphur dioxide, than formaldehyde, are emitted from the solution. 1 ml of Schiff reagent add to 1 ml of the hot mixture. Observe the change of the colour of the solution to violet.

QUANTITATIVE ANALYSIS

The content of metamizole sodium is evaluated by the iodometric method described in Polish Pharmacopoeia V.



Dissolve 200 mg of metamizole sodium (calculate the amount of tablet mass) in 5 ml of water, add 5 ml of 0.02M hydrochloric acid, and titrate the mixture with 0.05M solution of iodine. Add to the titrating mixture the solution of starch before the end of titration process. Make a blind test.
1 ml of 0.05M solution of iodine corresponds to 16.67 mg of anhydrous metamizole.

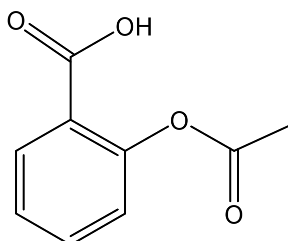
EXERCISE No 5.4. (2 meetings)

ASCODAN

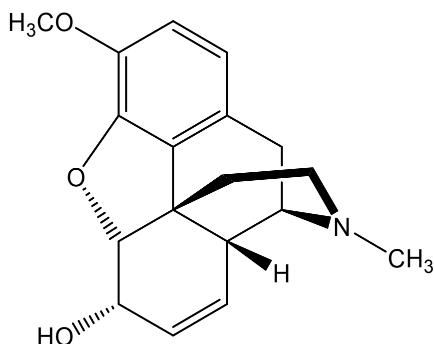
(**ACETYLSALICYLIC ACID** + **CODEINE PHOSPHATE**)

REAGENTS		EQUIPEMENT	
ASCODAN	3 p. (31.5 tab.)	tubes	
10% NH_4OH	35 ml	beaker	250 ml
HCl dil.		heating mantel	
diethyl ether	155 ml	pipette	5 ml
chloroform	155 ml	measuring cylinder	50 ml
H_2SO_4 conc.	5 ml	conical flasks	2 x 100 ml
0.25M H_2SO_4		burette	
10 % $FeCl_3$		mortar with pestle	
25% HNO_3		evaporator	
2 % silver nitrate (V)	0.15 ml	test papers	
Marqis reagent (1 drop of formaldehyde in 20 ml of conc. H_2SO_4)		separator	
0.1 M NaOH	5 ml	condenser	
0.5 M NaOH	80 ml		
glacial acetic acid	30 ml		
0.05 M chloric acid (VII)			
anhydr. Na_2SO_4			
crystal violet			
phenolphtaleine			

Ascodan is a two-component drug. It contains 400 mg of **acetylsalicylic acid** and 8 mg of **codeine phosphate**.



Acetylsalicylic acid (acetosal, aspirin) is a drug in the family of salicylates, often used as an analgesic (against minor pains and aches), antipyretic (against fever), and anti-inflammatory. It has also an antiplatelet (“blood-thinning”) effect and is used in long-term low-doses to prevent heart attacks and cancer. Low-dose long-term aspirin irreversibly blocks the formation of thromboxane A2 in platelets, producing an inhibitory effect on platelet aggregation, and this blood-thinning property makes it useful for reducing the incidence of heart attacks. Aspirin produced for this purpose often comes in 75 or 81 mg dispersible tablets and is sometimes called “Junior aspirin” or “Baby aspirin.” High doses of aspirin are also given immediately after an acute heart attack. These doses may also inhibit the synthesis of prothrombin and may therefore produce a second and different anticoagulant effect.



Codeine or **methylmorphine** is an opiate used for its analgesic, antitussive and antidiarrheal properties. It is marketed as the salts codeine sulfate and codeine phosphate. Codeine hydrochloride is more commonly marketed in continental Europe and other regions. Codeine is an alkaloid found in opium in concentrations ranging from 0.3 to 3.0 percent. While codeine can be extracted from opium, most codeine is synthesized from morphine through the process of *O*-methylation.

EXTRACTION

Dilute the powdered tablet mass of ascodan, which contains 200 mg of codeine phosphate, in 150 ml of water. Next add 50 ml of 25% NH_4OH, and extract the solution with 50 ml of chloroform in the separator. Repeat the extraction process two times. Combine the organic phases together and add a drying substance to this mixture, to dry it from some rests of water. Filter the organic phase straight into a round bottom flask, evaporate this chloroform extract to dryness and weigh the residue which should contain codeine phosphate. Add 3 M HCl to the water part of extract, by controlling it with a test paper, to receive acidic mixture. In these conditions acetylsalicylic acid should precipitate from the solution. Next, extract the water solution with 50 ml of diethyl ether. Repeat the extraction process two times. Combine the organic phases together and add a drying substance to this mixture, to dry it from some rests of water. Filter the organic phase straight into a round bottom flask, evaporate the ether part of the extract to dryness and weigh the received residue.

