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Laboratory practice

Manual

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Introduction

This manual was edited in the framework of the EU Erasmus+ project "«Improving skills in laboratory practice for agro-food specialists in eastern Europe" /Ag-Lab/. The authors' team consists of the universities professors and the specialists of agro-food laboratories from Slovenia, France, Italy, Poland, Ukraine, Georgia and Moldova.

This edition is destined to master students of veterinary, agronomy, animal husbandry and food technologies faculties specialized on laboratory practice, to young specialists starting their carries at laboratories of agro-food sector as well as to specialists already working at laboratories for updating their professional knowledge.

The manual covers all areas of agro-food production and includes general information related to laboratory practice such as the bases of the work at chemical and biological laboratories, laboratory equipment, laboratory analyses methods, sampling techniques for different matrixes, international norms related to laboratory practice, quality management and metrology issues. Two specific parts are devoted to genetic analyses applied in animal husbandry and sensory analyses applied in foodstuffs control.

The manual provides the essential knowledge necessary to the work at laboratories and is edited in the form of guide. The additional sources should be used for deeper learning of some specific scientific, legal, technical questions.

This English version of the manual is supposed to be used internationally and contains the norms, rules, techniques, methods that are applied in EU countries and in the countries harmonizing their legislation and control systems to the European standards.

Chapter 1. Basics of Working in a Chemical Laboratory

1.1 Preparation of the laboratory for testing. Security rules

Working in a chemical laboratory for profans sometimes resembles magic, but for others it is not differ from the usual actions in the kitchen. And indeed, a chemist in the lab is like a chef in the kitchen. But the chemist does not work with ordinary dishes, but special ones and mixes not products, but chemical reagents.

In order to carry out chemical experiments successfully, it is necessary to know clearly what equipment is in the chemical laboratory, what utensils are used by chemists and what it is needed for. Chemical methods are usually based on the chemical reactions of the test substance with certain reagents in the presence of appropriate indicators using weight or volumetric analyzes.

Measuring in a chemical laboratory is associated with a number of hazardous and harmful factors. Safety is based on security and fire safety.

The laboratories constantly conduct research using chemicals. Improper handling of chemical substances may cause poisoning of workers, chemical burns, development of occupational diseases.

The rules of labor protection regulate the requirements for the microclimate, the content of harmful substances, noise and vibration levels, illumination in chemical laboratories.

The facilities of the laboratories provide natural, artificial and combined lighting, depending on the characteristics of the visual work. Local lighting should be used in combination with general lighting. The use of only local lighting is forbidden. Local lighting fixtures must be appropriate for the category and group of explosive substances and be fixed so that the worker can change the direction of the light beam if he wants.

The microclimate indices in the working area of the chemical laboratories shall meet the requirements of the State Sanitary Norms for the Microclimate of the Industrial Premises. In the work area of chemical laboratories, the content of dust, gases and vapors of harmful substances should not exceed the MPC established by applicable regulatory documents.

Laboratory work should only be carried out with proper ventilation, automatic ventilation and blocking must be provided. If any ventilation defects are found, the worker must inform the laboratory manager and the Department for Occupational Safety.

The facilities of chemical laboratories intended for work with extremely hazardous (1st class hazard) and highly dangerous (2nd class hazard) substances must be isolated from other laboratory premises, have a separate entrance and ventilation hoods which are not connected with other premises. All work with caustic, poisonous, with a sharp smell, flammable and explosive substances is carried out in isolated (from the general premises of the laboratory) and equipped with effective ventilation devices in the premises or in the hoods. Ventilation hoods are equipped with suction pumps.

To protect employees of chemical laboratories from the effects of hazardous and harmful factors, it is necessary to use the means of collective protection in accordance with the requirements of current regulatory documentation. The premises of the chemical laboratories shall be equipped with primary fire extinguishers (dry sand boxes, fire extinguishers, fire blankets made of non-combustible insulating material, etc.), the location of which shall be indicated by signs in accordance with ISO 6309: 2007 "Fire protection. Safety signs. Form and color "(ISO 6309: 1987, IDT).

Workers of chemical laboratories shall be provided with special clothing, special footwear and other personal protective equipment (PPE) in accordance with the requirements of the "Regulation on the procedure for providing workers with special clothing, special footwear and other personal protective equipment".

During the performance of the duties, the laboratory worker is obliged to observe the requirements of sanitary norms and personal hygiene: to work only in personal protective equipment; to keep the workplace clean and in proper order.

In order to neutralize spilled acids or alkalis, glasses with pre-prepared neutralizing solutions (baking soda for acids and acetic acid for alkalis, etc.) should be in the chemical laboratory. Solid wastes accumulated in the chemical laboratory should be collected in separate packaging and disposed in locations agreed with the sanitary and fire control authorities.

Works with violence of reaction, pressure increasing, overheat the glass unit or damage it by spraying hot or caustic products, as well as work under vacuum must be carried out in the hoods on special sheets. Transparent safety shields should be installed at the site of such work. Heat-resistant glass or porcelain utensils should be used when mixing or diluting substances accompanied by heat. Glass heat-resistant dishes are forbidden to heat on an open flame without heat-resistant mesh; thin-walled glass bottles and bulbs of ordinary glass cannot be heated over an open fire and hotplates.

Chemicals are stored in chemical laboratories in accordance with the certificate of terms and storage conditions of the manufacturer. The bulk (spare) amount of chemicals is stored in a special isolated room outside the chemical laboratory. Each receptacle shall be labeled with the exact name of the substance and with an inscription indicating the presence of toxic, flammable properties in the substance: red is for "Flammable", yellow is for "Poison", green is for "Keep away from water" or others. It is forbidden to store chemicals with illegible labels and without labels. Substances in non-labeled glasses are to be eliminated. When storing flammable and explosive substances, taking into account their physicochemical properties, additional precautions should be observed, namely: diethyl ether (sulfuric) should be stored isolated from other substances in a cold and dark place; metallic sodium should be stored in thick-walled glass jars with wide necks that are tightly closed with a cork under a layer of dry (without moisture) kerosene, paraffin or transformer oil in sand boxes; peroxide hydrogen, perchloric acid (concentrated) and other oxidants cannot be stored together with reducing agents, i.e. coal, sulfur, starch, etc.; metallic sodium and phosphorus cannot be stored with bromine and iodine. A glass dishes for the storage of flammable liquid substances with a capacity more than 1 liter should be placed in a hermetic metal case.

When performing a set of work in a chemical laboratory pressurized gas are used. These can be inert gases (e.g. argon, nitrogen, helium, carbon dioxide) or explosive which are capable of burning (hydrogen, oxygen). Gases are usually contained in cylinders under pressure up to 250 atm. Depending on the gas content, the cylinders are colored in a certain color.

The gases are taken from the cylinders using reducers that are colored in a color that matches the color of the balloon. The selection of gases without a suitable gearbox is strictly forbidden.

When using compressed gas (in some cases liquefied gas), first of all it is necessary to check carefully the proper condition of the cylinder and the gearbox and that the shelf life of the cylinder is not expired. It is forbidden to use them after the expiration date of the of the cylinder, as well as damage to the cylinder body or gearbox.

Gas cylinders should be protected from shocks, mechanical damage and heat. They are placed in metal boxes outside the premises, and gas is supplied to the laboratory by a special pipeline, which is installed according to certain rules.

It is strictly forbidden to leave the gas cylinder unattended with the open valve.

1.2 Chemical laboratory glassware

Chemical laboratory glassware can be divided into groups.

By the purpose:

- general;
- special;
- measuring.

By material: simple glass, special glass, quartz.

General glassware includes those items that should always be in the laboratory and without which most work cannot be done. These are: test tubes, simple and measuring funnels, glasses, flat-bottomed flasks, continuous cast dyes, conical flasks (Erlenmeyer's), Bunsen flasks, refrigerators, retorts, distilled water flasks, tees, taps and more.

Specialty glassware includes items used for a specific purpose, for example: Kipp apparatus, Soxhlet apparatus, Kjeldahl apparatus, fractioning columns, Woolf glasses, Tyschenko glasses, pycnometers, hydrometers, round bottom flasks, special refrigerators, melting and boiling temperature measuring devices and the so on.

Measuring dishes include: measuring cylinders and tubes, pipettes, burettes and measuring flasks. They are designed to measure fluid volume.

1.2.1. Pipettes.

A pipette is an empty tube with an elongated spout and it is an indispensable tool for dispensing liquids used in chemical, environmental, microbiological and medical laboratories.

Types of pipettes:

- graduated pipettes,
- Mohr pipettes,
- Sally pipettes,
- Pasteur pipettes,
- Panchenkov's pipettes,
- dropper pipettes,
- Electronic or mechanical pneumatic dispensers.

As a rule, they are made of chemically resistant glass or polymeric materials.

In analytical chemistry, graduated pipettes (with graduation lines) and Mohr pipettes (one division) are most commonly used. Among the graduated pipettes, micro pipettes are singled out, they are designed for dispensing liquids of 0.1 and 0.2 ml.

Pipes made of rubber or PVC are used to collect liquid into the pipette. There are also special pears for pipettes with valves.

Graduated pipettes were invented in the mid-XIX century and are still an indispensable tool for accurate measurement of a given volume of liquid. The pipette scale is graduated in cubic centimeters (cm3) at 20 °C. Graduated pipettes produce two accuracy classes (1st and 2nd) Graduated pipettes are manufactured of several types:

1 - straight pipettes with graduation lines;

1a - pipettes with graduation lines with a spare fountain;

2 – extended pipettes with graduation lines with extension;

2a - extended pipettes with graduation lines and spare fountain.

Besides, pipettes are divided into types. The three most popular types of pipettes are:

Type 1. Drain pipettes from the top zero to any mark. The lower mark on the pipette corresponds to the nominal capacity. Such pipettes are called incomplete drain pipettes. Pipettes of this type can be of the 1st and 2nd class of accurateness;

Type 2.Pipettes for draining liquid from any mark to the drain tip. The upper mark corresponds to the capacity value. These are pipettes for full downpour. Pipettes of this type can be of the 1st and 2nd class of accurateness;

Type 3. Drain pipettes from the top zero to the drain tip. The lower part of the drain tip corresponds to the nominal volume. Pipettes of this type can only be of 2ndclass of accurateness.

Nowadays, several types of automatic pipettes (dispensers) are used in all modern laboratories, namely:

- mechanical dispensers of variable volume;
- mechanical fixed volume dispensers;
- electronic dispensers.

Mechanical dispensers can be in different modifications, have different dosing volumes, different number of channels (usually 1, 8, 12 or 16), some models can be subjected to full or partial autoclaving.

Variable and fixed volume mechanical dispensers have a similar principle of work; the main distinguishing feature is that in the first case, you can choose the volume required for this analysis in the set parameters of the device (for example, from 100 to 1000 µl), and in the second one only the volume that is provided by this model (for example, 100 µl) can be chosen. The dosage volume is selected by rotating a special adjusting wheel (drum) on the housing of the tool, the value of the selected volume being displayed. Manufacturers usually place the adjusting drum under the main operating button of the device.

It is recommendedto use the dispenser tips once, and dispose them after usage. If you are dosing the same liquid, you cannot change the tip during operation, but after shift to other solutions, it is necessary to change the tip. If the work is done carefully, the tips can be reused by pre-rinsing, sterilizing and drying them. The tips are made of polypropylene, which withstands autoclaving at 121^0C.

There are two main types of tips: sterile and non-sterile.

Sterile tips should be used for polymerase chain reaction (PCR) and other medical analyzes that are important for the purity and exclusion of foreign DNA and RNA. The tips can be packaged in bulk packs or packaged in special tripods. The number of tips in a package is formed depending on the volume of one tip, ie the larger the volume, the less they are contained in the package. For each company and type of dispensers there are their own, unique tips, but there are also universal, and their size should correspond to the size and volume of the dispenser used.

The lower part of the dispenser is equipped with a so-called "landing cone" to which it is necessary to attach tightly the tip. Do not put on the tip with your hands, especially if you are working with sterile tips. You can use special tripods for your convenience.

Avoid temperature variations between the appliance, thetip and the dispensed liquid during operation to avoid damage of the device. Also, the temperature difference may affect the dosage accuracy.

Mechanical pipettes have a high advantage over glass in dosing speed, accuracy and convenience. But along with the benefits, mechanical pipettes (dispensers) also have disadvantages. For example, most of these devices cannot be used to select corrodent substances (concentrated acids, alkalis, solvents, etc.). During the work with such substances, the dispensing mechanism deteriorates and the pipette breaks down.

1.2.2. Glass flasks

Chemical laboratory glassware is made of various natural and artificial materials: glass, porcelain, corundum, chamotte, quartz, metal, plastics, etc. General-purpose utensils made of glass are the most common in the laboratories. Due to its thermal stability, glass is a suitable material for making chemical utensils. In cases where its chemical or thermal resistance is not sufficient, dishes made of other materials are used.

Thermal resistance means the ability of glass to withstand (without destroying) sharp fluctuations in temperature. In terms of thermal stability, glass is divided into groups based on their coefficients of thermal expansion (CTE) in the temperature range of 20–300 ° C.

Chemical resistance means the ability of glass to withstand the destructive effects of water, acids, alkalis and other chemical reagents.

The flask is a glass vessel with a round or flat bottom and an elongated neck. The flasks come in different capacities - from 50 cm^3 to several dm^3, with and without grinders:

- flat-bottomed,
- conical,
- round-bottomed
- pear-shaped.

Flat-bottomed and conical flasks are commonly used as receivers for the distillation of liquids, for solution preparation and crystallization. They cannot be used when the substances are heated to high temperatures and used under reduced pressure.

Round-bottom flasks are used for distillation of substances, including under vacuum. The length and diameter of the mouth of round-bottomed flasks may be different. Such flasks are two-, three-necked, etc. Round-

bottomed flasks with a discharge tube are called Würtz flasks. They are designed for distillation at atmospheric pressure. Kleisen flasks areusedfor distillation under reduced pressure.

Suction flasks (Bunsen) are used for vacuum filtration.

1.2.3. Measuring flasks

Usually these flasks are flat-bottomed with long necks, intended for the preparation of solvents of a certain concentration, dissolution of substances, dilution of solutions, have an annular mark on the cylindrical part of the neck.

Rules for working with measuring flasks. The flask is taken over the upper part of the throat, avoiding touching the arms with its convex part. From the heat transmitted by the hands of the walls of the flask, the capacity of the flask and, therefore, the volume of fluid in it increases. Before filling the flask is placed on a flat, well-lit table surface.

To dissolve the solid in a measuring flask, it is placed in a flask, which is filled with solvent no more than 1/2 or 2/3. Then the content of the flask is shaken in a smooth circular motion until complete dissolution of the substance. Only then add to the flask new solvent. The last portions of the solvent are added dropwise by means of a pipette equipped with a rubber cap. When adding the last drops of liquid, the eyes of the experimenter must be on a level with flask mark. The concave meniscus of the liquid surface with its lower part should merge with the mark line, and the convex meniscus should merge with the mark line with its upper part. The solvent droplets retained on the inner surface of the neck of the flask above the mark are carefully removed using a tube of filter paper. The solution is stirred very carefully after closing the flask with a cork.

The lower end is immersed in the solution almost to the bottom of the flask when selecting from the measuring flask of the solution with a pipette. If the pipette is lowered to the bottom of the flask, the air will leak when the solution is sucked in with the liquid.

It is not recommended:

a) to fill the measuring flasks with difficult-to-wash solutions or hard-to-remove gel on the walls of the flasks

b) to store in measuring flasks prepared solutions for a long time

c) to heat measuring flasks, mix residual reagents, use them improperly.

Maintenance of chemical utensils

The first and the main requirement for laboratory staff is the prohibition of the use of dirty dishes and equipment.

The skill to wash chemical utensils is a part of the laboratory technics, the knowledge of which is obligatory for every employee of the laboratory.

It is possible to remove dirt from the walls of dishes by various methods: mechanical, physical, chemical, physicochemical or combined.

In order to choose the method of washing dishes in each case, the following information should be available:

1. to know the properties of contaminants of dishes
2. to use the solubility of contaminants in water (cold or hot), in solutions of alkalis, various salts and acids;
3. to use the properties of oxidants, to oxidize in certain conditions organic and inorganic contaminants, to destroy them with the formation of easily soluble compounds;
4. all substances having surface-active properties (soap, synthetic detergents, cleaning clays, etc.) can be used for washing;
5. mechanical cleaning (using rust, etc.) can be used to remove sediment that contaminates dishes if it is chemically stable;
6. only cheap reagents should be used for washing;
7. 7. Always keep in mind the safety and the possibility of accidents when washing dishes. Every new lab worker should be familiar with the safety rules.

The following rules should be observed during washing chemical dishes:

1. You should wash dishes immediately after use, as a last at the end of the working day. Do not delay the washing of contaminated dishes the next day;

2. Choosen method of purification must be based on the nature of the pollution , i.e. its solubility in water or aqueous solutions, organic solvents, the ability to oxidize;
3. If you do not know which method of cleaning to prefer, start with the simplest and most affordable method - washing with hot or soapy water. Stronger agents such as hot solvents, concentrated acids and alkalis, chromium mixture should be used only when contamination is not washed with water;
4. Always put on rubber gloves while washing dishes, and when using harsh liquids, especially chromium mixture, concentrated alkalis, etc., put on protective glasses or a mask;
5. it is desirable that the dishes be cleaned by worker working with it. Other persons may do it only when they use the same substance in the laboratory, the contamination is non-aggressive, non-toxic and easily washed off by any mean. If the contamination properties of the laboratory assistant are unknown, he or she should receive an instruction before washing dishes.
6. utensils intended for particularly accurate operations and analytical purposes should be rinsed several times with distilled water after washing with tap water.

Measuring flasks, pipettes are considered to be pure only if they do not contain any visible contaminants and if distilled water flows from the inner walls of the vessel without leaving droplets. If there are water droplets on the glass, it indicates contamination of its by fatty substances. Fatty contaminants severely distort the results of measuring the capacity of all dishes, and therefore their presence is unacceptable. To remove fat pollution of the flask, pipettes washed with chromium mixture, alkaline solution of permanganate, a mixture of alcohol with ether, an alcohol solution of caustic potassium, a hot solution of trisodium phosphate (washing powder), etc. potassium and concentrated sulfuric acid.

The unmeasured measuring utensils should not be used in the analytical laboratory. Validation of a measuringdishes is to determine its true content (Vvalid.). As a result of the check, they find the ΔV correction to its nominal volume, which is indicated on the measuring dishes ΔVnom. = V valid - Vнам. Measuring dishes are thoroughly washed and dried before checking, then filled to the mark with distilled water at a certain temperature and barometric pressure. The water is poured into a pre-weighed measuring dish with an accuracy corresponding to the capacity of the measuring vessel so that the weighing error does not exceed 0.1% of water weight.