IDENTIFICATION ANALYSIS

Codeine phosphate:

1. Dissolve 10 mg of the substance (or a very small quantity, because of their paste-like consistency) in 5 ml of concentrated H_2SO_4, add 1 drop of 10% solution of $FeCl_3$ and heat the mixture in the water bath to receive blue-violet colour (hydrochloride morphine; the solution of morphine should be rose). After adding 1 drop of 25% solution of HNO_3 the colour should change to red.
2. Add 1 drop of Marquis reagent to a small quantity of the substance. The colour of the receiving mixture should change to violet (presence of codeine).
3. Dissolve 30 mg of the substance (or a very small quantity, because of their paste-like consistency in 5 ml of water, and add 0.15 ml of 2% solution of silver nitrate (V) to receive the yellow precipitate of silver phosphate, which is easy to dissolve in 10% solution of NH_4OH and 10% solution of HNO_3.

Acetylsalicylic acid:

1. Add 10 ml of water to 100 mg of the substance, and heat the mixture to boiling. After 1 minute cool the solution, and add 1 drop of 10% solution of $FeCl_3$. The colour of the mixture should change to dark violet.
2. Add 5 ml of the solution of 0.1M NaOH to 100 mg of the substance and heat the mixture in 3 minutes. Next cool the solution, and add the diluted sulphuric acid to receive acidic solution. The white crystals of salicylic acid should precipitate from the solution.

QUANTITATIVE ANALYSIS

1.
The content of codeine phosphate is evaluated by the acidimetric method described in Polish Pharmacopoeia V.
Dissolve 75 mg of the substance in 10 ml of glacial acetic acid by heating the mixture. Next cool the mixture, and titrate it with the solution of 0.1 M chloric acid (VII) to receive a colour change. As an indicator of the titration process use crystal violet. Make a blind test. Repeat the titration process.
1 ml of the solution of 0.1 M chloric acid corresponds to 39.74 mg of anhydrous codeine phosphate.

2.
Acetylsalicylic acid is evaluated by the alkalimetric method, which is described in Polish Pharmacopoeia V. At first the hydrolysis of acetylsalicylic acid is necessary to receive sodium salt of salicylic acid and sodium acetate in the solution of NaOH at high temperature. The non-reacted residue of NaOH is titrated. The blind test is very important, because during heating the solution of NaOH, it could react with CO_2 from air.
2 mol of NaOH are needed to hydrolyze 1 mol of acetylsalicylic acid.

Weigh out the dry acetylsalicylic acid after extraction process (the expected mass is 12.0g). Take only 0.025 part of the whole mass (2.5 % of the dry mass) which corresponds to about 300 mg of a pure substance. Dissolve it accurately in 20 ml of 0.5M NaOH and heat the mixture under reflux for 15 minutes. Cool the solution and titrate the non-reacted residues of NaOH with 0.5M H_2SO_4. As an indicator use phenylphtaleine. Do this procedure 3 times. Make a blind test by repeating the procedure, also with heating the solution of NaOH.

1 ml of the solution of 0.5M H_2SO_4 corresponds to 0.0909g of acetylsalicylic acid.

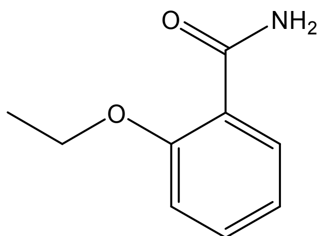
EXERCISE No 5.5. (2 meetings)

ETOPIRYNA

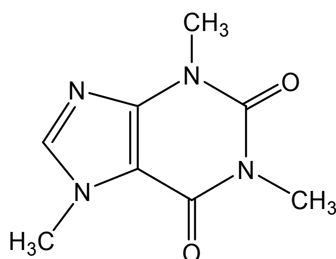
(ACETYLSALICYLIC ACID + ETHENZAMIDE + CAFFEINE)

REAGENTS		EQUIPEMENT	
ETOPIRYNA	2 g (3 tab.)	mortar with pestle	
distilled water		tubes	
25% NH_4OH		beaker	
concentrated HCl	1 ml	separator	
$KClO_3$	0.05 g	heating mantel	
Marqis reagent (1 drop of formaldehyde in 20 ml of conc. H_2SO_4)		evaporator	
		pipette	
Mandelin reagent (1 g of NH_4VO_3 dissolved in 1.5 ml of H_2O, and diluted in 100 ml of conc. H_2SO_4)		conical flasks	5 ml
		burette	50 ml
1M NaOH	2 ml	funnel	3 x 100 ml
0.5M NaOH	80 ml	balance	
0.1M NaOH		test papers	
chloroform	160 ml	measuring cylinder	
solution of Na_2CO_3 in water (1:15)	15 ml		
sodium benzoate	0.2 g		
concentr. H_2SO_4			
2M H_2SO_4	5 ml		
1M H_2SO_4	1.5 ml		
0.5M H_2SO_4			
0.1M iodine	60 ml		
0.1 M $Na_2S_2O_3$			
ethanol			
starch solution			
phenolphtaleine			

Etopiryna is a three-component drug. It contains 300 mg of **acetylsalicylic acid**, 100 mg of **ethenzamide** and 50 mg of **caffeine**.



Ethenzamide is a common analgesic and anti-inflammatory drug that is used for the relief of fever, headaches, and other minor aches and pains. It is a major ingredient in numerous cold and flu medications and many prescription analgesics. Ethenzamide (2-ethoxybenzamide) is a white to cream-white, odourless, powder. Soluble in ethanol and in methanol, practically insoluble in water and in solutions of alkali hydroxides.



Caffeine (1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione) is a xanthine alkaloid compound that acts as a stimulant in humans. Caffeine is called **guaranine** when found in guarana, **mateine** when found in mate and **theine** when found in tea. Overall, caffeine is found in the beans, leaves and fruit of over 60 plants, where it acts as a natural pesticide that paralyzes and kills certain insects feeding on the plants. Caffeine is a central nervous system (CNS) stimulant, having the effect of temporarily warding off drowsiness and restoring alertness. Beverages containing caffeine, such as coffee, tea, soft drinks and energy drinks enjoy great popularity; caffeine is the world's most widely consumed psychoactive substance, but unlike most other psychoactive substances, it is legal and unregulated in nearly all jurisdictions. In North America, 90% of adults consume caffeine daily. Caffeine forms odourless, white needles or powder. It is soluble in ethyl acetate, chloroform, pyridine, pyrrole, tetrahydrofuran solution; moderately soluble in alcohol, acetone; slightly soluble in petroleum ether, ether, benzene.

IDENTIFICATION ANALYSIS

1. Dissolve 200 mg of powdered tablet mass in 5 ml of hot water. Then cool the solution, filter it, and evaporate the filtrate do dryness. Add 0.05g of potassium chlorate ($KClO_3$), 5 drops of concentrated HCl, shake it, and again evaporate to dryness. Cool it and add 1 drop of concentrated NH_4OH (25%). The red colour (it could be also rose) of the received solution is responsible for the presence of caffeine.
2. Dissolve 200 mg of powdered tablet mass in 10 ml of chloroform. Divide the solution into two equal parts. Add 1 drop of Marquis reagent to the first solution. Its colour should change to red or orange (presence of ethenzamide). Add 1 drop of Mandelin reagent to the second solution. The green-brown colour of the solution is the result of the presence of ethenzamide.
3. Add 2 ml of 1 M NaOH to 200 mg of powdered tablet mass and heat it to boiling. After 2 minutes cool the mixture. The solution should be acidic. Do it with 1M H_2SO_4, control this process by using test papers. The white crystals of salicylic acid should precipitate from the solution.

EXTRACTION AND QUANTITATIVE ANALYSIS

1.

Put the 1.4g of powdered tablet mass into a separator, add 30 ml of chloroform, and shake the mixture in 5 minutes to dissolve all ingredients. Then add 15 ml of water solution of Na_2CO_3 (1:15 m/v). Extract this mixture in 15 minutes and leave it to settle. In the water fraction there should be sodium salt of acetylsalicylic acid, and in the chloroform fraction – caffeine and ethenzamide. Save the water fraction. Extract the water fraction with another 2 portions of chloroform (2 x 20 ml). Put the chloroform fractions together, dry them from the rests of water using the drying substance, filter it and put it into a dry, weighted flask. Do not forget

about washing the precipitate of drying substance and the filter paper with a small volume of chloroform. Evaporate the chloroform extract to dryness. The weight of the received precipitate is the sum of caffeine and ethenzamide mass.

2.
Add 200 mg of sodium benzoate to the dry chloroform extract, then add 5 ml of water to dissolve caffeine. Shake the mixture in 5 minutes and filter it. Wash the filter paper with another 5 ml of water. Save the filter paper with the precipitate. Put the water filtrate into separator, add 0.5M NaOH to make an alkali solution. Control it with test paper. Then extract the water solution with 10 ml of chloroform. Repeat the extraction process two more times. Dry the chloroform extract with the drying substance to absorb some rests of water, filter it and put into dry, weighted flask and evaporate it to dryness. To receive the weight of caffeine monohydrate multiply the mass of the evaporated rest by 1.093.
The content of caffeine is evaluated by the iodomeric method described in Polish Pharmacopoeia V. Dissolve the received caffeine in 45 ml of water, acidize this solution with 5 ml of 2M H_2SO_4 and add 50 ml of 0.1M iodine solution, shaking all the time. After sedimentation, filtrate the precipitate. Put 3 x 20 ml of the supernatant to the 3 different flasks and titrate each the excess of the rest of non-reacted iodine with 0.1M sodium thiosulfate solution ($Na_2S_2O_3$) in the presence of starch. Do not forget about a blind test using 20 of the solution (9 ml of water + 1 ml of 2M sulfuric acid + 10 ml of 0.1M iodine solution).
1 lm of the solution of 0.1M iodine corresponds with 4.853 mg of caffeine and with 5.305 mg of caffeine monohydrate.