If ΔV goes beyond the permissible errors, then the dishes are corrected by implementation in the calculation of the error or drawing a new mark.

1.3 Solvents

Solvent is an individual chemical compound or mixture capable to dissolve different substances, that is, to form homogeneous systems of variable composition, consisting of two or more components, i.e. solutions.

Requirements for solvents:

Any substance can be a solvent for any other substance. However, in practice, only substances that meet certain requirements are classified as solvents. For example, solvents should have good, active solubility, being sufficiently chemically inert to the soluble substance and apparatus.

Chemical classification of solvents

The most commonly used chemical classification according to which all solvents are subdivided is:

• inorganic
• organic

Inorganic solvents

Water is the most common inorganic solvent used for a large number of inorganic and organic compounds. Inorganic solvents include: liquid ammonia which is a good solvent for alkali metals, phosphorus, sulfur, salts, amines, etc. substances; liquid sulfuric anhydride is a solvent for many organic and inorganic compounds.

Organic solvents

Organic solvents have great importance. These are solvents of petroleum (including their halogenated), alcohols, ethers and esters, ketones, nitro compounds. As solvents, mixtures of various individual substances, such as gasoline, petroleum ether, mixtures of alcohols and ethers, also diethyl ether, acetone, turpentine, carbon tetrachloride, and other substances are common.

Solvents are classified by characteristics:

• by boiling point, low-boiling solvents (e.g. ethanol, methyl acetate) and high-boiling solvents (e.g. xylene);
• by evaporation rate: the following types are distinguished:
- low volatiles (turpentine);
- medium volatility (kerosene);
- highly volatile (gasoline, white spirit, etc.).

It is important to know! The higher the volatility of the solvent, the more flammable and explosive it is.

• by polarities: non-polar (hydrocarbons, carbon disulfide) and polar (e.g. water, alcohols, acetone).

Specifications on the solvent usually contain data on the flash point, the limits of explosive vapor concentrations in the air, the vapor pressure at standard temperatures, and the solubility - for what type of substances can be used this solvent (for dissolving oils and fats, resins, natural and synthetic rubbers, etc.).

1.3.1.1. Rules for working with organic and inorganic solvents

All solvents are used in the liquid state. The method of application depends on its type and density and can be: brush, jet, as well as immersion, vaporization, electrodeposition and sputtering (pneumatic/airless /electrostatic).

It is important always to take into account the possibility of inflammation and therefore to adhere to certain rules during the cargo transportation processes, storage and during work.

In addition, improper handling of the solvent can adversely affect human health (the severity of this effect depends on the type of substance). Damage to the skin, mucous membranes, digestive tract, nausea, arrhythmia, tinnitus, excessive sweating are only part of the possible detrimental effects. To avoid poisoning or at least to minimize the toxic effect, it is necessary to strictly adhere to safety and use of PPE (glasses, masks, respirators). Avoid contact with the skin and enter the respiratory tract. If it does get on the skin, wipe and rinse the contact area with running water as soon as possible.

Ensure that the work area is well ventilated so that the air temperature in the work area does not exceed the permissible limits, since some of the solvents are explosive. Also avoid hot saurses in this regard.

Storage and transportation should be carried out in cool conditions, with the obligatory vertical position of the container.

According to the degree of danger, the solvents used in laboratories belong to three groups:

- solvents that predominantly cause acute poisoning with the predominant phenomenon of anesthesia: gasoline, ethyl and butyl alcohols, acetone;
- Solvents are more toxic, causing acute poisoning; this solvent group includes methyl alcohol (methanol), dichloroethane, etc.;
- solvents with high toxicity, in addition to acute poisoning, cause persistent changes in the functions of the circulatory organs and nervous system; this group includes benzene, toluene, xylene and others.

In terms of fire safety, most of them are flammable. When working with solvents, a worker must always be very careful, even a slight negligence cannot be tolerated, as this can lead to an accident.

The basic safety rules for work with organic solvents are:

- Always work with solvents in the ventilation hood.
- When working with flammable solvents, all burnersmust be switched off and the open heater electric heaters switched off in the ventilation hood where the test is carried out.
- Glass ware used for the organic solvent test should be clean and dry before filling.
- Work connected with the risk of fire, flash or explosion should be performed standing.
- You cannot leave your workplace unattended during solvent experiments.
- Heating and distillation of flammable and combustible organic solvents may only be carried out in a water or air bath using closed-circuit electric heaters.
- Do not pour organic solvents into drains. Waste liquids must be collected in a sealed container for this purpose and disposed in an approved sanitary and fire inspection area.

1.3.2. Strong and weak acids

All acids are divided into:

- Strong
- Weak

According to Bransted the strength of the acid is determined by the ability to protolysis, which depends on how strongly expressed the readiness of the substance to give or receive protons. The reagent determines what function the substance performs in the reaction – acids or bases. In this case, an important reagent is water because it acts as a base or acid:

Acids that dissociate without residue in aqueous solutions are called strong.

Examples of strong acids

• HCl - hydrochloric acid;
• HBr - hydrobromide;
• HI - iodide;
• HNO3 - nitric acid;
• HClO4 - hydrochloric acid;
• H2SO4 - sulfuric acid.

All the acids (except sulfuric acid) presented in the list above are monoprotonic because their atoms give one proton; Sulfuric acid molecules can give off two protons, so sulfuric acid is diprotonic.

Weak acids have incomplete dissociation. As a result, there are undissociated molecules in the solution.

1.3.3. Concentration of solutions and dilutions

Aqueous solutions of chemical compounds (reagents) are mainly used in the practice of chemical analysis.

A solution is a homogeneous system in which solute molecules are distributed between solvent molecules. The solutions are saturated and unsaturated. In a saturated solution a certain temperature the substance no longer dissolves. In unsaturated solution at a certain temperature the substance can still dissolve.

The concentration of a solution is the amount of chemical compound contained in a given mass or volume of a solution.

The concentration can be mass (in parts or percent) or volumetric (molar or normal).

Solubility is the mass of a substance that is capable to dissolve in a certain mass or volume of solvent forming a saturated solution. Most often, solubility is expressed by the number of grams of a substance that can dissolve at 100 g of solvent at a certain temperature, forming a saturated solution. Sometimes the weight of the solvent is 1000 g. For soluble substances, solubility is expressed in grams per cubic decimeter.

For most substances the solubility increases with increasing temperature. If the solution is saturated at higher temperature is cooled to a lower temperature at which the solubility of the substance is less, then the excess of this substance crystallizes out.

By appropriation solutions are:

• Working (with approximate concentration) are used for general preparatory analysis operations. They can be quite concentrated.

• titrated (with exact concentration) are used in the final stage of analysis to obtain quantitative indicators. These solutions are quite dilute.

Most often, the solutions are prepared by mixing a certain mass of reagent (sample) with the calculated volume of distilled water (or other solvent): the reagent is weighed on a technochemical or analytical waigts, and the solvent is measured with a measuring cylinder, pipette or burette. Since working solutions are prepared from “h” classification reagents, it is recommended to filter them.

For titrated solutions, the reagent sample must be weighed on the analytical balance.

Preparation of solutions with percent concentration

Percentage concentration (%) is numerically equal to the number of grams of dissolved chemical compound contained in 100 grams of solution.

Preparation of solutions with normal concentration

Normal concentration is expressed by the amount of equivalent masses of the substance contained in 1l (1000 ml) of solution.

The equivalent of a chemical compound is numerically equal to its equivalent mass in the reaction, but is expressed in grams.

The equivalent mass of the element is equal to its molar mass divided by the valence.

The equivalent mass of the oxide is equal to its molecular weight divided by the number of oxygen atoms multiplied by 2 or the sum of the equivalent masses of oxygen and element. For example, the equivalent mass of aluminum oxide is:

$$\mathrm{E}_{Al_2O_3} = \frac{102}{3\times 2} = 17 \quad \text{or} \quad \mathrm{E}_{Al_2O_3} = 9+8 = 17$$

The equivalent mass of the acid is equal to its molecular weight divided by the basicity of the acid in this reaction, or the sum of the equivalent masses of the acid residue and hydrogen. For example, the equivalent mass of sulfuric acid is:

$$\mathrm{E}_{H_2SO_4} = \frac{98}{2} = 49 \quad \text{or} \quad \mathrm{E}_{H_2SO_4} = 48+1 = 49$$

The equivalent mass of the base is equal to its molecular weight divided by the number of hydroxyl groups involved in this reaction. For example, the equivalent mass of $Al_2(OH)_3$ is:

$$E_{Al(OH)_3} = \frac{78}{3} = 26 \quad \text{or} \quad E_{Al(OH)_3} = 9 + 17 = 26$$

The equivalent mass of the ion is equal to its molar mass divided by the value of its charge without taking into account the sign of the charge. For example, the equivalent masses of aluminum ion and sulfate ion are equal to:

$$E_{Al^{3+}} = \frac{27}{3} = 9 \quad \text{or} \quad E_{SO_4^{2-}} = \frac{96}{2} = 48$$

The equivalent mass of a salt is equal to its molar mass divided by the product of the number of metal atoms in the molecule by its valence in that salt, or by the sum of the equivalent masses of cation and anion. For example, the equivalent mass of aluminum sulfate is:

$$E_{Al_2(SO_4)_3} = \frac{342}{2 \times 3} = 57 \quad \text{or} \quad E_{Al_2(SO_4)_3} = 9 + 48 = 57$$

Preparation of solutions with molar concentration

The molar concentration (M) is numerically equal to the number of moles of the substance contained in 1 liter (1000 ml) of solution. The mole (molar mass) is numerically equal to the molecular weight of the substance, but it is expressed in grams. For example, M_{KOH} = 56.11 g / mol, M_{HCl} = 36.46 g / mol.

Conversion of solution concentrations from one unit to another

When calculating the percentage concentration in molar and vice versa, it should be remembered that the percentage concentration is calculated for a certain mass of the solution, and the molar and normal respectively for volume, so you need to know the density of the solution. If we denote: c as a percent concentration; M is the molar concentration; N is the normal concentration; e is the equivalent mass, r is the density of the solution; m is the molar mass, then the formulas for the percentage concentration will be as follows:

$$M = (c \cdot r \cdot 10) / m$$
$$N = (c \cdot r \cdot 10) / e$$

The same formulas can be used to convert normal or molar concentrations to percent one.

For example

What is the molar and normal concentration of a 12% sulfuric acid solution whose density is r = 1,08 r / см^3?

Solution

The molar mass of sulfuric acid is 98. Therefore,

m (H2SO4) = 98 and e (H2SO4) = 98: 2 = 49.

Substituting the necessary values into the formula, we get:

a) The molar concentration of 12% sulfuric acid solution is

M = (12 · 1,08 · 10) / 98 = 1,32 M

b) The normal concentration of 12% sulfuric acid solution is

N = (12 ·1,08 ·10) / 49 = 2,64 H.

Sometimes in laboratory practice it is necessary to convert the molar concentration to normal one and vice versa. If the equivalent mass of the substance is equal to the molar mass (For example, for HCl, KCl, KOH), then the normal concentration is equal to the molar concentration. So, 1 n. hydrochloric acid solution will be simultaneously 1 M solution. However, for most compounds the mass is not equal to the molar and, therefore, the normal concentration of solutions of these substances is not equal to the molar concentration.

For conversion from one concentration to another it is possible to use formulas:

$$M = (N \cdot e) / m$$

$$N = (M \cdot m) / e$$

For example
Normal concentration of 1 M of sulfuric acid solution is
$N = (1 \cdot 98) / 49 = 2$ H.

For example
Molar concentration 0,5 n. Na_2CO_3
$M = (0,5 \cdot 53) / 106 = 0,25$ M.

Preparation of solutions out of fixanals

Fixanal is an ampoule into which the precisely weighed substance is soldered.
On each box and ampoule, the formula of substance and its concentration (0,1N, 0,01N, etc.) is written. The content of the ampoule, which is quantitatively transferred and dissolved in a 1liter volumetric flask, gives exactly 0.1N, 0.01N, etc. solution.

1.4. Analytical equipment and principles of it detection

Physical and chemical research is currently being implemented by means of analytical technique which is a large area of measuring equipment. With its help, both the latest scientific and routine research in physics, chemistry, biology, medicine and other fields are carried out. Analytical tools are used in agriculture, in environmental control systems, in medicine.

Modern provision of laboratories with analytical equipment is the result of a long selection of measuring instruments and installations that have been used in various fields of science and technology, as well as years of development of specialized methods and means of analysis.

Theoretical bases and practical possibilities of physicochemical methods of analysis:

- determination of the physical (chemical) value on which the method of analysis is based;
- relationship between chemical composition and physical properties of a substance;
- methods of conversion of the analyte into concentration or mass: calibration graph method, standard (sample) method, additive method, calculation method.

Classification of instrumental methods of analysis:

- Direct
- Indirect
- Inversion.

1.4.1. pH meter: characteristics, usage and maintenance

pH is an indicator of the acidity of the solution, i.e. the ratio of acid to alkali.

Potential Hydrogen/Hydrogen power/pH is a hydrogen index that characterizes the concentration of free hydrogen ions in water.

To simplify the concept, the pH is determined by the quantitative ratio of H+ and OH- ions in water. For example: the more H+ in water, the more acidic the water is. The more OH- (ie less H+), the more alkaline water is.

The pH level tends to rise on its own.

In perfectly clear distilled water, these ions will balance each other. In such cases, the water is neutral and pH = 7.

If various substances are being dissolved in water, this balance can be disturbed, which leads to a change in pH.

NOTE! Do not try to keep the pH in a strict figure. Suppose the pH of the solution = 6.0, and we at least change, calibrate it back to 6.0. DO NOT DO IT! Only extreme values of this range of 5.5 - 6.5 should be maintained. Therefore, no element will be preferred.

Very often, the pH is confused with the acidity and alkalinity of water. It is important to understand the difference between them. pH is an indicator of intensity, but not quantity. That is, the pH reflects the degree of acidity or alkalinity of the medium, while the acidity and alkalinity characterize the quantitative content of substances in water capable of neutralizing respectively to alkali and acid. For example, putting your hand in water, we can say what kind of water - cold or hot, but we will not be able to determine how much heat in it (that is, how long this water will be cooling down).

Depending on the pH, the rate of chemical reactions, the degree of corrosive aggressiveness of water, the toxicity of pollutants, etc., may vary.

Methods for determining pH.

Several methods are widely used to determine the pH of solutions. The hydrogen index can be estimated approximately by means of indicators or determined analytically by carrying out acid-base titration.

- Acid-main indicators. Acid-basic indicators - organic substances - dyes, the color of which depends on the pH of the medium are widely used to roughly estimate the concentration of hydrogen ions. The most well-known indicators include litmus, phenolphthalein, methyl orange and others. The color change of each indicator occurs in its own acidity range, usually it is 1-2 units.
- Ionometric method. Determination of pH is based on the measurement of a millivoltmeter - EMF ionometry of a galvanic chain, which includes a special electrode whose potential depends on the concentration of H+ ions in the surrounding solution. The method has the convenience and high accuracy, especially after the calibration of the indicator electrode in the selected pH range, allows to measure the pH of opaque and colored solutions and it is therefore widely used.
- Analytical volumetric method. Acid-main titration also gives precise results of determination of acidity of solutions. A solution of known concentration (titrant) is added dropwise to the test solution. When they are mixed, a chemical reaction takes place.

How to calibrate the pH meter correctly

pH electrodes are not an ideal systems. They can have different lengths, imperfect geometric shape, defection in the composition of the internal electrolyte, etc. All this affects their characteristics and, at the same time, it is quite normal, as there are certain tolerances in any production. Therefore, each pH meter requires calibration, which helps the instrument establish the relationship between the electrode signal and the pH value in the solution.

Calibration is a very important moment! It is necessary to understand the impossibility of measuring pH to an accuracy greater than the standards used. For example, if you want to work with an accuracy of 0.01pH, then the following conditions are required: the total error of the pH meter and the electrode should not exceed 0.005 pH and the calibration should be carried out with special attention to special high-precision buffer solutions. You cannot buy these solutions because they are not stored. They have to be prepared on your own, using specially prepared reagents and water.

If it is not possible to prepare a buffer with an accuracy of +/- 0.005 pH, then we work with buffer solutions, the accuracy of which is ensured at the level of +/- 0.02 pH. With calibration by such standards, the total error will not exceed 0.04 - 0.03 pH, on condition that the error of the instrument is at the level of 0.01 pH.

Modern pH electrodes are generally combined, that is, there are the pH electrode and the reference electrode. In addition to ease of use, it provides faster response and reduces overall error.

The isoelectric point for such electrodes is at pH = 7 (0 mV). Therefore, the instrument should be calibrated against a buffer with a neutral pH (eg 6.86 or 7.01). The second point should be selected at a distance of

approximately 3 pH units, ie pH = 4 or 10. If the instrument is calibrated on only two buffers, then the choice of the second point depends on the range in which you usually work. If these are alkaline solutions, then a buffer of pH = 10 should be used, if acidic - pH = 4. This is due to some difference in the slopes of the calibration lines in the acidic and alkaline area. There will be no problem if the device can be calibrated for three or more points. In this case, the calibration order is not important as the pH meter monitors it on its own.

The most advanced models of pH meters have the so-called GLP support, which in addition to the date of the last calibration, allows to estimate the state of the electrode on the basis of data on the ratio of the slope of the calibration curve to the theoretical values (59.16 at 25 ° C) in%. If the instrument does not have GLP support but has a mV measurement mode, the slope can be calculated independently by measuring the mV values in the buffer pH = 7 and pH = 4.

For example:

pH 7 = -10 мВ
pH4 = +150 мВ
slope = 150 - (-10) /59.2x3 = 90.1%
95 - 102% - an electrode in working state,
92 - 95% - an electrode needs cleaning,
less than 92% - you need to change the electrolyte or replace the electrode.

An important point in the proper work of the pH meter is compensation of changes in temperature. Thisproblem is one of the most important and most difficult to solve in the pH measurement.

Measurement error occurs for three reasons:

- the Nernst equation includes temperature;
- equilibrium concentrations of hydrogen ions in buffer and samples vary with temperature;
- the characteristics of the pH electrode depend on the temperature.

According to the Nernst equation, the theoretical slope of the calibration curve changes with temperature. If the instrument does not take this change into account, then an error of 0.003pH per degree Celsius and each pH unit from the isopotential point is added to the measurement error.