3.
Dissolve the precipitate of ethenzamide (the precipitate after filtration of the caffeine solution – the rest on the wall of the flask and on the filter paper) in 5 ml of chloroform. Wash the filtration paper with another 5 ml of chloroform. Repeat it three times. Dry the chloroform solution with the drying substance, filtrate it, wash the filter paper with another few ml of chloroform. After drying, using drying substance put the chloroform fraction into dry, weighted flask and evaporate the solution to dryness. Weigh the mass of ethenzamide.

4.
Acidize the water solution of sodium salt of acetylsalicylic acid with 1M solution of H_2SO_4, control it with test papers. Extract this solution with 20 ml of chloroform. Repeat this process. Dry the chloroform extract with the drying substance from the rests of water. Put the chloroform fraction into dry, weighted flask and evaporate the solution but not to dryness. Dry the wet precipitate under fumehood (dygestorium) and weigh it to receive the mass of acetylsalicylic acid.
To verify the mass of acetylsalicylic acid dissolve it accurately in 60 ml of 0.5M NaOH, divide it into 3 equal parts. Heat each mixture under reflux for 15 minutes. Cool each solution and titrate the non-reacted residues of NaOH with 0.25M H_2SO_4. Usee phenylphtaleine as an indicator. Make a blind test by repeating the procedure, also with heating the solution of NaOH.
1 ml of the solution of 0.25M H_2SO_4 corresponds to 0.0909g of acetylsalicylic acid.

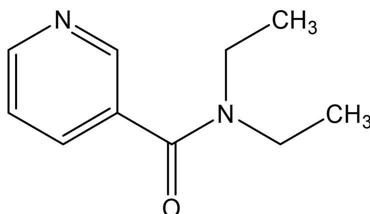
EXERCISE No 5.6.

CARDIAMIDUM

(CARDIAMIDE, NIKETHAMIDE)

(drug in drops)

REAGENTS		EQUIPEMENT	
CARDIAMIDUM 25% (in drops)	8.5 ml	tubes	
2% $CuSO_4$		beaker	
0.1 M ammonium thiocyanate		separator	
1 M NaOH	3 ml	heating mantel	
0.1 M H_2SO_4		evaporator	
concentr. H_2SO_4		cooler	
0.1 M $KMnO_4$		measuring cylinder	25 ml
glacial acetic acid	18 ml	conical flasks	3 x 100 ml
acetic acid anhydride	20 ml	burette	
0.05 M chloric acid (VII)		funnel	
crystal violet		balance	
distilled water		Petri dish	
0.1 M HaOH		pipette	5 ml
phenolphtaleine			



Nikethamide is a stimulant which affects mainly the respiratory cycle. It was formerly used as a medical countermeasure towards tranquilizer overdoses. It is a central nervous system stimulant. Nikethamide was formerly used in the treatment of barbiturate overdose but is now considered to be of no value for such purposes and may be dangerous. In sports, nikethamide is listed by the World Anti-Doping Agency as a banned substance.
Cardiamide (other names: diethylamide nicotinic acid, nicethamidum, nicorine, nicotinoyldiaethylamidum, nikethylamide; proprietary names: Cardiamidum, Coramin(e), Cormed, Juvacor, Kardonyl; chemical name: *N,N*-diethyl–3–pyridinecarboxamide). A colourless or slightly yellow substance, oily liquid or crystalline solid. Miscible with water, acetone, ethanol, chloroform, and ether. pH of 25% w/v solution is 6.0-7.8.

IDENTIFICATION ANALYSIS

1. Put 1 drop of cardiamide solution (25%) on a Petri dish (small glass dish) and add 1 drop of 2% solution of $CuSO_4$. The colour of solution should change to deep blue. After adding 1 drop of 0.1 M solution of ammonium thiocyanate forms green crystalline precipitate. It is a complex of cardiamide with copper and thiocyanate.

2. Add 3 ml of 1 M NaOH to 5 drops of cardiamide solution (25%) and heat the mixture. The fumes of diethylamine are emitted after the degradation of cardiamide.
3. Add 5 drops of concentrated H_2SO_4 to 0.5 ml of the solution of cardiamide (25%). Mix the solution and then add 3-4 drops of 0.1M solution of $KMnO_4$. The mixture should become brown or violet. Then after heating, the colour should change to colourless because of the reduction of the level of oxidizing of manganese ions. Lactic acid reacts to give CO_2 and acetic aldehyde (the characteristic smell).

QUANTITATIVE ANALYSIS

1.
Add 5 ml of glacial acetic acid to 0.5 ml of cardiamide solution (25%). Next add to the solution 5 ml of acetic acid anhydride and again 5 ml of glacial acetic acid. Heat the mixture in 5 minutes under reflux. After cooling the solution add a small pinch of crystal violet and titrate this mixture with 0.05 M chloric acid (VII) to change the colour of solution from violet to deep blue. Carry out this procedure three times. Make a blind test and instead of the solution of cardiamide use water.
1 ml of 0.05M solution of chloric acid (VII) corresponds with 8.91 mg of cardiamide.

2.
Add 20 ml of water to 2 ml of cardiamide solution (25%). Titrate this solution with 0.1M NaOH solution. As an indicator use phenolphtaleine. Carry out this procedure three times. Make a blind test and instead of the solution of cardiamide use water.
1ml of 0.1M NaOH solution corresponds with 9.008 mg of lactic acid.

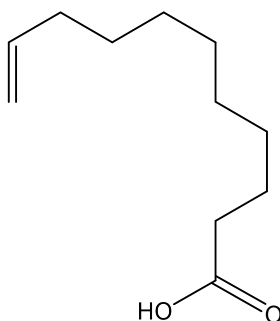
EXERCISE No 5.7. (2 meetings)

UNGUENTUM UNDECYLENICUM

(**UNDECYLENIC ACID AND ITS ZINC SALT**)
(drug in ointment form)

REAGENTS		EQUIPEMENT	
UNGUENTUM UNDECYLENICUM (in ointment)	7 g	tubes	
distilled water		beaker	
concentrated NH_4OH	1 ml	separator	
thioacetamide (TAA / AKT)		heating mantel	
10 % H_2SO_4	25 ml	evaporator	
diethyl ether	70 ml	cooler (condenser)	
aniline	3 ml	measuring cylinder	25 ml
powdered zinc		conical flasks	100 ml
ethanol	140 ml	burette	
active carbon		funnel	
concentrated HCl	12 ml	balance	
hexane	270 ml	pipette	
0.1 M HaOH		evaporator	
phenolphtaleine		Büchner funnel	

Undecylenic Acid (9-undecylenic acid, Ω-hendecenoic acid, 10-undecenoic acid, undec-10-enoic acid, 10-hendecenoic acid, 10-henedecenoic acid) is an organic unsaturated fatty acid derived from natural Castor oil (ricinoleic acid). It is used in manufacturing pharmaceuticals, cosmetics and perfumery including anti-dandruff shampoos, anti-microbial powders and as a musk in perfumes and aromas. Undecylenic acid is produced by cracking of Castor oil under pressure.



Undecylenic acid and its salts exhibit fungicidal effects against dermatophytes (*Epidermophyton floccosum*, *Trichophyton* and *Microsporum* genus) and weak antibacterial effects. It results from the formation of an environment with pH that is unfavourable for the development of microorganisms.
It exerts an effect not only on mycelium but on fungal spores as well**.** Undecylenic acid has been shown to be effective in preventing fungal overgrowth associated with vaginal and gastrointestinal candidiasis via its fungicidal activity. Undecylenic acid has long been known

to be fungicidal against *Candida albicans*, thus helping achieve a healthy balance of normal vaginal and intestinal flora. Undecylenic acid has been shown to have antibacterial and antiviral properties *in vitro* and is effective topically against the herpes simplex virus in both animals and humans. The ointment may irritate mucous membranes. It may cause nausea, vomiting, diarrhoea, headache, smell disturbances and, when overdosed, swelling of joints and lymph nodes.
1g of the ointment of Unguentum Undecylenicum contains 50 mg of undecylenic acid, 200 mg of zinc undecylenate, white petrolatum, anhydrous eucerin, Aseptine A and Aseptine M.

IDENTIFICATION ANALYSIS

1. Add 10 ml of water to 0.5g of the ointment, and 1 ml of concentrated NH_4OH, mix it well and filter. Then add a few drops of the fresh solution of thioacetamide (TAA/AKT) to the filtrate. A white flocculent precipitate should arise in the solution (the presence of zinc).
2. Add 20 ml of water to 5g of the ointment, next add 25 ml of 10% solution of sulfuric acid, and shake the mixture in a separator with 25 ml portion of diethyl ether, repeat the extraction process once. Put the ether fractions together and dry the ether fraction with a drying substance, and filter the mixture to the dry round-bottom flask. Wash also the precipitate and the filter paper with 10 ml of diethyl ether and evaporate the ether fraction to dryness. Measure the refraction coefficient of received paste-like substance (about n_D^{20}=1.4475-1.4485).
3. Add 3 ml of fresh distilled aniline to the received undecylenic acid, a small pinch of zinc powder and heat it to boiling for 10 minutes, under reflux. After cooling the solution add the mixture of 10 ml of ethanol and 10 ml of diethyl ether. Dissolve the yellow-orange liquid and put the solution without rests of zinc powder and non-dissolved waxes into the separator. Then add a portion of 20 ml of water into the separator and shake this mixture in 10 minutes, change water phase and repeat the extraction twice. After extraction process dry the organic fraction using the drying substance, filter this solution. Add a few milligrams of active carbon to the filtrate, shake the mixture and filter it on a simple funnel. Then evaporate the organic fraction to dryness, and crystallize the received anilide in 70 % ethanol. To do it add 7 ml of ethanol to the dry rest, heat the mixture a little to dissolve anilide. Then cool the mixture in an ice bath and add 3 ml of water in small portions. Cool the solution in a flask in a refrigerator to receive white crystals of anilide. Filter the precipitate under low pressure using Büchner funnel, dry it, and measure the melting point of the received anilide (it should be 66-67.5 ºC).