For example:

The instrument is calibrated to buffer pH = 7 at 25 ° C.
Sample with pH = 5 at 20 °C, error = 0.003 x 5 x 2 = 0.03
Sample with pH = 2,5 at 2 °C, error = 0.003 x 23 x 4.5 = 0.31
Sample with pH = 12 at 80 °C, error = 0.003 x 55 x 5 = 0.82

2. It is much more difficult to compensate changes in the equilibrium concentrations of hydrogen ions in samples with changes in temperature. The problem is that without knowing the exact chemical composition of the sample, it is impossible to predict the nature of these changes. When the temperature changes by 25-30 degrees, the pH can change by 0.5 - 1 unit. The usual general laboratory pH meters do not take this factor into account, and it cannot be taken into account, since the solutions are very different. Buffer solutions are not exceptions:

TemperaturepH value					
C	4,01	6,86	7,01	9,18	10,01
0	4,01	6,98	7,13	9,46	10,32
10	4,00	6,92	7,07	9,33	10,18
20	4,00	6,88	7,03	9,22	10,06
25	4,01	6,86	7,01	9,18	10,01
30	4,02	6,85	7,00	9,14	9,96
50	4,06	6,83	6,98	9,01	9,82
70	4,12	6,85	6,99	8,93	9,75

To reach the best results, the pH meter must be calibrated in such cases:

- At regular use, at least once a week
- If it is not used, once a month
- If you assume that the measurements are incorrect
- If aggressive liquids are tested (very acidic or very alkaline ones)
- If fluids are tested over a wide measurement range
- After electrode replacement

Proper pH meter maintenance

Although there are common methods of pH meters maintanence, there may be specific requirements for each brand and model. It is always necessary to follow the instructions for the pH meter and then it can be used for a longer time.

In addition to calibration frequency, proper care of the pH electrode will ensure its longer life and more accurate results. Many pH meter electrodes consist of a glass bulb with an internal sensor which should be contained in a special solution. When using a portable pH meter, the storage solution should be in the protective cap of the device. Pouring this solution out of the cap is not allowed ... it is really necessary! For most electrodes of the pH meters it is very important to be stored in a humid environment of a suitable storage solution.

To clean most of the electrodes of pH meters, it is sufficient to wash them in distilled (deionized) water. Tke away the excess water and return to moist storage solution. If measuring solutions can contaminate the surface of the electrode, use a detergent solution or even leave the electrode there for a long time.

1.5 Optical methods of analysis

Optical methods of analysis are based on the use of the phenomenon of emission of electromagnetic radiation by atoms or molecules of the test substance, or the interaction of electromagnetic radiation with matter. Since the nature of the radiation depends on the qualitative and quantitative composition of the substance, it allows to analyze the substances.

Optical Range Electromagnetic Radiation - Optical (light) radiation. The term combines visible light, infrared and ultraviolet radiation.

Photometric methods of analysis are based on the absorption of radiation in the visible range of the spectrum, ie in the wavelength range of 400 - 780 nm, have become most widespread. This is due to the possibility of obtaining many intensely colored organic and inorganic compounds, suitable for their photometric determination in the visible region of the spectrum with the help of quite simple and relatively inexpensive devices.

Depending on the nature of the interaction of the substance with electromagnetic radiation optical methods are divided into:

- absorption (based on the measurement of absorption by a substance of light radiation). These include colorimetry, photocolorimetry, spectrophotometry, atomic absorption methods;
- Emission (based on the measurement of the intensity of light emitted by a substance). These include fluorimetry, emission spectral analysis.

Optical methods of analysis cause the use of modern devices of varying complexity, which gives a number of advantages in comparison with classical chemical methods: speed, simplicity of the technique, the use of a small amount of substance for analysis, the ability to analyze compounds of any nature. In addition, they increase the sensitivity, accuracy and reproducibility of the results.

1.5.2 Photoelectric colorimeters

Photometric analysis is based on the selective absorption of electromagnetic radiation of different sections of the spectrum of one system. Photometric analysis with the use of monochromatic radiation is called the method of absorption spectrophotometry.

Depending on the structure, photocolorimetric and spectrophotometric methods of analysis are distinguishedin photometric analysis.

Photocolorimetric methods measure the absorption of the color of solutions, involve the use of simple instruments and measurements.

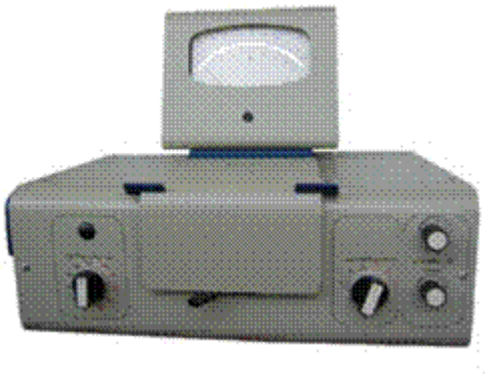
Photoelectric concentration colorimeters (PCC) are designed to measure transmittance and optical density of transparent liquid solutions and solid samples, as well as to determine the concentration of substances in solutions after pre-calibration of consumer devices.

The PCC spectral range is from 315 to 990 nm. Radiation source is halogen lamps; radiation receiver are photodiodes.

The principle of work of colorimeters of this type is based on a comparison of the light flux passed through the solvent or control solution in respect of which the measurement is made and the light flux passed through the test medium.

The light streams of the photodetectors are converted into electrical signals, which are processed by the microcomputer of the colorimeter and are presented on the digital board in the form of indexof transmittance, optical density, concentration.

Measurement of the concentration of the test solution is possible with abidance to the basic law of light absorption, ie, with a linear dependence of the optical density on the concentration.



Measurements on the colorimeter should be carried out at an ambient temperature of 10 to 35 ° C, with an air humidity of 50 - 80%.

There shouldn't be powerful sources of electrical, magnetic fields, powerful light sources and heating devices near the the colorimeter.

It is not allowed the incidence of direct sunlight on the colorimeter.

Setting the wavelengths must be done by bringing up the short waves to the longer ones.

The working surfaces of the cuvette must be thoroughly wiped with the alcohol-ester mixture before each measurement.

When installing the cuvette into the cuvette holder, it is forbidden to touch the work areas. The liquid is poured into the cuvette to the mark on the side of the cuvette. When installing the cuvette holder, do not tilt the cuvette with the liquid. Close the cuvette with the lid. After changing a photofilter, and after finding the colorimeter with the lid of the cuvette section open for more than 5 minutes, the measurements begin after 5 minutes of holding the photodetector with the lid of the cuvette section closed.

An instrument must be switched off after work.

Choosing a filter

The presence in the colorimeter of the node of the filters and the cuvette set allows to choose such a combination of them, in which the error in determining the concentration will be smallest.

The selection of the filter is carried out in such way:

- pour the solution into the cuvette and determine the optical density for all colorimeter light filters;
- according to the obtained data, construct a curve, postponing along the horizontal axis the wavelengths corresponding to the maximum transmittance of the filters, and along the vertical axis - the corresponding values of the optical density of the solution;
- mark the section of the curve for which the following conditions are true: the optical density has a maximum value; the course of the curve is approximately parallel to the horizontal axis, ie the optical density is little dependent on the wavelength.

The wavelength corresponding to the maximum transmittance of the filter falls on the spectral curve of the test solution, for which the abovementioned conditions are fulfilled.

Choosing a cuvette

The preliminary choice of the cuvette is made visually, according to the intensity of the color of the solution. If the solution is intensely colored (dark), use a cuvette with a small working length (1 - 3 mm). In the case of poorly colored solutions, it is recommended to work with cells with a longer working length (30 - 100 mm).

Pour the solution into the pre-selected cuvette and measure its optical density by entering appropriate filter into the ray.

When measuring a series of cuvette solutions, fill with a medium concentration solution. If the resulting optical density is approximately 0.3 - 0.5, select this cuvette to work with this solution. If this condition is not met, a different cuvette should be tested. If the value of the measured optical density is greater than 0.5 - 0.6, take the cuvette of a smaller working length, if the value of the optical density is less than 0.3 - 0.2, a cuvette with a larger working length should be selected.

Measurement of concentration of a substance in solution

You should do the following operations: select the wavelength; to choose a cuvette; build a calibration graph for the substance; measure the concentration of the substance in solution.

To construct a calibration graph, it is necessary to prepare a number of standard solutions of this substance with known concentrations, covering the range of possible changes in the concentrations of this substance in the test solution.

Measure the optical density of all solutions and make a calibration graph, by plotting the known concentrations along the horizontal axis, and the values corresponding to the optical density corresponding to the vertical axis.

It should be ensured that the concentration dependence on the optical density is linear, that is, expressed in the graph by a straight line.

Pour the test solution into the cuvettes of the same working length with which the calibration was performed, set the appropriate wavelength λ and measure the optical density of the solution.

1.5.3 Spectrophotometry

An analysis method based on the determination of the absorption spectrum or the measurement of light absorption at a certain wavelength, which corresponds to the maximum absorption curve of the test substance. The analysis is performed by absorption of monochromatic radiation substances in the visible, ultraviolet and near infrared regions of the spectrum.

Spectrophotometry is used to identify compounds, investigate the composition, structure and quantitative analysis of individual substances and multicomponent systems. The absorption curve of the wavelength or wavenumber is called the absorption spectrum of the substance. This curve is a specific characteristic of a particular substance. Qualitative analysis of substances by their absorption spectra is carried out in two ways:

- by the known parameters of the absorption spectrum of the test substance;
- by comparing the absorption spectrum of a standard substance solution and a solution of the test substance of the same composition.

Unlike photocolorimetric determinations, not only colored but also colorless solutions can be analyzed in spectrophotometry. In this case, the analysis is performed not in the visible but in the UV or IR regions of the spectrum.

The main type of instruments for spectrophotometry are spectrophotometers, in which unlike photoelectrocolorimeters, monochromatization is provided not by filters, but by special optical devices, so called monochromators, which allow to change continuously the wavelength of electromagnetic radiation passing through the solution. Types of monochromators (photofilters):

- adsorption: weak the light by absorbing the light flux by the filter material;
- dispersion: reduce light by changing the refractive index of the filter substance;
- interference: the work is based on the phenomenon of interference of a parallel beam, which is reflected by two mirrors, which are located at a wavelength.

Monochromators are optical systems that emit narrow sections of frequencies or wavelengths of the optical spectrum. Monochromators are characterized by:

- bandwidth - the range of wavelengths in which the light intensity passed through the monochromator is greater than half the light intensity at the maximum peak wavelength;
- Slit width - the range of wavelengths that fall on a monochromator (input slit) and exit it and fall a sample (sample slit).

It is necessary to carry out research at the minimum narrow slits. The resolution of the spectrophotometer is significantly enhanced by the use of a dual monochromator: that is, if there are two consecutively connected monochromators in one device, in which the output gap of the first serves as the input gap of the second. This can significantly reduce the scattered light.

To work in the visible are of the spectrum use glass cuvettes, in the near UV use quartz ones. For continuous analysis of solutions use flow cuvettes.

When making measurements, it should be taken into account that the smallest measurement error of optical density is observed at a numerical value of optical density of about 0.4. At an optical density greater than 0.8 it is necessary to use cuvettes with a shorter absorption layer length or dilute the test solution. If the optical density is less than 0.1, it is necessary to use longer cuvettes.

When calculating in spectrophotometry use the method of calibration graph. Prepare a series of dilutions of standard solution (C_{st}), measure their optical density (A_{ST}), make A_{ST} - C_{ST} plot. Then measure the optical density of the analyzed solution and graphically determine its concentration.

In spectrophotometric analysis, as in photocolorimetry, it is necessary to create optimal conditions for achieving certain accuracy and reproducibility of results.

1.6 Atomic spectroscopy

Atomicspectroscopy methods are methods of analysis based on the measurement of the energy state of atoms of substances. They differ in the way the signal is received and recorded, and the need for pre-atomization of samples is common. Atoms of substances that evaporate in a flame emit or absorb light of a certain wavelength. By the intensity of the bands in the spectrum, the number of individual chemical elements in the sample can be determined.

• Atomic absorption spectroscopy based on the atomic absorption of radiation from an external source.

• Atomic emission spectroscopy is based on the atomic emission of radiation excited by the use of different external sources (arc, spark, flame, plasma).

• Atomic fluorescence spectroscopy method of quantitative elemental analysis on atomic spectra of fluorescence. The sample of the analyte is converted to an atomic vapor and irradiated to excite fluorescence by radiation that is absorbed by the atoms of only the element being determined (the wavelength of the radiation corresponds to the electron energy of these atoms). Part of the excited atoms emits light (analytical signal) recorded by spectrophotometers. Usually used resonance fluorescence (the wavelengths of absorbed and emitting light are the same). Flame, inductively coupled plasma or electrothermal atomisers (graphite tubes, filaments, electrically heated rods) are used to atomize the solutions.

Atomic absorption spectral analysis (flame) is a method of analysis that is carried out by the selective absorption of light by the atoms of a substance converted to an atomic gaseous state. Radiation from a light source passing through gases of matter at frequencies coinciding with the frequency of transition of the electron from the basic level to the closest to it is absorbed (resonance line), and the degree of attenuation of the spectral lines of the element under study its concentration in the sample is determined. The intensity of light absorption by the atomic absorption method is determined by the *Behr-Lambert-Bouger law*:

$$D = lg(I_0/I) = klC,$$

where D is the optical density (absorption); I_0 is the output intensity of the exciting light; I is the intensity of light passing through the sample; k is the absorption coefficient; l is the thickness of the absorption layer; C is the concentration of the element to be determined. The absorption coefficient k, proportional to the probability of the resonance transition, it doesn't depend on temperature. The sample atomization requires a temperature of ~ 2000–3000 ° C. Flame atomisers, electrothermal atomizers as well as HF and microwave discharge are used in this temperature range, but most often are flame retardants operating on a mixture of acetylene nitrous oxide. In this temperature range, more than 90% of atoms are not in the excited state, so other atoms and molecules cannot

affect the absorption coefficient. This fact along with the small number of absorption lines, makes this method highly selective.The light source emits a linear spectrum that contains the required line of the element being determined. Hollow cathode lamps, electrode-free gas discharge lamps, a tunable laser are used as radiation sources. A significant disadvantage of the method of atomic absorption, compared with the method of atomic emission spectral analysis, is the inability to detect several elements in the sample simultaneously and the need for their sequential determination. The technique of carrying out atomic absorption analysis, in comparison with other methods of atomic spectral analysis, is much simpler and allows to determine up to 70 elements with a sensitivity of ~ 10^{-4}–10^{-9}% of weight of not only low but also high concentrations in the samples. Today, the Atomic Absorption Spectral Analysis method is considered to be one of the most selective, rapid, productive, accurate and at the same time relatively cheap method.

Atomic emission spectral analysis (spectrometry), AESA is a set of elemental analysis methods, which are based on the study of the emission spectra of free atoms and ions in the gas phase. Typically, emission spectra are recorded in the most convenient optical range of wavelengths from ~ 200 to ~ 1000 nm (vacuum spectroscopy is required to register spectra in the <200 nm region to eliminate short-wave absorption by air; to record spectra in the region> 1000 nm special infrared or microwave detectors are needed).

The sample of the test substance is introduced into the radiation source, where its evaporation occurs. Dissociation of molecules and excitation of atoms (ions). Atoms emit characteristic radiation that enters the recording device of the spectral device.

At high-quality AESA, the spectra of the samples are compared with the spectra of known elements shown in the corresponding atlases and tables of the spectral lines, and thus establish the elemental composition of the analyte. In quantitative analysis, the amount (concentration) of an element in the analyte according is determined to the magnitude of the analytical signal (blackness or optical density of the analytical line on the photographic plate; light flux to the photoelectric receiver) of this element from its content in the sample.

Excitation of atoms occurs when one or more electrons move to a more distant energy level. In the non-excited state the atom has the lowest energy E_0. In an excited (unstable) state, an atom can stay for a very short time (≈10^{-7}-10^{-8} s) and always tends to occupy a normal state. In this case, the atom gives off excess energy in the form of photon radiation.

The sensitivity and accuracy of AESA depends mainly on the physical characteristics of the sources of excitation of the spectra, such as temperature, electron concentration, the residence time of the atoms in the excitation zone of the spectra, the stability of the source mode, etc.

$$I = a + c$$

where *a* is the coefficient that depends on the process conditions;
c is the concentration of the element in the sample.

Inductively coupled plasma atomic emission spectroscopy. Elements emit a quantum of light with a certain wavelength when they are excited and ionized with the subsequent transition to a stable state. Determining the wavelength, you can perform a qualitative analysis, and determining the intensity of the emission – quantitative one.

The main components of the device for this analysis are: sample feed system, atomizer, sample atomization unit (quartz plasma torch), optical camera and detector. The source of atomization and excitation in this method is inductively coupled plasma.

The advantage of the method is the ability to regulate smoothly the conditions of atomization and excitation, that is, it is possible to find conditions that ensure the simultaneous determination of many elements. The disadvantage of this method is the very high consumption of argon gas (plasma burner). Also argon with a purity of at least 99.99% is required.

The principle of operation of all methods of atomic spectroscopy:

2. Carry out sampling (select part of the substance from the object of analysis, which reflects its chemical composition as fully as possible).
3. Select a sample from a solid sample, dissolve it in suitable solvents to convert the test element in solution. A liquid aliquot is taken from the liquid sample and a working solution is prepared for analysis according to the same principles.
4. A series of working calibration solutions are prepared that cover the required calibration range.
5. Spectrometers are prepared for recording the signal under optimal conditions of the element under study.
6. Introduce the analyte into the atomizer, create an absorbent layer of atomic vapor and make measurements.
7. The calibration solutions are sequentially introduced into the atomizer and the calibration characteristic (functional relationship between the analytical signal and the concentration of the element in the calibration solution) is obtained.
8. Using it, determine the concentration of the test element in the sample solution and in the original sample.

1.7 Chromatographic methods of analysis

Chromatography is a large area of physico-chemical methods of analysis that combines both methods of concentration and separation as well as methods of identification and quantification of various substances.

Chromatographic methods have a special place among the effective methods of analytical analysis because they are very widely used due to their versatility. They allow to analyseacomplex inorganic and organic substances in the gas, liquid and even solid state. The latest chromatographic methods can be used to analyze gaseous, solid and liquid substances with a molecular weight of which is from 1 to 106. This is one of the most important analytical methods.

Chromatography is a hybrid analytical method that combines separation and determination.

Unlike other methods based on the distribution of components between phases, chromatography is a dynamic method that ensures the multiplicity of sorption-desorption acts of components that are separated because separation occurs in the mobile phase flow. This is due to the high efficiency of the chromatographic method in comparison with the methods of sorption and extraction.

The most common definition of chromatography is the physicochemical method of separation of substances, which is based on the distribution of components between two phases - stationary and mobile.

The stationary phase is a solid adsorbent with a developed surface or a film of liquid adsorbed on a solid carrier; the function of the stationary phase is to adsorb, retain substances. The mobile phase is the flow of gas or liquid that flows (filtered) through the sorbent layer, the function of the mobile phase is to dissolve substances and move them.

Chromatographic analysis is a criterion for the homogeneity of a substance. If in any chromatographic manner the analyte is not separated, it is considered to be homogeneous (without impurities).

The fundamental difference between chromatographic methods and other physicochemical methods of analysis is the ability to separate closely related properties of substances. After separation, the components of the substance can be identified (determined by nature) and quantified (mass, concentration) by any chemical, physical and physico-chemical methods.