QUANTITATIVE ANALYSIS

Add 3 ml of concentrated HCl to 0.5g of the ointment and mix it well. Then add 50 ml of water and heat it a little to dissolve the ointment better. Put the dissolved mixture into a separator. Extract it with 30 ml of hexane, repeat it twice. Dry the hexane fraction from rests of water using the drying substance, and the filtrate evaporate to dryness. Measure the mass of the received rest (undecylenic acid and its zinc salt). Dilute the dry rest in 30 ml of ethanol and titrate the solution with 0.1M solution of NaOH. Use a few drops of phenylphtaleine as an indicator. Repeat all steps of this process 3 times. Make a blind test.
1 ml of 0.1M solution of NaOH corresponds with 18.43 mg of undecylenic acid (the free form of acid and also in the form of zinc salt).

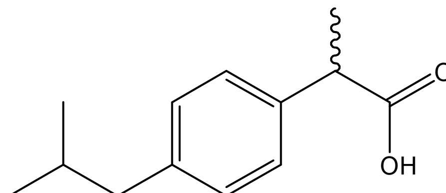
EXERCISE No 5.8.

IBUPROFEN

(drug in suspension form)

REAGENTS		EQUIPEMENT	
IBUPROFEN, in suspension	30 ml	tubes	
Marquis reagent		beaker	
(1 ml of 40 % formaldehyde in		separator	
100 ml of concentrated H_2SO_4)		heating mantel	
Libermann reagent		evaporator	
(1g of $NaNO_2$ or KNO_2 dissolved in		measuring cylinder	25 ml
10 ml of H_2SO_4, cooled and mixed		conical flasks	100 ml
to absorbe brown fumes by the solution)		burette	
concentrated H_2SO_4		funnel	
diethyl ether	150 ml	balance	
water		pipette	
ethanol	120 ml		
0.1 NaOH			
phenolphtaleine			

Ibuprofen is a non-steroidal anti-inflammatory drug, originally marketed as Nurofen and later under various trademarks including Ibum, Ibuprofen, Bolinet, Ibufen, Ibuprom, Nurofen, and multicomponent drugs like Modafen, Nurofen Plus. It is used for relief of symptoms of arthritis, primary dysmenorrhoea, fever, and as an analgesic, especially where there is an inflammatory component.



Ibuprofen was made available under prescription in the United Kingdom in 1969 and in the United States in 1974. In the years since, the good tolerability profile along with extensive experience in the community (otherwise known as Phase IV trials) has resulted in the rescheduling of small packs of ibuprofen to allow availability over-the-counter in pharmacies worldwide and indeed in supermarkets and other general retailers.

Ibuprofen is believed to work through inhibition of cyclooxygenase (COX), thus inhibiting prostaglandin synthesis. There are at least 2 variants of cyclooxygenase (COX-1 and COX-2). Ibuprofen inhibits both COX-1 and COX-2. It appears that its analgesic, antipyretic, and anti-inflammatory activity is achieved principally through COX-2 inhibition; whereas COX-1 inhibition is responsible for its unwanted effects on platelet aggregation and the GI mucosa.

Ibuprofen can have the following effects even if as few as four tablets are taken: common adverse effects include: nausea, dyspepsia, gastrointestinal ulceration/bleeding, raised liver enzymes, diarrhoea, headache, dizziness, salt and fluid retention, hypertension. Infrequent adverse effects include: oesophageal ulceration, heart failure, hyperkalaemia, renal impairment, confusion, bronchospasm, rash. Very infrequent adverse effects include Stevens-Johnson syndrome.

Ibuprofen has been reported to be a photosensitizing agent. However, this only rarely occurs with ibuprofen and it is considered to be a very weak photosensitizing agent when compared with other members of the 2-arylpropionic acids. This is because the ibuprofen molecule contains only a single phenyl moiety and no bond conjugation, resulting in a very weak chromophore system and a very weak absorption spectrum which does not reach into the solar spectrum. Ibuprofen has been implicated in elevating the risk of myocardial infarction, particularly among those chronically using high doses.
It is a white-yellow powder with a characteristic smell, very well dissolved in ethanol and in diethyl ether, also in alkali solutions. In water practically non-dissolved. Melting point is 72 – 78 °C.

IDENTIFICATION ANALYSIS

1. Add 1 drop of Marquis reagent (1 ml of 40 % formaldehyde in 100 ml of concentrated sulfuric acid) to small quantity of ibuprofen suspension. The brown colour should turn out at least??/last (presence of ibuprofen). After heating to boiling, the colour should change to orange.
2. Add 2-3 drops of Libermann reagent (1g of $NaNO_2$ or KNO_2 dissolved in 10 ml of H_2SO_4, cooled and mixed to absorb brown fumes by the solution) to a small quantity of ibuprofen suspension. The colour of mixture should change to brown-orange. This test must be repeated, but use concentrated H_2SO_4 instead of Libermann reagent.

QUANTITATIVE ANALYSIS

Add 10 ml of the syrup to 20 ml of water. Mix it well and add 20 ml of diethyl ether. Extract this mixture in a separator in 10 minutes. Extract the water fraction with another portion of diethyl ether and repeat it. Combine diethyl ether fractions and dry the solution with a drying substance, filter it and evaporate it to dryness. Measure the receiving mass of ibuprofen. It should by about 0.2 g. Repeat it two times to receive three similar extracts.

Dissolve ibuprofen extracts each time in 30 ml of ethanol. Titrate each solution with 0.1 M NaOH. As an indicator use a few drops of phenolphtaleine. Make a blind test.
1 ml of 0.1 M solution of NaOH corresponds with 20.63 mg of ibuprofen.

EXERCISE No 5.9. (2 meetings)

GUAIAFENESIN

(WILLIAMSON ETHER SYNTHESIS AND ISOLATION FROM TABLETS)

REAGENTS		EQUIPEMENT	
guaiacol	2,55 ml	tubes	
GUAJAZYL (Espefa), tablets 100 mg	2 tab.	beaker	250 ml
ethanol	16 ml	heating mantel	
NaOH solid	1 g	pipette	5 ml
water		condenser	
3-chloro-1,2-dipropanodiol	2 ml	glass funnel	
ethyl acetate	90 ml	Büchner funnel	
$MgSO_4$ (drying substance)		filter flask	200 ml
hexane	95 ml	crystalizator	
concentr. HNO_3	3 ml	round-bottom flask	250 ml
diethyl ether	10 ml	evaporator	
$FeCl_3$		separator	
chloroform	15 ml		
Marquis reagent (1 ml 40% formaldehyde in 100 ml conc. H_2SO_4)			
Mandelin reagent (1g NH_4VO_3 dissolved in 1.5 ml H_2O and added to 100 ml conc. H_2SO_4)			
Libermann reagent (1 g $NaNO_2$ or KNO_2 dissolved in 10 ml H_2SO_4, cooled and mixed to absorb brown fumes).			

Guaifenesin (3-(2-methoxyphenoxy)-1,2–propanediol) is a natural substance that was isolated around the early 1500's for purported uses to cure rheumatism. Though it has since been proven ineffective on rheumatism, at present it is a widely used cough expectorant in nearly every cough, cold, flu, and allergy medication on the market. Various preparations containing this compound include Tylenol Cold®, Robitussin® and Guai-Aid® as either tablet or syrup form. The tablets are the best source for the laboratory extraction as they include the highest concentration of the target with the fewest interferences. One stereogenic centre is present within the molecule, resulting in two enantiomers. Guaiafenesin is administered as a racemic mixture in both syrup and tablet form, although it has been implied in the literature that the (S)-(+) enantiomer is the more potent expectorant.

The extraction from the natural source involves a very simple steam distillation process followed by a series of acid-base extractions to remove the guaifenesin resin from the other substituents in the distillate. As with most natural ingredients, the natural extraction processes are not efficient enough, or the compound simply is not abundant enough from its natural sources for a large-scale distribution. Thus, synthetic processes must be designed to obtain the needed compound. For the laboratory synthesis of guaifenesin, a simple Williamson ether synthesis reaction will provide the desired product, retaining the stereochemistry of the original stereo reactant, 3-chloro-1,2-propanediol. The Williamson ether synthesis reaction

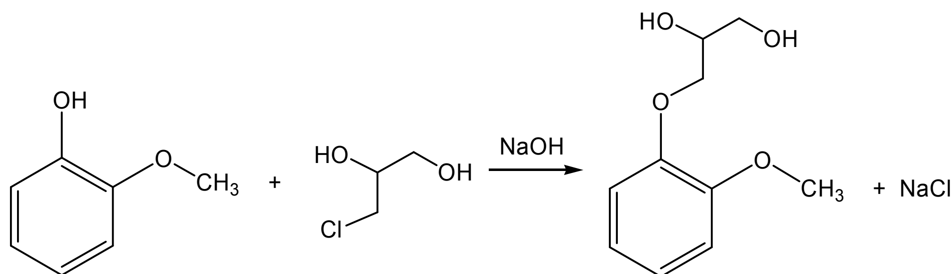
between an alkoxide or phenoxide anion and a sterically unhindered alkyl halide has been extensively studied for 150 years. The reaction proceeds by an S_N2 mechanism and is a subject of discussion.