Chromatography is used in laboratories and industry for the qualitative and quantitative analysis of multi-component systems, production control, especially in connection with the automation of many processes, as well as for preparative (including industrial) separation of individual substances (eg, precious metals), separation of liquid and scattered elements.

In some cases, chromatography in combination with other physico-chemical and physical methods, such as mass spectrometry, IR, UV spectroscopy, etc., is used to identify the substances. The main advantages of chromatographic analysis:

- express determination;
- high efficiency;
- the possibility of automation and obtaining objective information;
- combination with other physicochemical methods;
- a wide range of concentrations of compounds;
- ability to study the physicochemical properties of the compounds;
- performing qualitative and quantitative analysis;

•application for control and automatic regulation of technological processes.

There are different types of classification of chromatographic methods:

1. By the aggregate state of the phases (the first word in this classification characterizes the aggregate state of the mobile phase).
   - Gas - liquid chromatography
   - Gas - solid (gas adsorption) chromatography
   - Liquid - liquid chromatography
   - Liquid-solid (liquid-adsorption) chromatography
   - Liquid - gel chromatography
2. By the nature of the interaction, which causes the distribution of components between the eluent and the stationary phase (ie the nature of the interaction between the sorbent and the sorbate).

• Adsorption chromatography. A separation is based on differences in the adsorption capacity of separable substances by solid adsorbent (solid body with developed surface);

• Separation chromatography. Separation is based on differences in solubility of the substances in the stationary phase (gas chromatography) and on differences in the solubility of the substances in the mobile and stationary phases;

• Ion exchange chromatography. Separation is based on differences in the ability of separating substances to ion exchange;

• Gel filtration or exclusive chromatography (gel permeation, gel filtration chromatography). It is a type of chromatography during which the molecules of substance are separated by size due to their different ability to penetrate into the pores of the stationary phase. The largest molecules (larger molecular weight) are the first to leave the column, able to penetrate into the minimum number of pores of the stationary phase. Substances with small sizes of molecules are the lattest to able to penetrate freely into the pores. Unlike adsorption chromatography, in the case of gel filtration, the stationary phase remains chemically inert and does not interact with the substances that are separated.

• Sediment chromatography is based on the different ability of the components to precipitate on the solid stationary phase.

• Adsorption-complexing chromatography. Separation is based on the formation of coordination compounds of different strengths in the phase or on the surface of the adsorbent.

3. By the aggregate state of the eluent:
   - gas chromatography (GC);
   - high performance liquid chromatography (HPLC).

Gas chromatography is a method of separating volatile components, in which inert gas is the mobile phase (carrier gas), which flows through the stationary phase with a large surface. Hydrogen, helium, argon, carbon dioxide are used as the mobile phase. The carrier gas does not react with the stationary phase and the substances being separated. There are:

- gas-solid chromatography - the fixed phase is a solid carrier (silica gel, coal, aluminum oxide).
- gas-liquid chromatography - the fixed phase is the liquid on the surface of an inert carrier.

Separation is based on differences in volatility and solubility (or adsorption) of the components of the separable mixture. This method is used for gas separation, determination of impurities in air, water, soil, industrial products; determining the composition of the products of basic organic and petrochemical synthesis, exhaust gases, drugs, as well as in forensics, etc.

Liquid chromatography is one of the most advanced chromatographic methods for multicomponent analysis. Its distinctive features are expressiveness, high precision, and the possibility of automation. The basis of

chromatographic separation is the participation of the components of the separable mixture in a complex system of van der Waals interactions (preferably intermolecular) at the interface. HPLC is a group of methods which include the preliminary separation of the original complex mixture into relatively simple ones. Then the obtained simple mixtures are analyzed by conventional physicochemical methods or using liquid chromatography.

A feature of HPLC is the use of high pressure and fine-grained sorbents (typically 3-5 μm, even up to 1.8 μm). According to the mechanism of separation of the substances being analyzed, HPLC is divided into:

- adsorption chromatography
- distribution chromatography
- ion-exchange chromatography
- ligand exchange chromatography

However, in practical work, the separation often takes place not by one, but by several mechanisms at the same time. This method is used to analyze, separate and purify synthetic polymers, drugs, detergents, proteins, hormones, and other biologically important compounds. The use of highly sensitive detectors allows to work with very small quantities of substances, which is extremely important in biological research.

4. By the nature of the technique:

- Column chromatography. The separation of substances is carried out in special columns. The sorbent is filled with special tubes - columns, and the mobile phase moves inside the column due to pressure drop. A kind of column chromatography is capillary, when a thin layer of sorbent is applied to the inner walls of the capillary tube.
- Planar chromatography. Planar chromatography is divided into thin-layer and paper. In thin layer chromatography, a thin layer of granular sorbent or porous film is applied to a glass or metal plate; in the case of paper chromatography, special chromatographic paper is used. Thin layer (TLC) and paper chromatography are used for the analysis of fats, carbohydrates, proteins and other natural substances and inorganic compounds.

Chromatographs are used for analysis and for preparative (including industrial) separation of mixtures of substances. In the analysis of separation in the chromatographic column of the substance together with the eluent fall into a special device installed at the outlet of the column, so calleddetector, it registers the concentration over time.The resulting curve is called the chromatogram. For qualitative chromatographic analysis, the time from the moment of introduction of the sample to the exit of each component from the column at this temperature and using a determined eluent is determined. For quantitative analysis, determine the heights or areas of the chromatographic peaks, taking into account the coefficients of sensitivity of the detection device used to the analytes.

1.8 ELISA - enzyme immunoassay

Enzyme-linked immuno sorbent assay (ELISA) is an immunological method for determining the presence of certain antigens by antigen-antibody reaction. It is widely used in research and clinical laboratory diagnostics.

This method is mainly used for large-scale epizootic surveillance and research in the implementation of disease control programs. It is a highly sensitive, specific, simple, fast and economical laboratory test method that makes it easy to reproduce and interpret the result.

there are many variants of this method, a variety of binding principles and the diversity of ELISA conditions due to the variety of objects of study (from low molecular weight compounds, peptide and steroid hormones, pharmacological preparations, pesticides, to viruses and bacteria, as well as to other antibodies). With only one option for the registration of enzymatic activity, photometric, fluorometric, bio- and chemiluminescent methods

can be used, and electrochemical and microcolorimetric sensors have been successfully used in a number of cases (especially related to technological problems).

1.8.2 Experimental methods for determining enzymatic activity

- Photometric method

Photometric method of recording the activity of enzymes is the most commont in ELISA. As the substrates of the enzymes, substances the products of which are the colored compounds or, conversely, the coloring of the substrates themselves changes during the reaction are used. The colored compounds absorb visible light, ie electromagnetic radiation with wavelengths of 400-700 nm. The absorption of light obeys the Beer–Lambert–Bouguer law, according to which the optical density of a solution in a certain range is directly proportional to the concentration of the substance. A spectrophotometer is used to measure optical density.

- Fluorometric method

Recently, ELISAs have become widespread substrates, which form products registered by the fluorometric method. The molecule in the absorption of the photon goes from the basic electronic state to excited one. The excited molecule can return to the basic state, with the excess energy going into heat, but there can be a reverse process of electron transition to the ground level, which is accompanied by the release of a quantum of light, which is called fluorescence. Due to the partial loss of energy in the transition of the molecule from the excited state to the fundamental wavelength of light, which is emitted always more than the wavelength of the absorbed light. The fraction of molecules that have switched from the excited state to the basic one with the emission of light is determined by the quantum yield ϕ. The fluorescence intensity is proportional to the amount of light absorbed by the sample. Thus, it is directly proportional to the concentration of the solute and the absolute value of the initial light intensity, while in photometry the relative intensities adsorbed by the sample are compared. This fact allows to increase the sensitivity of determination of a substance in solution for 1-2 ranks of magnitude by a fluorometric method in comparison with a photometric one.

- Bioluminescence and chemiluminescence

Enzymatic reactions, the energy of which is realized in the form of light radiation - the reaction of bio- and chemiluminescence have been used as detection systems in ELISA. The speed of such reactions is monitored by the intensity of the luminosity of the reaction system, which is recorded by a luminometer. Bioluminescence reactions are catalyzed by the luciferases of scions and bacteria, and the oxidation reaction of cyclic hydrazides with hydrogen peroxide (chemiluminescence reaction) is catalyzed by horseradish peroxidase.

- Electrochemical method

Electrochemical methods for determining the activity of enzymes are also known, they are used as labels in immunoassay. Such sensors allow you to determine the rate of enzymatic reactions in turbid environments and they are convenient for creating flow-through enzyme cells.

In enzyme-linked immunosorbent assays, both the enzymes and their substrates can be used as the label of antigens and antibodies. If the enzyme molecule serves as the label, then the detection method chosen should ensure that the signal is proportionally dependent on the enzyme concentration, and in the case of the substrate label, on the substrate concentration. In the first case, the enzyme acts as a marker (it is covalently bound to an antigen or antibody molecule), in the second one it is a detector (free enzyme). After carrying out all the immunochemical stages of any ELISA method, it is necessary to establish the concentration of the enzyme-labeled component of the immunochemical reaction, ie to determine the catalytic activity of the enzyme in the sample. The rate of reaction is judged on the concentration of the enzyme marker in the system. It should be noted that ELISA is always based on a comparative determination in identical conditions of the standard and measured sample, and therefore the requirement of proportionality of speed and concentration is more desirable than mandatory. It is sufficient that there is a one-to-one correspondence between the amount of enzyme reaction product formed and the amount of enzyme in the system. However, the fulfillment of the proportionality condition in a certain concentration range provides greater accuracy of the experiment and allows the construction of a theoretical model describing the method for its optimization.

Enzymes used as labels in ELISA test.

The fundamental possibility of using enzymes as labels in ELISA is due to the extremely high sensitivity of enzyme registration in solution. If by conventional spectrophotometric or fluorometric methods it is possible to register the formation of the product at a concentration of 10-7 mol / l, then the concentration of the enzyme will be 10-13 mol/l.

There are a number of general requirements for selecting ELISA enzymes. The main ones are: 1. High specificity and specific catalytic activity, which allows to detect the label in low concentrations;

2. The availability of the enzyme, the ability to obtain sufficiently pure enzyme preparations that retain high activity after chemical modification upon receipt of a conjugate with antigens or antibodies; 3. Stability under optimal conditions for the interaction of the antigen with the antibody;

4. Simplicity and sensitivity of the enzyme concentration determination method.

1.8.3 Classification of ELISA methods

A large number of different ELISA variants have been developed that have both fundamental and minor differences. Typically, from the standpoint of separation ELISA methods are devided into heterogeneous and homogeneous, ie on the principle of carrying out all stages of analysis with the participation of solid phase or only in solution.

The primary process in ELISA is the stage of "recognition" of the analyzed compound specific to it antibody. Since the process of formation of immunochemical complexes occurs in a strictly quantitative ratio due to the affinity, concentrations of components and reaction conditions, it is sufficient to determine the initial concentration of the analyzed compounds is a quantitative assessment of the formed immune complexes. In the case of antigen analysis, there are two approaches to this evaluation:

1. Direct measurement of the concentration of formed complexes;
2. Determination of concentration of remaining free (unreacted) antibodies.

Classical ELISA methods are based on the formation of antibodies in the presence of the precipitate antigen (precipitate), however, for the visual registration of the precipitation process requires high concentrations of components and long reaction time. Moreover, the results of such an analysis cannot always be interpreted uniquely, and in most cases they are of a qualitative or semi-quantitative nature. In addition, for many monovalent antigens (hapten), such as hormones and drug compounds, these methods are not applicable. Indication of the formation of the antigen-antibody complex in solution can be accomplished by introducing a label in one of the initial components of the reaction system, which is easily detected by an appropriate highly sensitive physico-chemical method. Isotope, enzymatic, fluorescence, paramagnetic tags, which have made it possible to increase the sensitivity of immunochemical methods by millions of times and to reduce the time of analysis to several hours, proved to be very convenient for this purpose. Since the process of complexation is strictly quantitative, the concentration of the label that is part of the complex is related to the original concentration of the antigen.

- Heterogeneous ELISA in microplate format

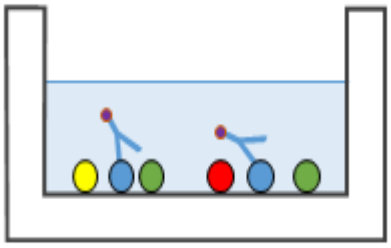
To analyze the complexation efficiency, it is necessary to clean the complexes of free components. This problem is easy to solve if one component of the antigen-antibody pair is firmly bound (immobilized) on a solid carrier. Immobilization allows to prevent aggregation in solution and to carry out physical separation of complexes formed from free components. The use of antibody immobilization on a solid carrier initiated the methods of solid-phase (heterogeneous) ELISA.

The development of antibodies and antigens of special polystyrene boards containing 96 wells as carriers for the sorption immobilization had particular importance for the widespread introduction of solid phase ELISA into practice.

Heterogeneous (solid-phase) ELISA in the microplate version has become the most widespread in test systems for clinical laboratory studies. As the solid phase the surface of the wells of polystyrene microplate is used, known antigens or antibodies (in this case - immunosorbent), which are part of the test system which is adsorbed. During the specific reaction of the immunosorbent with the antibodies or antigens specified in the test sample, immune complexes are formed, which are fixed on the solid phase. Substances that did not participate in the reaction, as well as excess reagents, are removed by repeated washing. This scheme allows to simplify the process of effective separation of the reaction components.

Direct ELISA. In direct enzyme immunoassay, the introduced material (antigen) is fixed during incubation on the surface of clean wells. The amount of test material is detected with antibodies up to finding antigen coupled to a specific label that provides an enzymatic reaction.

The principle of direct ELISA



multicolored circles - different antigens from the introduced serum into the hole
Y with purple dot - antibodies labeled with an enzyme that provides a color reaction (conjugate).

Brief scheme: **(Antigen) → Antibody**

Since the added specific label is related to antibodies, the concentration of the colored reaction product is equal to the concentration of antibodies. And the concentration of antibodies is equal to the concentration of antigens.

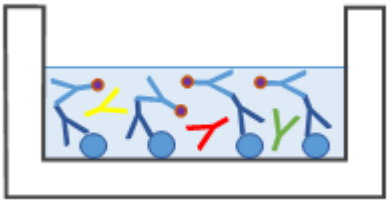
Indirect ELISA. In the indirect enzyme immunoassay, antibodies to the detected antigen associated with a specific label are used. This specific label is the substrate for the enzymatic reaction.

By the type of immunochemical interaction at the first stage of analysis (in which the binding of the substance that is determined is carrying out) among heterogeneous methods are distinguished:

2. Non-competitive method. If the system contains only the test compound and its corresponding binding centers (antigen and specific antibodies);
3. Competitive method. If a test compound and its analogue (enzyme-labeled or solid phase immobilized compound) are present in the system at the first stage, competing for a limited number of specific binding centers.

Indirect non-competitive ELISA. Test biological material (serum or human blood plasma) is introduced onto wells, on the solid surface of which pre-adsorbed antigen containing antibodies to the antigen. The sample is tested for antibody content.

The principle of indirect non-competitive ELISA



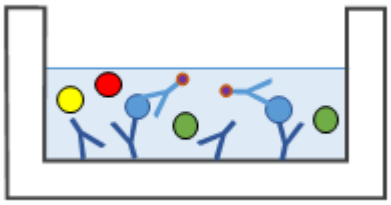
blue circles - immobilized antigen on the surface of the hole;
Y, Y, Y, Y- antibodies from the serum introduced into the well
Y with purple dot - antibodies labeled with an enzyme that provides a color response (conjugate).

Brief Scheme: **Antigen → (Antibody) → Antibody-K**

Thus, the difference from the direct method is that the tested antibodies are not glued to the surface of the clean well, and bind to the immobilized antigen.on the tablet

"Sandwich" is a variant of indirect non-competitive heterogeneous ELISA in which the antibody acts as an immunosorbent.

The principle of indirect non-competitive ELISA “sandwich”



multicolored circles - different antigens from the serum introduced into the hole;
Y - immobilized on the surface of the well antibodies;
Y with purple dot - antibodies labeled with an enzyme that provides a color response (conjugate)

Brief Scheme: **Antibody → (Antigen) → Antibody-K**

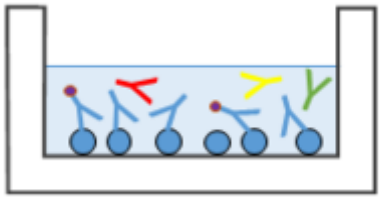
At the stage of detection of a specific immunocomplex, the antigen to be sandwiched between the molecules of immobilized and labeled antibodies, which led to the widespread distribution of the name "sandwich" method. Similarly, antibody test systems work but they use an antigen as an immunosorbent and the conjugate contains a solution of the enzyme labeled antigen.

Competitive. In case of competitive ELISAs, the antigens or antibodies that are detected compete with similarly labeled antigens or conjugate antibodies at the binding sites to the immunosorbent. Assays of this type are often used to identify antigens present in high concentrations or hormones having only one antigen-binding center.

There are two main formats for competitive ELISA schemes: direct and indirect.

Direct Competitive ELISA. Specific antigen immobilized on a solid phase is used, and enzyme-labeled and unlabeled antibodies compete for binding to the immobilized antigen.

The principle of direct competitive ELISAПринцип прямого конкурентного ІФА



blue circles - immobilized on the surface of the hole antigen;
Y, Y, Y, Y- antibodies from the serum introduced into the well;
Y with purple dot - antibodies labeled with an enzyme that provides a color response (conjugate).

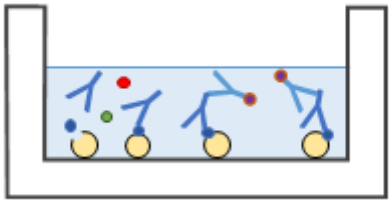
Brief Scheme: **Antigen → (Antibody) + Antibody-K**

Thus, the amount of detectable signal obtained by a direct competitive ELISA is inversely dependent on the concentration of the antigen.

The advantage of a direct scheme is the small number of steps that make it easy to automate the analysis. The disadvantages of the scheme include the complexity of the methods of synthesis of enzyme conjugates, as well as the possible influence of the components of the sample on the enzyme activity.

*Indirect Competitive ELISA.*Enzyme-labeled anti-specific antibodies (specific or secondary) and solid-phase conjugate antigen-protein carrier are immobilized in indirectly competitive ELISA. One of the most common ELISA schemes. Competition occurs at the stage of binding of antibodies or antigen from the serum of the subject (free, removed by washing), or with the antigen immobilized in the solid phase (not removed are not removed). Then conjugates of labeled antibodyis affiliated to antibodies and the optical density is determined. That is, if there is no measurable substance in the sample, all antibodies will bind to the antigen immobilized through the carrier protein on the surface of the well, while washing, they will remain in the well, and the detected signal will be high. If many of the measured substance is found in the serum of the test sample (it will be in the free state in solution), part of the antibodies will bind to that substance and some to be immobilized; antibodies in free state (bound to the antigen out of serum) will be removed by washing, and the detectable signal will give antibodies associated with immobilized in the hole antigen, it will be low, since some of the antibodies are removed during washing.

The principle of indirect competitive ELISA



big yellow circles - conjugate antigen-protein immobilized on the surface of the hole;
small red, green and blue circles are different antigens from the serum introduced into the hole (for example, drugs);
Y are unlabeled antibodies specific for a particular antigen;
Y with a purple dot is a secondary anti-species antibody labeled with an enzyme that provides a color response (conjugate).

Коротка схема: **Антиген-К → (Антитіло) + Антиген → Антитіло-К**

Brief Scheme**: Antigen-K → (Antibody) + Antigen → Antibody-K**

The magnitude of the detectable signal is inversely proportional to the concentration determined by the antigen.

- Homogeneous ELISA

In 1972, Rubenschetin and his staff developed a new approach to conduct an analisys without solid phase. The method was called homogeneous ELISA (English "EMIT" - enzyme multiplied immunoassay technique) and it was based on the consideration of differences in the catalytic properties of the enzyme label in free form and in the immunochemical complex. The principle of methodis the binding of a low molecular weight antigen to the

enzyme lysozyme near the active center. In combination with antibodies, the active center becomes sterile inaccessible for macromolecular substrate, which is the walls of bacterial cells. As the antigen concentration is determined, the concentration of the inactive antibody conjugate complex decreases, and therefore, the enzyme response parameter recorded increases. Based on this approach, kits have been developed to identify a wide range of toxic, drug and medicinal products. A significant advantage of EMIT analysis is the ability to use small volumes of the analyzed sample (5-50 µl) and high speed of determination (2-5 min) due to the absence of separation stage of free and labeled analyzed compounds. The disadvantages of the method include the lower sensitivity than in the heterogeneous ELISA (~ 1 µg / ml), and the ability to detect only low molecular weight antigens.

Recently new ELISA variants containing non-infectious reagents based on recombinant p32, p54 and pp62 virus proteins were developed. Also they show high sensitivity and specificity in the study of blood sera, even at low quality.

The basic principle of ELISA is the antigen-antibody reaction. If the antigen (target molecule) is a protein, then its purified preparation is usually used to produce antibodies, which then help to detect the target.

There are a number of approaches to determine whether antibody binding to the target antigen has occurred. One of them is enzyme-linked immunosorbent assay (ELISA), it is often used to diagnose a variety of antigens.

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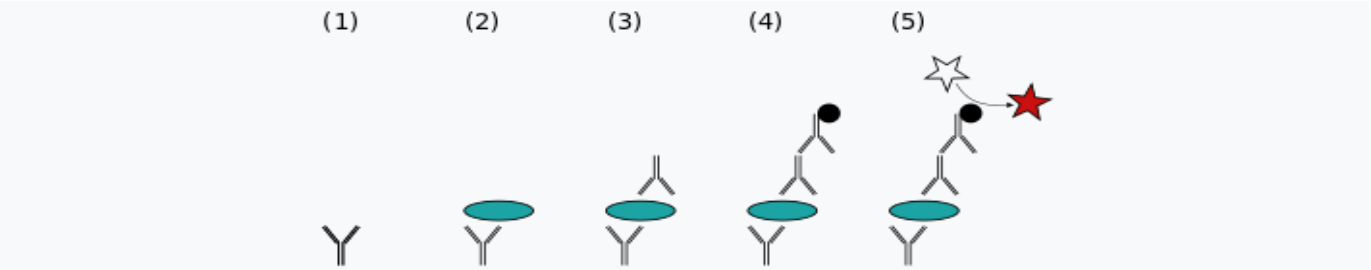


Scheme of analysis

1. Preparation of a pad for fixation of the sample;
2. The sample in which a specific molecule or microorganism is to be detected is fixed on a solid pad, for example, on a plastic microtiter plate, which has 96 wells;
3. An antibody specific to the marker molecule (primary antibody) is added to the fixed sample, then the well is washed to remove the primary antibody molecules that have not bound;
4. A secondary antibody is added that specifically binds to the primary antibody and does not interact with the marker molecule. An antibody (eg, alkaline phosphatase, peroxidase, or urease) is attached to this antibody, which can catalyze the conversion of a non-colored substrate into a colored product. The well is washed to remove unbound molecules;
5. Colorless substrate that is recognized and utilized by the enzyme is added;
6. Qualitative or quantitative determination of the colored product is carried out by means of optical density estimation.

Enzyme-linked immunosorbent assay is widely used for the diagnosis of various infectious diseases, cancers (mainly due to specific proteins and peptides), determination of the concentration of various low molecular weight compounds, such as toxins, drugs and others.

1.9 **Hygiene and safety in a chemical laboratory**

The rules on safety, labour protection, surveillance and regulation in the field of the safe handling of dangerous substances and industrial materials are regulated by a central executive body.

The requirements for safety regulations in the chemical laboratory and hygiene have been discussed earlier. Section 1.1 “Preparation of the laboratory for testing. Security Rules. "

1.9.1 Security equipment

Testing laboratoriesshould ensure the protection of the confidentiality of the information and property rights of its customers, including procedures for the protection of electronic storage and the transmission of results. The laboratories also have testing, measuring and auxiliary equipment and standards to perform the tests. Therefore, each director seeks to provide protection that would not impair confidence in the competence, integrity, and preservation of laboratory property by installing security systems.

Security system is an automated complex for the protection of various objects of property.

Classification of security systems:

1. by the interaction with the threat:

- Passive: a set of tools and actions aimed at attracting the attention of the property owner or security services
- Active: designed to prevent penetrationinto protected object or safe.

To organize active systems it is necessary to be guided by the current legislation of the country. If the health of the attacker is harmed, then a trial will take place and criminal proceedings may be brought.

1 By the method of transmission of information

- wired
- Wireless - security sensors transmit information to the receiving device by radio signal.
- Without feedback

Disadvantages are the presence of many ways to suppress the radio signal by special "Noise Generators" (and sometimes even this comes from ordinary household appliances).

- with a feedback with receiver: allows continuous monitoring of all sensors.
- Via GSM network: used for both remote control (the alarm is transmitted to the security company console) and to inform the owner of the protected object, which can receive information about various events (alarm, fire, malfunction, etc.). e.) as an SMS to your mobile phone. GSM-communicators are used for this purpose.

Wireless systems are usually used in cases where there is ability to make wiring. Sometimes it combines both wireless systems with wired one and passive systems with active ones.

1.10 Waste management

The problem of recycling is relevant in industry. Some enterprices seek help from specialized companies. Disposal must be waste-free and harmless to both humans and environment.

Chemical compounds and their mixtures are toxic, active and many of them flammable and explosive. Evaporations produced by these volatile substances cause irreparable harm to humans and nature. Therefore, the process must be approached in compliance with safety rules, including the use of personal protective equipment.

Classification of chemical wastes:

There are many types of chemical waste. In addition, the determination of waste of this kind often involves common organic substances contaminated with any substance.

- waste from the production and use of alkalis and acids;
- spent oil products and oil production waste;
- waste of the production of paints and solvents;
- expired chemical materials;
- expired medicines;
- products of activity of chemical laboratories;
- pyrotechnic and halogen wastes;
- mercury-containing products;
- wood waste containing chemical impregnation.
- food contaminated with pesticides.

By the degree of severity of impact on living organisms, all waste of the chemical industry is divided into 4 classes of hazard.

Why should we dispose chemical waste in proper way? Any group of chemical waste contains a dangerous set of both chemicals and by-products released during the production of a product.

Legal interpretation of the concept of hazardous chemical waste in addition to MPC (maximum permissible concentration) of hazardous substances widely uses such terms:

- irritable;
- toxic;
- explosive and flammable;
- carcinogenic;
- corrosive.

According to the legislation of almost all countries of the world, wastes containing such substances should be neutralized and disposed obligatory. If this does not happen, the harmful substances gradually decomposing can cause irreparable damage to all living things on the planet.

The proper disposal of hazardous chemical wastes begins with a thorough sorting process. Substances which can react in close contact must be separated. The second stage of the start of recycling is their separate storage in tightly closed containers, without the possibility of interaction with each other and the environment.

Будь-який виробник, який використовує для своїх потреб хімреактиви повинен мати документ, що підтверджує дозвіл на використання. Так званий паспорт небезпечного відходу (ПНВ). Згідно з вимогами цього документа можна вибрати безпечний спосіб утилізації. Для ситуацій, не обумовлених в ПНВ, застосовуються додаткові тести.

In addition to the disposal of waste reagents in the laboratories from time to time pipelines, sewage effluent should be cleaned from the taint of harmful waste.

The Law on Production and Consumption of Wastes sets out in detail the rules for the disposal of hazardous industrial waste and also about the chemical reagents that lost their working properties due to their expiration date.

The best solution for laboratories is to conclude an agreement to provide recycling services by specialized companies.

When disposing of waste in the laboratory, the rules for handling reagents must be strictly followed. Methods of waste processing:

1. Chemical neutralization for the conversion of a harmful chemical into a neutral mass.
2. Heat treatment in special chambers without air access.

Responsibility for improper disposal of chemical waste

Norms for the disposal of deleterious substanceare strictly regulated by the Law "On Environmental Protection". Violation of the law entails legal responsibility for the committed. And the consequences can be very serious, up to criminal penalties.

The stringency of the legislation is easily explained by the danger of disregarding the rules of disposal for human health and environment.

Fines for not meeting the requirements for the disposal of chemical waste.

1. Compensation for environmental damage and repair of health of affected people or animals.
2. Administrative penalty payments, depending on the caused damage.
3. Temporary business interruption for up to 3 months.
4. Asset freeze of laboratories with its subsequent confiscation.

The disposal of chemical waste is a serious and responsible deal. And it should be treated properly.

Chapter 2. The bases of the work at biological laboratory

2.1 Classification of laboratories according the levels of dangerousness

Levels of biological safety of microbiological laboratories and basic requirements for their work. WHO has offered to classify all microbiological laboratories with regard to their purpose, design, equipment, tools that they use, practices and operational procedures to work with microorganisms belonging to different groups of risk. There are 4 types of laboratories: Basic Biosafety Level 1 (BSL – 1), Baseline Biosafety 2 (BSL – 2), Isolation Biosafety Level 3 (BSL – 3), and Maximum Isolated Biosafety level 4 (BSL – 4). It is falsely believed that if the microorganism is assigned to a particular group, a laboratory with the same level of biosafety is needed for safe work with it. However, based on the requirements of certain procedures and other factors, it is better to use a higher or lower biosafety level.

Laboratory of the 1st Biological Safety Level (BSL-1). Biosafety level 1 is suitable for work with well-studied microorganisms that do not cause human disease and are of minimal potential danger to laboratory personnel and environmental. Most often BSL–1laboratories are educational laboratories. They maynot be separated from the premises of the building. The work in BSL–1 laboratories is usually carried out using standard microbiological methods. Special conditions for the maintenance of equipment or facilities are not required.

Common hygienic measures are used in BSL-1-laboratories. All laboratory workers should be in gowns. Closed footwear must be on feet. If there are fresh wounds on hands it is better to put om gloves during the manufacture of drugs, their staining, as well as working with hazardous chemicals. To perform standard laboratory procedures in the case of compliance with hygiene rules, gloves are not used. Hand hygiene must be carried immediately after the end of work and contact with microorganisms, by washing with soap and wateralcohol.

Floor, walls and surfaces of all furniture must be smooth and intact. The laboratory must contain sink for washing. The door must be locked. It is also necessary to prevent pests in the laboratory. Personal belongings are stored outside the working area. Autoclaves must be in the laboratory.

To work in the laboratory, you can only use cultures obtained from the reference laboratories, etc. It is prohibited to use microorganisms allocated from the environment as they may be organisms that require the practice and equipment of BSL-2.Availability of document informationabout the origin, properties and movement of museum microorganisms.

It is necessary to wash your hands before going out from the laboratory, tables should be treated with preservative before and right after work. For the movement of microorganisms in the laboratory, transport tubes and tripods should be used and the cultures should be kept in an airtight container after handling them.

Laboratory of the 2nd Biological Safety Level (BSL – 2). The Biosafety Level 2 is based on BSL–1. BSL – 2 is suitable for dealing with agents that pose a moderate danger to the staff and environment. Laboratory staff musthavespecial training on working with pathogenic agents.Access to the laboratory is limited. The procedures in which infectious aerosols or sprays can be generated are carried out in biosecurity boxes. Special procedures for disinfection and safe storage of biological agents are applied.

In BSL-2 laboratories only goggles and face masks when operating under the conditions of droplets and aerosols, which are performed outside the biological safety compartments are used. When working in a biosafety cabinet, goggles and face shields / masks are not used. Special clothing and footwear, gloves are available (only when working with micro-organisms or chemicals of the 2nd level of risk,when hand skin is damaged).

There must be a door with access control in BSL–2 laboratory. The windows that open outward should be equipped with protective screens against insects and rodentsIt is necessary to prevent pests from entering the laboratory. Personal belongings are stored outside the working area. There must be autoclave in the laboratory. Access to BSL–2 laboratories is limited. The laboratory personnel should undergo a health check, and immunization against agents with whom they work in the laboratory if it is necessary. All procedures with materials that may lead to aerosol formation should be carried out in biological safety boxes.

Laboratory of the 3rd Biological Safety Level (BSL – 3). The 3rdevel of bio-safety is used in clinical areas where work with local or exotic agents is performed, which can lead to severe or potentially fatal diseases with respiratory transmission mechanism. The laboratory staff should have special training to work with pathogenic and potentially life-threatening agents. All procedures related to infectious materials must be carried out in biosecurity boxes.

Biosafety Level 3 is based on BSL-2 and additional measures. The laboratory should be separated from other parts of the facility. Laboratory access is restricted and must be through a lockable two-door. Changing clothes takes place in a vestibule between two doors. The laboratory should have a separate area where clean equipment and supplies, showers are stored. There must be an observation window on the lab door. The lab should have self-operated hand wash basins located near the exit door, or. in each work area. The laboratory is designed to be easy to clean and disinfect. The floor is non-slip, waterproof, resistant to chemicals. The surface of walls and ceilings should be covered with sealed and smooth materials that are easy to clean and disinfect. Laboratory furniture must be covered with materials capable of withstanding the disinfectant and be arranged so that it can be easily removed. All windows in the laboratory should be deadlight. The biosafety boxes should not be in front of windows and doors. The presence of a tidal ventilation system. The ventilation system must be equipped with HEPA filters. Lab staff should change gloves after contamination/breach of their integrity (if it is necessary, personel wear two pairs of gloves) PPE are used in rooms where infected animals are kept.

Laboratory of the 4th Biological Safety Level (BSL – 4). Biosecurity Level 4 is used to work with hazardous and exotic agents that pose a high individual and social risk, they are transmitted respiratory, and the treatment and prevention of such diseases are not developed. Agents with a similar antigenic structure to agents requiring BSL-4 should also be handled in laboratories of this security level. All laboratory staff and supervisors must be licensed to work with BSL-4 agents.

There are two types of BSL-4 laboratories: 1) all manipulations with agents are performed in biosafety cabinets of the IIIrd class (boxing labs); 2) all manipulations with agents are carried out in protective suits, which are under positive pressure (suit-laboratory).

The workplace must be sealed with the possibility of its fumigation. Lab workers put on overalls. If necessary, the laboratory may be equipped with an oven for burning animal carcasses.

All persons entering the laboratory should be warned of the potential danger and rules for leaving the laboratory. Persons who are involved in scientific research or supporting work. Are allowed to enter the laboratory. Anyone who enters or leaves the labpratorymust register his person and time. Personnel should come in and exit the laboratory with the mandatory change of all clothing and taking shower. Laboratory staff and service personnel are subject to mandatory medical examination and vaccination. There must be an insulator for the workers within the laboratory. Materials entering the laboratory must be pre-decontaminated in a two-door autoclave / fumigation chamber. The check of all life support systems must br carried out and their condition have to be documented before starting work.

2.2 "Small" laboratory equipment for microbiological laboratories.

2.2.1 Microscopes.

Special optical devices, microscopes, are used to study the cells of microorganisms invisible to an unaided eye, (Greek micros - small, scopeo - I look), which increases studied objects in hundreds (light microscopes) and hundreds of thousands or more (electron microscopes) times. Morphology and structure of microorganism cells, their growth and development, primary identification of the studied organisms, development of microbial cenoses (communities) in soil and other substrates are studied using a light microscope in microbiology. submicroscopic structure of the cells of microorganisms, previously unknown forms of the smallest microorganisms are detected using an electron microscope in microbiology.

The structure of microscopes.

In microbiological practice, light microscopes of the following brands are used: MBR-1, BRM-1, BRM-2, BRM-3, BRM-6, Biolam R-1, etc. They are designed to study the shape, structure, size and other characteristics of various microorganisms, the value of which is not less than 0.2–0.3 microns. A light microscope consists of two parts - optical and mechanical. The mechanical part includes a tripod, a stage, a tube. The tripod consists of a base and a motionlessly screwed tube holder. The tripod is adjoined by a box of mechanisms, a system of gears for regulating the motion of the tube. The system is driven by the rotation of the macrometer and micrometer screws, respectively designed for coarse and accurate focusing on the sample. When the screws rotate clockwise, the tube moves toward the sample; when rotating counterclockwise - from the sample. The subject table serves to place the preparation on it with the object of study. The stage can be moved with screws in a horizontal plane. There is a round hole for illumination of the preparation from below with beams of light directed by a microscope mirror in the center of the table. Two pole clamps (terminals) are mounted on the table, spring metal plates intended for fixing the preparation.

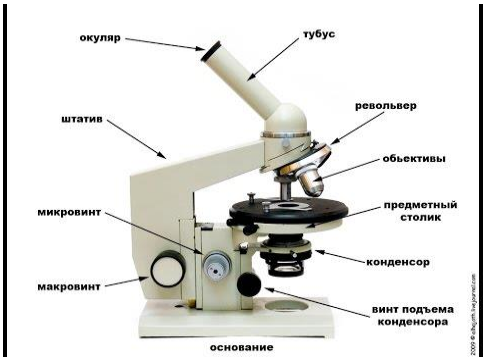


Fig. 1 **The structure of light microscope**.

A tube is a frame in which the elements of the optical system of a microscope are enclosed. A revolver (lens holder) with lens slots is attached to the bottom of the tube. An eyepiece is inserted into the upper end of the tube. Modern models of microscopes have an inclined tube with an arc-shaped tube holder, which ensures the horizontal position of the stage. The optical part of the microscope consists of a main optical unit (lens and eyepiece) and an auxiliary lighting system (mirror and condenser with iris diaphragm).