The first reactant, guaiacol (*o*-methoxyphenol), is dissolved in ethanol and refluxed with 6.25M solution of sodium hydroxide, a strong base. This serves to deprotonate the phenyl ring, to give the highly reactive phenoxide ion. The second reactant, 3-chloro-1,2-propanediol, is also dissolved in ethanol and then added to the reaction mixture after the period of refluxing. The ethanol is an aprotic solvent that aids in reducing the competing E2 mechanism, and is also a solvent that is easily removed by vacuum filtration. Washing the precipitated product with water and making several extractions of the aqueous layer serves to remove the formed NaCl and recrystallizing the organic layer from ethyl acetate with hexane provides the racemically pure product. Pure guaifenesin appears as a white crystalline powder, that with further analysis is revealed to be minute rhombic prisms.

HAZARDS

The reactant (±)3-chloro-1,2-propanediol is a highly toxic liquid by skin absorption, ingestion, and particularly inhalation and can target the central nervous system. This compound is also suspected to be carcinogenic. It is therefore essential that each one is dispensed wearing protective gloves, safety glasses and a laboratory coat. Guaiacol (2-methoxyphenol) is a toxic liquid that should be treated in the manner described for (±)3-chloro-1,2-propanediol.

SYNTHESIS



In a 250 ml round bottomed flask dissolve 2.2 ml (20 mmol) of guaiacol in 12 ml of ethanol in a fume hood and add 1g of crushed NaOH pellets dissolved in 4 ml of water (25 mM). Then heat the mixture under the reflux for 10 minutes. Cool the mixture and add dropwise to the phenoxide anion the ethanolic mixture of (±)3-chloro-1,2-propanediol (2 ml of (±)3-chloro-1,2-propanediol dissolved in 2 ml of ethanol, 23.92 mmol). Continue the heating process for 1 h. After cooling the mixture evaporate the rests of ethanol using a rotary evaporator. During the evaporation process the rests of NaCl, which is one of the products of this reaction, will crystallize in the flask. Add 10 ml of water to dissolve the precipitated salt. Then add a water solution of 30 ml of ethyl acetate. Extract the mixture for 10 minutes, then put it into a separator and separate fractions. Repeat the extraction process of the water phase with another portion of ethyl acetate. Combine the organic fractions and dry them using $MgSO_4$. Filter the solution on a glass funnel, and evaporate the filtrate to dryness to receive a pale yellow oil. It should be solidified by staying open in a locker for a week. Recrystallize

the crude product. To do it dissolve it in 10 ml of ethyl acetate, and then add 10 ml of hexane dropwise, cool the mixture to yield white crystals of guaiafenesin. Filter them using Büchner funnel, under reduced pressure, and wash with hexane. Dry the received crystals, measure the mass and melting point of them (78-79 ºC, 45-60% yield).

ISOLATION OF GUAIAFENESIN FROM TABLET MASS

Add 5 ml of ethyl acetate to the powdered tablet mass, containing 200 mg of guaiafenesin, and mix it well for 10 minutes. The insoluble compounds of tablet mass should be removed by gravity filtration, using a glass funnel. Wash the precipitate another portion of ethyl acetate. Add dropwise to the filtrate 20 ml of hexane to receive white crystals of guaiafenesin for a few minutes. Cool this mixture in an ice-bath and filtrate the precipitate using Büchner funnel under reduced pressure. Wash this precipitate with 5-10 ml of cold hexane, dry it, and measure mass and melting point of it (78-79 ºC). Calculate the yield of isolation in %.

IDENTIFICATION ANALYSIS

Guaiacol:

1. Add 0.1 ml of guaiacol and 3 ml of concentrated HNO_3 into a tube. The colour of the solution should change to red. This colourful product is very well dissolved in ethanol, chloroform and diethyl ether. Add 10 ml of diethyl ether to the red solution and shake this mixture very carefully. The colourful product is very well soluble in the organic solvent.
2. Dissolve 0.05 ml of guaiacol in a tube containing 2 ml of ethanol. Add a few drops of fresh prepared $FeCl_3$ to this solution. The solution should change its colour to light green.

Guaiafenesin:

1. Dissolve 100 mg of synthetized guaiafenesin in 20 ml of chloroform. Divide this solution into 4 equal portions (in tubes). Add one drop of Marquis reagent (1 ml of 40% formaldehyde dissolved in 100 ml of concentrated H_2SO_4) to the first solution. The colour of this solution should change to violet. Add one drop of Mandelin reagent (1 g of NH_4VO_3 – ammonium vanadate (V), dissolved in 1.5 ml of water and then in 100 ml of concentrated H_2SO_4) to the second tube. After that the solution should change the colour to green-grey. Add one drop of Libermann reagent (1g of $NaNO_2$ or KNO_2 dissolved in 10 ml of concentrated H_2SO_4 then cooled and mixed to absorb brown fumes) to the solution in the third tube. The colour of this solution will change to black. Repeat this test using the fourth solution but instead Libermann reagent use concentrated H_2SO_4. Heat the solutions to receive the right colours in a water bath if it is needed.

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