The **mirror** reflects the light incident on it and directs it into a condenser to illuminate the preparation. The mirror has two surfaces: flat and concave, folded back and enclosed in a single annular frame, mounted in a semicircular fork. With its help, the mirror moves in any direction. The flat side is used at any light source and at any amplification. The concave side is used at low amplifications without a condenser and, as a rule, with artificial lighting. In some cases, the mirror is replaced by a stationary illuminator.

A **condenser** is an optical system of two to three short-focus lenses to enhance the brightness of the illumination of the object. The condenser concentrates the beams coming from a flat mirror and directs them at a large angle to the object. Condenser lenses are mounted in a cylindrical frame, connected to a gear mechanism, which allows the condenser to be moved up and down along the axis of the microscope with a special screw. When lowering the condenser, the field of view of the microscope is darkened, when it is raised it is illuminated. To adjust the intensity of illumination, the condenser is equipped with an iris (petal) diaphragm, consisting of thin opaque sickle-shaped plates. When moving the diaphragm lever located in the lower part of the condenser frame, the plates can be moved together and apart, gradually changing the diameter of the active hole. In many modern types of microscopes, a condenser and a light source are mounted in a microscope.

Lenses (from the Greek. Objectum - the subject of research) are the most important part of the microscope, mainly the image of the object depends on their quality. They are screwed into the nests of the revolver and consist of a system of lenses enclosed in a metal frame. The front lens is the smallest and only one that gives amplification. The rest of the lenses in the object-glasses only correct the imperfections of the resulting image and are called corrective.

Lenses are divided into dry and immersion. When working with dry lenses, there is air between the front lens and the object of study. In cases of using immersion lenses, a liquid with a refractive index close to the glass

refractive index should be between the front lens of the lens and the object of study. The best for this purpose is immersion oil (cedar oil) with a refractive index of 1.515 (glass refractive index of 1.52). Due to this light beams do not refract upon transition from glass to a layer of cedar oil (since they remain in an optically homogeneous medium) and enter the lens without being reflected.

The eyepiece (from lat. Oculus - eye) is inserted into the upper end of the tube. The eyepiece is a system of two plane-convex lenses convex towards the lens. The lens facing the eye is called the ophthalmic, while the lens facing the preparation is called field or collective lens. The purpose of a field lens is to collect the beams coming from the lens so that they pass through a small hole in the eye lens. An eye lens, like a simple magnifier, magnifies the actual image given by the lens. The distance between the lenses is equal to half the sum of their focal lengths. The purpose of the eyepiece is to direct imaginary magnification of the actual reverse and enlarged image that the lens gives. Eyepieces are marked with numbers showing their own amplification × 5, × 7, × 10, × 15. When working with a microscope for a long time, you should use double eyepieces - a binocular nozzle.

Basic rules for working with a microscope

A place for the microscope is chosen away from direct sunlight. Working on a table with a dark surface contributes to less eye fatigue. It is recommended to look into the eyepiece with the left eye and not to close the right one. In the case of working with a binocular nozzle, the distance between the eyepieces is adjusted first in accordance with the distance between the eyes of the observer so that the visual fields of both eyepieces merge into one. It is necessary to carry the microscope with two hands: one hand should hold a tripod, the other one holds the base of the microscope. It is impossible to tilt the microscope to the side, since the eyepiece may fall out of the tube. The microscope should be protected from shocks, contact with potent substances such as acids and alkalis. Do not touch the surface of lenses, mirrors and filters with your fingers. It is not recommended to remove the eyepiece from the pipe so as not to make dirty the pipe and lenses with dust. During your work, it is advisable to protect the microscope from breathing, since condensation of vapors leads to its damage. The microscope should be protected from dust, moisture and stored in a case under a glass cover or covered with cloth.

During microscopy of preparations with a dry lens, you should take certain steps in the work:

1. the microscope is placed on the desktop with a tube holder at a distance of 3-5 cm from the edge of the table;
2. put a lens with a small amplification (×8) and at this magnification set the best lighting; the best lighting is achieved by adjusting the position of the mirror, condenser and diaphragm; when viewing unpainted preparations, a narrowed diaphragm and a lowered condenser are used; when observing colored preparations, an open diaphragm and a raised condenser are used. The field of view of the microscope with the correct installation of light will have the shape of a well and proportional lit circle.
3. place the preparation on the objective table under the lens, and fasten with clamps;
4. carefully put down the lens (× 8) with a macroscopic screw, observing the side of the lens, at a distance of about 0.5 cm from the objective table;
5. looking into the eyepiece, turn slowly the macroscrew towards, and raise the tube until an image of the object under study appears in the field of view. After that, the lens is focused by turning the micrometer screw so that the image of the subject is clear. The micrometer screw can be rotated no more than half a turn in one direction or another to prevent damage to the screw thread.
6. turning the rod, set the lens with medium magnification (× 20; × 40; × 60);
7. when the work is finished, you should remove the preparation from the objective table, put down the condenser, put the ×8 lens under the tube, wipe the microscope with a soft cloth and put it into the case.

When working with an immersion lens, the following rules and procedure must be observed:

1. set the mirror at flat side, raise the condenser and adjust the light under the small magnification of the microscope (×8 lens);
2. apply a small drop of immersion oil to the prepared and colored smear (without smearing it over the glass) and place the preparation on an object table;
3. turn the rod to the mark 90 of the immersion lens;
4. observing from the side, carefully put down the microscope tube until the front lens of the immersion lens is immersed onto a drop of oil;
5. looking into the eyepiece of the microscope and acting with a macroscrew, slowly raise the microscope tube until the studied object appears in the field of view;
6. do the final focusing of the preparation with a micrometer screw, rotating it within only one rotation;

7. at the end of microscopy, lift the tube, put down the condenser, move the rod to a small dry ×8 lens, remove the preparation and carefully wipe the front lens of the ×90 lens, first with a dry cotton cloth, and then with the same cloth, but slightly moistened with gasoline or alcohol

2.2.2 Autoclave

The autoclave is a vessel with thick walls operating under pressure, with a massive lid, closed with screw clamps or a special helm. The autoclave is equipped with a steam tube, a pressure gauge, a safety valve and a gauge glass. Water is heated using electric incandescent lamps in the interwall space of the autoclave, from where steam enters the work plane. Culture media, milk, dishes, rubber items, etc. are sterilized in autoclaves. Before sterilization, distilled or boiled water is poured into the autoclave through a special funnel (scum forms quickly when unboiled water is used) to the top mark on the gauge glass. The autoclave is loaded with the material to be sterilized and tightly closed with a lid, tightening the screws or turning the helm clockwise.

Turned on the autoclave, leaving the tube open to release air and steam. Do not close the tube until all of the air from the autoclave is displaced by the steam and the steam flows in a continuous stream. If air remains in the autoclave, the sterilizing effect will decrease, since the temperature of the mixture of steam and air is much lower than the temperature of saturated steam.

The gauge needle begins to rise after closing the tap. When the desired pressure is reached, the degree of heating of the autoclave is reduced so that the pressure gauge needle stops at the required level. The start of sterilization is considered the moment the arrow reaches the desired pressure. The pressure gauge indicates a specific temperature. At the end of the sterilization, the heating is stopped and the pressure gauge needle drops to zero. Only after that the steam vent valve is opened, then the lid is unscrewed and the sterilized material is removed.

Basic rules for working with Autoclave. Open the valve connecting the boiler to the sterilization chamber. The sterilization chamber of the autoclave is loaded with the material to be sterilized. Old water is poured from the autoclave through the lower valve connecting the boiler to the water tube, and water is poured through the funnel to the red line on the water tube. The lid of the autoclave is closed, screwed to the body with bolts and open the condensation and exhaust valves, turn on the autoclave. On the switchboard of the autoclave the toggle turned on the "Heating" position. Large air bubbles begin to come out with a soft gurgling sound when boiling water from the outlet valve through a drain pipe lowered into a bucket of water. Then, only a small number of small bubbles consisting of steam that did not have time to condense begin to come out with the steam. Then a stream of pure dry steam begins to break out of the tube with a sharp crack. The output of the steam jet corresponds to a boiling point of water - 100^0C. This point indicates the removal of air from the autoclave. After that, the outlet valve is closed, the outlet tube is removed from the bucket of cold water and the pressure is increased to the required value within 2 atm.

The beginning of sterilization is considered to be the moment when the pressure gauge needle indicates the required pressure. After this, the heating intensity must be reduced so that the pressure no longer rises. To do this, turn the switch to the "Sterilization" position. At the end of the sterilization time, the switch on the switchboard is set to the “off” position, the autoclave is turned off, the valve connecting the sterilization chamber to the boiler is closed, and wait until the pressure drops to atmospheric pressure and the arrow will show zero on the pressure gauge. Open the steam-discharge tap and release all the steam. Open the lid of the autoclave so that the remaining steam goes to the side and does not cause burns. The sterilized material is removed and condensate formed in the sterilization chamber is released through a condensation tap, after which the tap is closed. Do not open the valve before time, since hot media boil violently, moisten or even push cotton plugs, making the media non-sterile.

Do not open the tap slightly by immersing the drain pipe in a vessel of cold water. As a result of the created pressure difference, water from the bucket is sucked into the autoclave (sterilization chamber) and clogs it. Sterilized material should be left with a half-open autoclave lid for 3-5 minutes to dry cotton plugs in flasks or test tubes. After work, water is poured from the autoclave. It must be kept dry.

Regularly It is pnecessary to check whether the pressure gauge indications correspond to the temperature in the sterilization chamber. To do this, use powdered chemicals with a strictly defined fusion point, mix them with a small amount of paint (methylene blue, fuchsin, safranin). The mixture is poured into small tubes, well closed with a cork stopper and placed in an upright position between the material to be sterilized. When the powder is

distributed, an alloy is formed which is painted in the color of the added paint. The following substances are usually used as temperature indicators:

Material	***Fusion point, ^0C***
Benzonaphthol	110
Antipyrin	115
Flowers of sulfur	115
Pure resorcinol	118
Бензойная кислота	121

2.2.3 Thermostats

Types of laboratory thermostats

Laboratory thermostat is a device which main purpose is to obtain and maintain a stable temperature

These are special cabinets that maintain a constant temperature, used for growing microorganisms. Thermostats can be hot air bath and water bath. The first cabinet is heated with warm air passing through the pipes, or with electric heaters. In water thermostats between double walls there is water heated by electric current. To maintain a precisely set temperature, thermostats are equipped with contact thermometers.

The laboratory should have four thermostats: at a temperature of 25-30 °C for growing mesophilic microorganisms, 40-45 °C for growing thermophilic microorganisms, at temperature of 37°C and 43 °C for detection and identification of E. coli.

Air bath. The representative of laboratory air thermostats is TVL-K120. With its help, the device maintains the required temperature. Laboratory air thermostat TVL-K120 is actively used during bacteriological and serological studies. They are carried out by clinical diagnostic, environmental and research laboratories. For normal operation of the thermostat, the air temperature in the room should be in the range of 10-35 °C, and the relative humidity should not exceed 80%. The work of microprocessor control allows to achieve thermal stability with high accuracy. The laboratory thermostat TVK L with cooling is a device that has various models that differ in working volume, dims and weight.

Dry-air thermostat. The representative of dry-air thermostats is TSvL-160. It is necessary to obtain and maintain a stable temperature in the working volume, which is necessary for the study of bacteriological, microbiological, sanitary-bacteriological, virological, biochemical types.

Water thermostat. The laboratory thermostat reductazniltr 24 is water one. It has sufficient capacity to be used for research purposes. 24 test tubes, 24 butyrometers, 24 flasks, the diameter of which does not exceed 17 cm can be placed in it. It can maintain a temperature up to +95 °C. Milk samples are heated and then measured in a special analyzer in this type of water thermostat. It analyzes fat. The volume of the working chamber is 8 liters. Made of polished stainless steel. It also has a control unit with which to set the required temperature and the screen for changes or setting the operating mode.

Circulation thermostat. A circulation thermostat has air or water cooling. It is economical. It should be noted that the circulation laboratory thermostat is XP, therefore it can be used in rooms of various types.

Thermostat working principle

Laboratory thermostats operate on the principle of cooling the air in the interior using a refrigeration compressor. If we talk about dry-air models, they are characterized by forced air mixing, so that the temperature inside the chamber is evenly distributed.

Periodically thermostats are opened, which leads to the precipitation of various airborne microflora on the surface of their walls. They are inseminated with spores of microscopic fungi and actinomycetes when they are cultivated in Petri dishes in large quantities. All this leads to contamination of pure microbial cultures grown in thermostats and especially plating in cups made from different environmental objects: soil, water, air, milk, meat. These platings are spore-forming fungi - mucor, aspergillus, penicillas very often.

It is necessary to sterilize the thermostat once a week in order to prevent negative consequences and obtain undistorted results. Carry out wet cleaning in the thermostat and remove crops from it.

After drying the thermostat, take a long tweezers, cotton wool is wound on it by a spiral. Tweezers with cotton are dipped into alcohol and set on fire. This alcohol in the flame wipe all the walls of the thermostat and grillages. Without overheating you should work in the area of placement of the thermostat. In case of overheating, it may burst. The thermostat is closed after sterilization. Doors are also wiped with alcohol in a flame.

2.2.4 Desiccator

Desiccatoris used for many processes in a modern laboratory. Among these processes are drying, conditioning, vulcanization, easy firing and other operations associated with heat treatment or preliminary preparation of a sample or substance.

The desiccator is used to sterilize dishes, equipment, etc. with dry heat. The desiccator is loaded when it is unheated. The material to be sterilized is carefully wrapped with paper or placed in metal cases (pipettes, Petri dishes). The dishes should be dry, the necks of the flasks and tubes are closed with cotton plugs. The material is placed in a cabinet so that it does not touch the walls. The duration of sterilization at a temperature of 160-170 ° C is 2 hours (the start of sterilization is considered the moment when the temperature inside the cabinet reaches 160°C). The sterilized material is discharged from the desiccatorafter it has cooled.

2.2.5 Centrifuges

A centrifuge consists of three main components:

- Rotor
- Drive shaft
- Motor

The rotor is the rotating part of the centrifuge. Tubes, bottles or bags containing liquids that need to be centrifuged are placed in it. Rotors of various types and sizes are interchangeable. They are installed on the drive shaft, which is connected to the motor. The motor supplies the centrifuge with the energy necessary to rotate the rotor.

There are controls and indicators of speed and time on the wall. Most centrifuges have a braking system that puts the rotor at repose shortly after completing its work.

There are two configurations of centrifuges: floor-standing and desktop. The difference between the two is in capacity. The principles of work are the same.

The centrifugation method is based on the different behavior of particles in a centrifugal field created by a centrifuge. The sample in the centrifugation vessel is placed in the rotor, which is driven by a centrifuge drive. It is necessary to choose a set of conditions, such as rotational speed, centrifugation time and rotor radius to separate a mixture of particles. For spherical particles, the rate of deposition (sedimentation) depends not only on acceleration but also on the radius and density of the particles, as well as on the viscosity of the medium in which the sample is deposited.

Centrifugation can be divided into two types: preparative and analytical. Preparative centrifugation is used when it is necessary to isolate part of the sample for further research. This method is used to isolate cells from a suspension, biological macromolecules, etc.

Analytical centrifugation is used to study the behavior of biological macromolecules in a centrifugal field. This method allows you to obtain data on the mass, shape and size of molecules in relatively small volumes of the sample. Preparative centrifugation is most common in everyday laboratory work.

Preparative laboratory centrifuges, in turn, are divided into groups according to their purpose: preparative ultracentrifuges, general purpose centrifuges, and high-speed centrifuges. In all general purpose centrifuges the rotors are rigidly mounted on the drive shaft, so the centrifuged capacity must be accurately balanced. To avoid damage, do not load an odd number of tubes into the rotor; if the load is incomplete, the container should be placed opposite each other

High-speed centrifuges have a top speed of 25 thousand rpm and an acceleration of up to 89000g. The chamber in which the rotor and centrifuged samples are located is equipped with a cooling system to prevent heating caused by friction when the rotor rotates at high speeds. Typically, such centrifuges can work with volumes up to 1.5 liters and are equipped with angular rotors or rotors with interchangeable cups.

Preparative ultracentrifuges accelerate to 75,000 rpm and have a maximum centrifugal acceleration of 510000 g. They are equipped with refrigeration and vacuum units to prevent overheating of the rotor from friction against air. The rotors for these centrifuges are made of high-strength titanium or aluminum alloys. Unlike high-speed and preparative centrifuges the shaft of ultracentrifuges is flexible to reduce vibration when the rotor is out of balance. The capacities in the rotor must be carefully balanced to 0,1of a gram.

2.2.6 pH-meters

The pH-meter is used to measure the pH of nutrient media and reagents, as well as to verify their quality after sterilization.

The instrument can also be used to measure pH values of samples and sample suspensions. The use of a pH meter is provided for in the standard for a particular product to be analyzed, in which the conditions for measuring the pH value and the conditions for obtaining the desired pH value are defined.

The pH-meter is adjusted in accordance with the manufacturer's instructions at a standard temperature of 25 °C. The pH value is taken into account after the test value stabilizes. The pH value is recorded to two decimal places.

Ph-meters must be calibrated. Calibration of the pH-meter is carried out using ready-made solutions with a given ph level. Depending on the model of the device, it is possible to calibrate using one or several points. The more calibration points, the more accurate the readings and the less measurement error of the ph-meter.

Used links:
https://www.laboratorii.com/stati/vidy-laboratornykh-tsentrifug.html

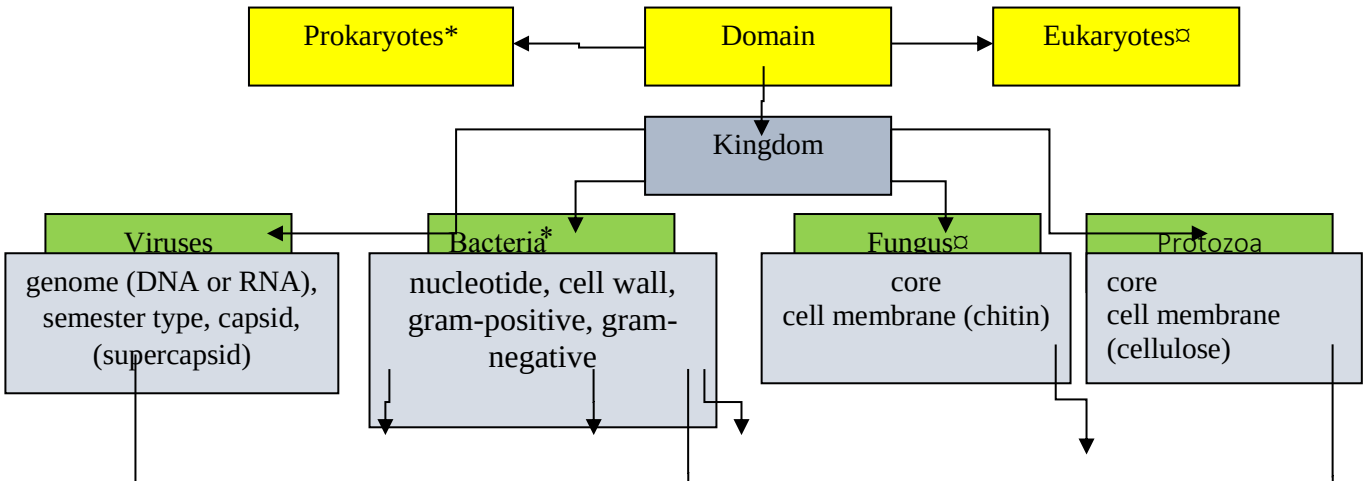
https://chem21.info/info/1279477/
http://www.activestudy.info/oborudovanie-mikrobiologicheskoj-laboratorii-specialnoe-oborudovanie/

http://old.stttrk.ru/book/page10.html

2.3 Classification of microorganisms and their dangerousness

Classification of bacteria

Microorganisms are organisms that are invisible to the naked eye because of their small size. This criterion is the only one that unites them. *Systematics of microorganisms* is a science whose task is to describe and organize the various microorganisms, their division (classification) into certain systematic groups (taxa). This allows us to develop a classification that is used to establish the pathogen (Fig. 1).



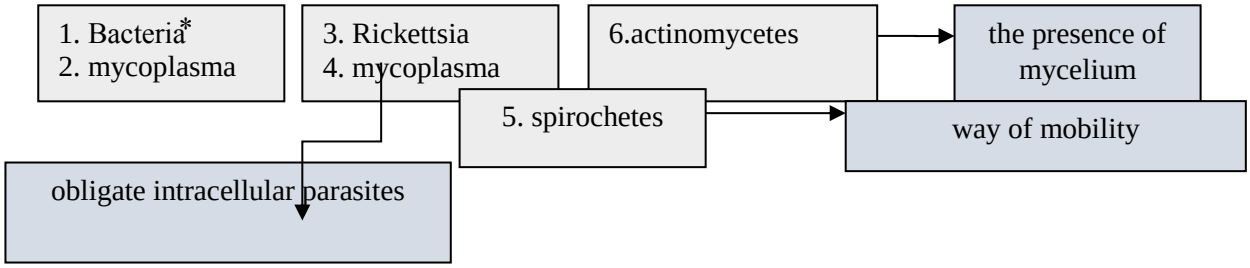


Fig. 1. Classification of pathogenic microorganisms

Note:*the sizes of prokaryotes and eukaryotes is from 0.01–0.4 μm to 20.0 μm; ¤ - sizes of viruses from 10 to 400 nm.

The principles of classification are studied in a separate section of systematic – taxonomy. For microorganisms the following categories of taxonomic hierarchy (taxa) have been adopted:

Classification of prokaryotes: Species are united into genera, genera (Genus) into families, families (Familia) into orders, orders (Ordo) into classes; classes (Classis) in divisions (Divisio), - in kingdoms (Regnum). The main taxonomic unit in microbiology is species.

*Species*is an evolutionary set of individuals that have a single type of organization and who, under standard conditions, exhibit similar phenotypic features (morphological, physiological, biochemical, antigenic structure), have their own gene pool and may cross. Microorganisms with little hereditary properties are called *variants*.

A strain is a group of bacteria of the same species isolated from different sources or from one source at different times. The strains may differ in some features that do not go beyond the species characteristics.

Serovar (Serotype) is a group of microorganisms of the same species, united by the common antigenic structure and determined by serological methods of diagnosis. Serovar is not a taxonomic category and allows the pathogenic opportunisticmicroorganisms to be systematized, which is necessary for epidemiological studies. Their systematization is based on virulence, lipopolysaccharides, Gram staining, the presence of exotoxins, genetic features or other factors that allow to distinguish two samples of the same species. A group of serovars with the same antigens is called a *serogroup*.

A clone is a collection of individuals formed from a single mother cell

Culture is the collection of individuals of one species or variant in a phase of separation or rest in a certain volume of nutrient medium.

All microorganisms are divided into *prokaryotes* (cells that do not have a nucleus) and *eukaryotes* (cells that have a nucleus).

2 principles of classification of microorganisms are applied:

• Phylogenetic principle: microbial belonging to a particular group is determined according to genome structure.

• Phenotypic principle is the unification of microorganisms with similar properties (pathogenicity, morphology, physiology, enzymatic properties, antigenic structure).

The kingdom of prokaryotes includes the cyanobacteria division and the eubacteria division, which is subdivided into orders: the bacteria (Gracilicutes, Firmicutes, Tenericutes, Mendosicutes); actinomycetes; spirochetes; Rickettsia; chlamydia.

The main taxonomic criteria for attributing bacterial strains to one or another group are: the morphology of microbial cells (Coccus, bacilli, spirilla); attitude to Gram staining, ie. tinctorial properties (gram-positive and gram-negative); the ability to sporogenesis. All microorganisms are divided into aerobes and anaerobes by physiological properties.

By the type of respiration, microorganisms are classified into:

1. *Obligate aerobes* are microorganisms that require 21% oxygen for optimal growth. These include the agents of tuberculosis, plague, cholera vibrios. Usually they grow in the form of a membrane on the surface of liquid nutrient media;
2. *Obligate anaerobes* are bacteria that grow in the absence of free molecular oxygen due to fermentation processes. They produce oxygen from organic compounds during their metabolism. Some of them do not tolerate even a small amount of free oxygen. Such bacteria are the agents of tetanus, botulism, gas anaerobic infection, bacterroids, fusobacteria and others. Some clostridia can be aerotolerant. To

cultivate them special nutrient media and apparatus (anaerostats) in which anaerobic conditions are created are used;

3. *Optional anaerobes (optional aerobes)* have adapted, depending on environmental conditions (the presence or absence of oxygen), to switch their metabolic processes using molecular oxygen for fermentation and vice versa. The group of optional anaerobes is formed by numerous representatives of the family of intestinal bacteria (Escherichia, Salmonella, Shigella), staphylococci and others bacteria;
4. *Microaerophils*is a special group of microbes for which the oxygen concentration during cultivation can be reduced to 2%. Higher concentrations are capable of holding back growth. This group is represented by lactic acid, nitrogen-fixing bacteria;
5. *Capneic* is a microorganism that in addition to oxygen requires up to 10% of carbon dioxide. Typical representatives are bovine brucellosis pathogens.

By the form, microorganisms are divided into three groups: spherical (cocci), rod-shaped and spirals. The spherical ones: monococci (placed individually), micrococci (small), diplococci (two microorganisms), tetracocci (four), sarzines (packages of 8-16 and more), streptococci (in the form of chains) and staphylococci (the accumulation of microorganisms in the form of grapes). (Fig.2).

The rod-shaped forms are divided into: bacteria - rods, bacilli - spores with rodss and clostridia - rodss with spores, the diameter of which exceeds the diameter of the microorganism. They are placed solely, in pairs, in chains, at an angle or in clusters. The ends of the rods may be rounded, rectangular, pointed or thickened. The spores are in the center of the cell, closer to the end (subterminally) or at the end (terminal). Some bacteria are branched, others looklike interwoven threads.

The tortuous forms of bacteria are divided into three groups: coma-shaped vibrios, spirals having 2-4 large curls and spirochetes having more than 5 curly, curvaceous forms.

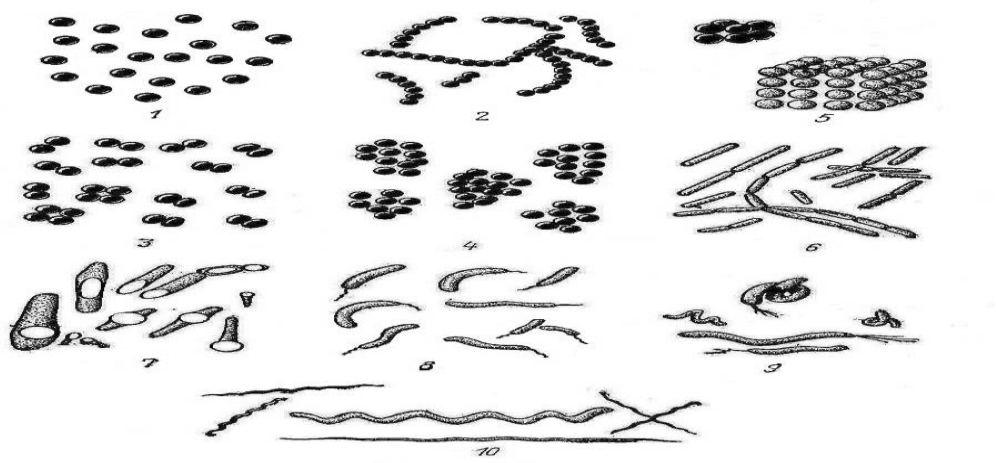


Fig. 2Forms of bacteria

Spherical (cocci):	**Rod-shaped:**	**Tortuous:**
1- monococci	6- bacteria (rod without spores)	8- vibrios
2- streptococcus		9- spirilla
3- diplococci and tetracocci	7- bacilli, clostridia	10- spirochetes
4- staphylococci	(rod with spores)	
5- Sarcina		

There are other forms of bacteria that do not meet the criteria of the main morphological groups described above. *Mycobacterium tuberculosis, M. leprae* - straight or curved rods, sometimes filamentous or mycelium-shaped, can be distinguished as a separate morphological group. The last two structures easily break down into rods or cocci. There are bacteria which are called filamentous. Filamentous bacteria are mainly rod-shaped unicellular and multicellular organisms, their filaments are formed by many cells connected by mucus, covers, plasmodesmus and so on.

Bacteria that look like a closed or open ring (toroidal shape), a regular hexagonal star, a triangle, flat square plates, dumbbells and bacteria that form outgrowths - prostheca. These are mainly unicellular organisms of triangular or other shape. Ray symmetry is revealed in some of them. They are motionless, they do not produce spores. The shape of prokaryotic cells is determined by the solid (rigid) shell. The form is a permanent feature for

most bacterial cells. In the cycle of development of a number of bacteria there is a change in the shape of the cells, for example in members of the genus *Arthrobacter.*

Mycoplasma and L-forms of bacteria do not have a dense shell but surrounded by a membrane, have the ability to take different forms, that is, pleomorphism istheir distinctive feature. In many cases, a form depends on the environment of the microorganism. Many types of bacteria grown on artificial nutrient media are significantly different from those found in humans or animals (causative agent of anthrax, etc.).

Bergey's Classification of Bacteria is also common. All bacteria are united in the kingdom of Procariotae, which is divided by the structure of the cell wall and Gram staining into 4 divisions: *Gracilicutes* (gram-negative); *Firmicutes* (gram-positive), *Tenericutes* (do not have cell wall) (mycoplasma), *Mendisicutes* (archebacteria (they are non-pathogenic).

The Division Gracilicutes combines bacteria, oval, straight or curved rods, spirals, long filaments that are stained gram-negatively. They multiply mainly by binary division, budding, multiple internal divisions with the formation of small spherical cells (beocytes (nanocytes).

Motile and fixed microorganisms are distinguished. The organs of motion of the bacteria are flagella, which look like spirally curved filamentous formations and, due to the helical rotation, cause chaotic movement forward, although capable of taxis. They are derivatives of the cytoplasmic membrane, departing from its basal body and consist of subunits of flagellin protein, which is a complete antigen. They are built of spiral filament, hook and basal corpuscle they look like thin (diameter 12–18 nm) and long cylinders, which can sometimes significantly exceed the length of the microorganism (from 10 to 20 microns, and in some cases up to 80-90 microns). Therefore, they are not visible in a simple light microscope and for staining methods of impregnation are used aimed at layering paints or reagents to artificially increase the diameter. The number of flagella and their location are characteristic of certain microorganisms.

Depending on this, the bacteria are divided into: Lophotrichous having one polar flagellum, Amphitrichous have flagellates at 2 edges of the cell (one or more); Lophotrichous - a bundle of flagella is located at one end of the cell; *Peritrichous* - flagella are located on the entire surface of the cell (Fig. 3).

To determine the movement of germs, young 18-24 hour broth cultures or agar condensates are used; and washedout in saline from the surface of the daily cultures of beef-extract agar.

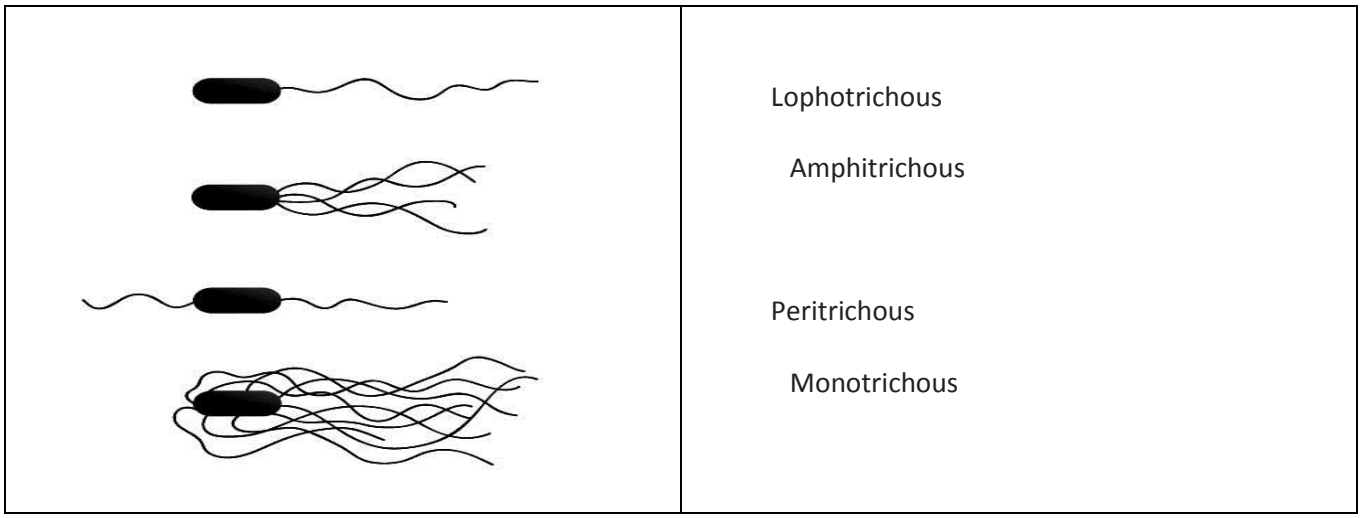


fig. 3. **Flagella of bacteria**

This division includes 1-16 of 35 systematic groups:
Group 1. Spirochetes;
Group 2. Aerobic (microaerophilic, motile, tortuous) viral gram negative microorganisms;
Group 3. Fixed (sometimes motile) grams-negative curved bacteria
Group 4. Gram-negative aerobic rods and cocci;
Group 5. Optional anaerobic Gram-negative rods;
Group 6. Gram-negative, anaerobic, straight, curved and spiral bacteria;
Group 7. Bacteria that carry out the dissimilative reduction of sulfur or its sulfate;

Group 8.Anaerobic Gram-negative Cocci;
Group 9. Rickettsia and chlamydia;
Group 10. Anoxygenic phototrophic bacteria;
Group 11. Oxygenic phototrophic bacteria;
Group 12. Aerobic chemolithotrophic bacteria and related microorganisms;
Group 13. Bacteria that bud or / and grow;
Group 14. Bacteria that have a cover;
Group 15. Non-photosensitive gliding bacteria that do not form fruiting bodies;
Group 16. Sliding bacteria that form fruiting body: myxobacteria.

The Firmicutes division includes eubacteria, asporogenic and spore-forming bacteria, actinomycetes, which are stained gram-positive. Systematic groups**:**

Group 17. Gram-positive cocci.
Group 18. Gram-positive rods and cocci forming endospores;
Group 19. Gram-positive rods of the correct form that do not form spores;
Group 20. Gram-positive rods of irregular shape donotform spores;
Group 21. Mycobacteria;
Group 22–29. Actinomycetes.

The Tenericutes division includes eubacteria (mycoplasmas) that do not have a cell wall, and the cytoplasm is surrounded by a membrane only. Polymorphic, predominantly fixed, gram-negative cells that multiply by budding, binary division, filamentous fragmentation, or by morphological transformations. The department includes only one group:

Group 30. Mycoplasma (mollicutes).

The Mendisicutes Division combines prokaryotes in the cell wall of which peptidoglycan is absent. Gram-positive bacteria are different in shape (rod-shaped, spherical, tortuous). Thermoresistant (grow at temperatures above 100 ° C and multiply in hypersaline water) are distinguished. Groups 31-35 include:

Group 31. Methanogens;
Group32. Sulfate-reducing archaea;
Group 33. Extreme halophilic aerobic archebacteria (halobacteria).
Group 34. Archebacteria devoid of cell wall.
Group 35. Extreme thermophiles and sulfur metabolizing hyperthermophiles.

The Gracilicutes division combines 3 classes:

1 class – *Scotobacteria* – bacteria that do not require light, there is no photosynthesis process;
2 class – *Oxyphotobacteria* – bacteria that require light and release oxygen;
3 class – *Desoxyphotobacteria* – bacteria that require light and do not release oxygen.

Classification of infectious microorganisms (**WHO**) by groups of risk:

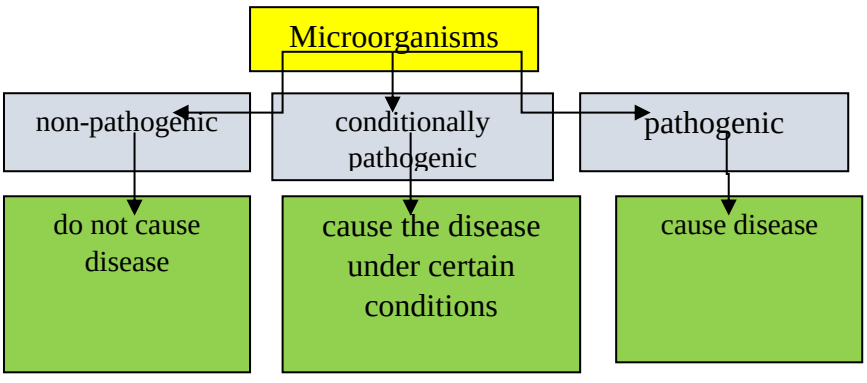
Risk group I (*low individual and social risk*). Microorganisms are not known as etiologic agents of human or animal diseases (*Bacillus subtitles*);

Risk group II (moderate individual and limited social risk). A pathogenic agent that can cause disease of humans or animals, however, does not pose a serious risk to laboratory personnel, society, domestic animals, or the environment. Incautious work in the laboratory can cause an infection that can be eliminated by therapeutic and prophylactic agents and the risk of its spread is limited (*Salmonella typhymurium*);

Risk group III (high individual risk and low social risk). The pathogenic agent causes serious diseases of humans, however, as a rule it does not spread from the patient to the healthy person (*Brucella, Lassa virus*);

Risk group IV (high individual and social risk). The pathogenic agent causes serious diseases of humans or animals and spreads easily from the patient to the healthy people (*Ebola-Marburg virus, FMD virus*).

Microorganisms are divided by pathogenicity (Fig: 4).



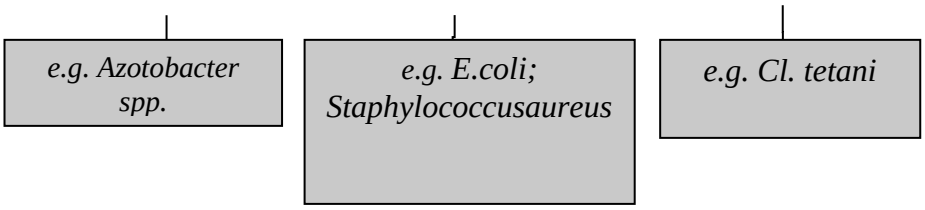


Fig. 4 Classification of microorganisms by pathogenicity

In the CIS countries, pathogens are classified into 4 groups according to their degree of danger:

Group I - agents of especially dangerous infections (glanders, plague, anthrax, black pox);

Group П – pathogens of highly contagious epidemic bacterial, viral, rickettsial, fungal diseases (brucellosis);

Group III – pathogens of bacterial, viral, rickettsial, fungal, protozoal infectious diseases, which are isolated in independent nosological forms (diphtheria);

Group IV – pathogens of bacterial, viral, fungal septicemia, meningitis, pneumonia, enteritis, toxic infections, acute bacterial poisoning.

2.4 Different milieu of culture. Peculiarities of microorganisms' growth on culture media

2.4.1 Types of culture media

There are two main types of culture media:

- synthetic ground, the main components of which are well known (for example, glucose-salt breeding ground);
- empirically selected breeding ground of natural origin, the content of which is not known exactly (for example, peptones prepared from partially hydrolyzed protein).

The choice of the nutrient medium depends on the purpose of the experiment.

Practical inconvenience for the use of synthetic nutrient media:

- Microorganisms grown on such culture media differ in the phenotype from microorganisms grown on natural culture media (e.g, composition and division rate).
- the reproduction of microorganisms in such media is easily suppressed by excessive aeration or toxic cations; microorganisms are also more sensitive to imbalance between some of the constituent parts of the nutrient medium, especially amino acids.

Synthetic culture media made up of chemical compounds is used primarily for the study of metabolism in a microbial cell. Culture media made from products of plant and animal origin, despite the fact that they can not be standardized and have no definite chemical composition are commonly used in applied microbiology.

2.4.2 Requirements for culture media

Culture media must:

- Keep nutrients necessary for the nutrition of the microbe.
- Have a pH reaction that is optimal for the grown of the microbe.
- Have sufficient moisture, since microbes feed on the laws of diffusion and osmosis.
- Have isotonicity.
- Be sterile that is necessary to ensure the possibility of growing pure cultures of microbes.

The composition of the grounds used for bacteriagrowth includes nitrogen, carbon, hydrogen, oxygen, phosphorus, potassium, sulfur, sodium, magnesium and microelements such as iron, cobalt, iodine, manganese, boron, zinc, molybdenum, copper and others that are necessary for construction of proteins of the cytoplasm. These elements should be in the breeding ground in compounds available for microorganisms, and the requirements of different microbes to them are not the same. The need for oxygen and water bacteria are met mainly through the flow of water into their cell.

Pathogenic microorganisms can absorb nitrogen from simple ammonium compounds, others require amino acids, and others split macromolecular substances , so called peptones, which are products of incomplete enzymatic digestion of proteins. Strictly parasitic species of bacteria multiply only if there is native protein.

The source of carbon for pathogenic bacteria is the various carbohydrates, polyhydric alcohols, organic acids and their salts.

Mineral elements of bacteria are obtained by adding salts such as NaCI, KH_2PO_4, K_2HPO_4, etc. to the breeding ground.

The microelements acting as catalysts of chemical processes, enter the breeding ground with peptone, inorganic salts and water.

In addition to organic and inorganic elements, bacteria need growth drivers. They fall into the culture media from products of plant and animal origin, containing nicotine, pantothenic, parabenzoic acids, vitamins A, B, C, and others.

Nutrients can be digested by microbes only after a certain reaction of the breeding ground since the permeability of the shells of microbial cells varies depending on the pH of the medium. Due to different requirements for nutrients and pH of the media for different types of microbes, the possibility of creating a universal breeding ground is ruled out.

2.4.3 Culture media of general and special purpose

To prepare breeding ground use:

- products of animal origin (meat, casein, milk, eggs, blood and others);
- products of plant origin (potatoes and others);
- organic and inorganic compounds of a certain chemical composition.

Dense and liquid culture media are distinguished. Dense nutrients are prepared from the liquid by adding glue substances to them such as agar or gelatin. Agar-agar (from Malay-Algae) is a product of plant origin derived from seaweed. Agar-agar dissolves at a temperature of 80-86 °C, sets at 36-40 °C in water, Gelatin is a substance of the protein nature of animal origin. In warm water at a temperature of 32-34 °C it swells and dissolves, and at a lower temperature it becomes a jelly. However, at pH below 6,3 and above 7,0, the density of gelatin decreases and it does not set well.

The ability of agar and gelatinous grounds to maintain a density of 37 °C made it possible to grow pathogenic microbes in optimal temperatures on dense grounds.

All culture media are divided into general and special ones.

General culture media include meatpeptone bouillon, meatpeptone agar, meatpeptone gelatin. General culture media are used to grow many pathogenic microbes and are used as a basis for the preparation of special grounds by adding to them blood, sugar, milk, whey and other ingredients necessary for the reproduction of this or that type of microbe.

Special nutrient media include selective and differential-diagnostic grounds.

Selective grounds. The principle of creating a selective nutrient medium is based on providing the basic biochemical and energy needs of the type of microbe that they are intended to cultivate. A certain composition and concentration of nutrient microelements, growth factors and the corresponding pH provide optimal conditions for the growth of one or more types of microorganisms. When plate on them a material containing a mixture of different microorganisms, the growth of the species for which the medium has been elected would be the best.

Differential-diagnostic grounds.

Differential and diagnostic nutrient media are used to determine the species of the studied microbe, based on the features of its metabolism. Differential-diagnostic culture media are divided into:

- ✓ media for the detection of proteolytic and hemolytic ability of microbes containing protein substances: blood, milk, gelatin, coagulated blood serum, and so on;
- ✓ media with indifferent chemical substances which serve as a source for nutrition of certain types of microbes and are not absorbed by other species;
- ✓ media with carbohydrates and polyhydric alcohols to detect appropriate enzymes;
- ✓ media for determining the reduction capacity of microbes.

Indicators such as neutral red, methylene blue, litmus, asid fuchsin, bromotymol blue, aqueous blue dye and azole acid are introduced into the composition of differential diagnostic grounds for the detection of sugrolytic and oxidative-reducing enzymes. Changing its color at different values of pH, the indicator shows presence or absence of splitting, oxidation or recovery of the ingredient introduced into the media. However, the indicator is not an

obligatory part of enzymes' detection media. Thus, the presence of gelatinase and other proteolytic enzymes in the culture is determined by the dilution of gelatin, coagulated egg or whey protein.

Dry breeding ground.

Preparation of culture media is one of the most critical and difficult areas of bacteriological laboratory work. In this regard, the biological industry produces standard, canned, dry nutrient media for the cultivation of microorganisms.

Preparation of general nutrient media

The basis for the preparation of general nutrient media is meat water containing extractive substances.

Meat water

1. 100 g of fresh lean beef or veal are freed from bones, fat and tendons, passed through a meat grinder, stuffed with 1 liter of tap water, stir well. Leave for a day in a cool place or placed for 2 hours into the thermostat.

2. The meat mass is pressed through the gauze, boiled for 5 minutes. For coagulation the proteins are cooled. Filter through a cotton filter, add water to the original volume. Meat water is poured into bottles, sterilized for 20-30 minutes at 120 °C and stored in a dark place. It is transparent yellowish liquid of a weakly acidic reaction (pH 6,2-6,4), without proteins. Meat water contains a small amount of amino acids, salts, carbohydrates, growth factors and extractives. Dry peptone which is the primary product of the protein hydrolysis and consists of a mixture of polypeptides and amino acids obtained by peptic or trypthic digestion is added to the meat water for preparation of the usual nutrient medium.

Fibrin, blood and other waste are used for the production of dry peptone at meat-processing plants. The peptone is dried in a spray vacuum dryer. A liquid peptone can be prepared in the laboratory by peptic digestion of proteins.

Martin's Peptone

Pork stomachs (not washed with water) with abundant mucous layer are cleaned of membrane, fat and passed through a meat grinder. Add water and hydrochloric acid to the farcemeat in a ratio: 250 g of farcemeat from pork stomachs, 1000 ml of tap water heated to 50 °C, 10 ml of hydrochloric acid (specific gravity 1,18). The mixture is kept in a thermostat at 50 °C. After 24 hours, the peptone is heated in an autoclave at 100°C and filtered, 10% NaOH solution to an alkaline reaction of the litmus is added to the filtrate. After that, the filtrate is poured into the flasks and sterilized at 115 °C for 30 minutes.

Meat peptone broth

To prepare the meat peptone broth, Martin's Peptone is mixed with an equal volume of meat water, set the required reaction, boiled for 30 minutes, filtered and sterilized at 115 °C for 30 minutes.

With the use of trypsin for the digestion of meat Hottinger's broth is obtained. Such digestion couses more complete use of meat proteins, which split up into peptones, polypeptides and free amino acids. During the tryptic digestion of meat, it is received 10 times more broth than the usual method.

Hottinger's broth

Peeled meat is cut into small pieces, placed in a saucepan with boiling water (ratio 1:1.5) and boiled for 15-20 minutes, then the meat is taken out of the liquid and passed through mincing-machine. Liquid is cooled down to 40-45 °C, pH is 8, and the water is poured onto minced meat. To the resulting mixture add dry pancreatin in the amount of 0.5-1% or pancreas at 100 g of gland per 1 liter of fluid. Then, the mixture is added with alkaline up to pH 8 and poured into a bottle. Then add 2-3% of chloroform, closing the bottle with a rubber stopper, shake the contents several times. The booth is placed for 7-14 days at a temperature of 37 ° C.

As a result of digestion, minced meat is converted into a homogeneous grayish precipitate, and completely clear straw-yellow liquid is over it. A good quality Hottinger'sbrothgives a positive reaction to tryptophan and contains 560-860 mg% of amine nitrogen. To prepare the broth, add 6 to 7 parts of water to 1 part of the broth.

Tryptich Hottinger's broth

This medium contains a large number of amino acids, therefore, increases its buffering, and due to this the indicator of the active reaction of the medium is more stable.

To make a broth take one kilogram of meat without tendons and fat, cut into small pieces app. 1-2 cm, put in a pan with a double volume of boiling water, and boil for 15-20 minutes, till meat becomes gray, indicating coagulation of proteins. It is removed from the liquid and passed through a meat grinder. In the remaining liquid, set the pH 8.0, put minced meat there and cool it to 40 °C. Then add 10% (to the volume of liquid) of the fresh pancreas, pre-purified from the connective tissue, fat and twice passed through a meat grinder. Instead of the gland use a dry pancreatin (0.5%). The resulting mixture is thoroughly shaken and adjusted to pH 7.8-8.0. 30 minutes later check the pH. If the active reaction of the medium does not change to the acid this indicates the poor quality of the

enzyme. When the pH of the medium stabilizes, the mixture is poured into large bottles, filling them by 1/3. Add up to 3% chloroform, cover the dishes with rubber corks and shake vigorously to mix the liquids. Deflate chloroform excess. 1-2 hours later check again the pH of the media, setting it at 7.4-7.6. The resulting mixture is left at room temperature for up to 16 days. During the first 3-4 days, check daily and adjust the pH of the medium, and shake the vials at least 3 times a day. Later, this procedure can not be carried out and shake the medium not so often. 1-2 days before the end of the cycle of digesting the shaking of the medium is stopped.

Completed quality digestion is evidenced by the enlightenment of the liquid, which turns a straw-yellow color, as well as the formation at the bottom of a dustlike precipitate. The liquid is easily filtered, it is checked for tryptophan using a bromine water sample (3-4 drops of bromine water is added to 3-4 ml of filtrate). If thre is tryptophan (up to 2.0-3.0 g / l) the color of the medium changes to pink-purple. Determine the total nitrogen, which normally reaches 11.0-12.0 g / l, and amine nitrogen (up to 7.0-9.0 g / l).

The hydrolyzate is filtered through a paper or cloth filter, bottled and autoclaved at 120 ° C for 30 min. Like this it can be stored for a long time.

Tryptic Hottinger's digestion is used to obtain the Hottinger's broth. For this, 800-900 ml of distilled water, 0.5% sodium chloride and 0.2% monosubstituted sodium phosphate are added to 100-200 ml of hydrolyzate. Adjust the pH to 7.4-7.6, pour into vials and sterilize for 20 minutes at 120 °C.

2.4.4 Microorganisms growth on culture media

Microorganisms on dense culture media grow in the form of colonies that differ in: (table. 1).

Differentiation of colonies on the basis of growth on culture media

Colonies	A feature of growth in culture media
Size (diameter)	big (4-6 mm and more),
	medium (2-4 mm),
	small (1-2 mm),
	tiny or dotty (less than 1 mm).
Form of a colony	correctly round,
	wrong (amoeboid),
	rhizoid.
Transparency	transparent transmitting light,
	turbid.
Relief and contour of the form in vertical section	flat,
	convex,
	domed,
	droplike,
	conical,
	flat convex,
	flat on the surface of the medium,
	with a impress center,
	with raised center in the form of a nipple.
Surface	matte or shiny,

	with gloss, dry or wet, smooth or rough. Smooth colonies are referred as S-forms and rough - R-forms. The shape of rough surfaces can also be varied: wrinkled, warty, shagreen, having a radial tinge and others.
Color of the pigment that bacteria synthesizes	white, cream, yellow, gold, blue, red, and more.
Consistency (determined by contact with the colony by a bacteriological loop)	pasty, viscous or mucous, dry, fragile

In liquid culture media the following cultural characteristics are taken into account:

- surface growth character - the presence of a wall ring, mesh, delicate, dry, wrinkled, mucous or coarse membrane;
- intensity of turbidity - weak, moderate, strong;
- volume and structure of the presipitate is significant and insignificant;
- if there is presipitate, its structure is registered - granular, brittle, powdery, spongy, mucous, in the form of a wad of cotton wool, etc.),
- change of the medium color - not changed, changed when the pigment is formed.

2.5 Analytical technics and their detection principles

Methods of analytical chemistry can be classified based on various principles.

1) Depending on the mass of the substance that is used for analysis such methods are distinguish:

- macro methods where at least 0.1 g of substance are required for analysis;
- semi-micromethods where 0.1-0.01 g of substance are required;
- micromethods where 10-2 - 10-3 g of substance are required;
- ultramicromethods where ~ 10-6 g of substance is required;
- submicromethods, where ~ 10-9 g of substance is required.

2) Depending on the type of analysis, separation methods and determination methods are distinguished.

In *separation methods* the main task is to separate the interfering components or to isolate the component to be determined in a form suitable for quantification.

In *determination methods*, the content of the analyzed component is found in the sample without prior separation.

3) Chemical and physico-chemical methods of analysis are distinguished, sometimes physical methods are distinguished as a separate group.

Chemical methods include gravimetric and titrimetric methods, physicochemical (instrumental) methods include spectrophotometric, electrochemical, chromatographic, etc.

2.5.1 Gravimetric analysisis the extraction of a substance in its pure form and its weighing. Extraction is carried out by precipitation or isolated in the form of a volatile compound (distillation method).

The essence of the method: mass is the analytical signal in gravimetry. The mass is found by comparing with another known mass using weights.

Principle of the method: mass is the analytical signal in gravimetry. The mass is found by comparing with another known mass with a help of weights.

The gravimetric method is used for:

- determination of inorganic substances (inorganic cations, anions, neutral compounds of I_2, H_2O, CO_2, SO_2 type can be quantified). In agronomy, the phosphorus content in phosphorus fertilizers and soils is determined (in this case, PO^{3+} is precipitated in the form of NH_4MgPO_4 salt, which turns into magnesium pyrophosphate $Mg2P2O_7$ after calcination). Crystallization water in salts, hygroscopic water in the soil, fertilizers, and plant material are determined by the distillation method. During distillation method, a specific component is isolated in the form of a volatile compound by the action of temperature and acid. Dry matter content in fruits, vegetables, fiber, "raw" ash in plant material are determined.

- determination of organic substances has limited use (for example, salicylic acid is determined by reaction with iodine: the yellow precipitate is filtered off, dried, weighed).

Methods of taking an average sample depend on the characteristics of the analyzed material and on the purpose of the determination. In production it is necessary to determine the average chemical composition of a large batch of heterogeneous material (pesticide, fertilizer, soil).

2.5.2 The titrimetric analysisis based on the accurate measurement of the amount of reagent used on the reaction with the analyte. The following definitions will be required to review the material in this chapter.

Titrated or standard solution is a solution which concentration is known precisely.

Titration is adding titrated solution to the analyzed one to determine exactly the equivalent amount.

The titration solution is often called a *working solution or titrant*. For example, if an acid is titrated with alkali, the alkali solution is called a titrant.

The moment of titration, when the amount of titrant added is chemically equivalent to the amount of titrated substance, is called the *equivalence point.*

Not all chemical reactions can be used in titrimetric analysis. The reactions used in titrimetry must satisfy the following basic requirements:

1) the reaction should proceed quantitatively, i.e. the equilibrium constant of the reaction should be sufficiently large;
2) the reaction must proceed at a high speed;
3) the reaction should not be complicated by the occurrence of side processes;
4) there must be a way to determine the end of the reaction.

If the reaction does not satisfy at least one of these requirements, it cannot be used in titrimetric analysis.

In direct titration methods, the analyte directly reacts with the titrant. One working solution is sufficient for analysis by this method.

In the methods of reverse titration (or as also they are called methods of titration by residue), two titrated working solutions are used: the main and auxiliary. For example, it is widely known the reverse titration of chloride ions in acidic solutions. A deliberate excess of the titrated silver nitrate solution is added firstly to the analyzed chloride solution (basic working solution). In this case, the reaction of formation of oil-soluble silver chloride: Ag + + Cl- → AgCl. The excess unreacted AgNO3 is titrated with a solution of ammonium thiocyanate (auxiliary working solution): Ag + + SCN- → AgSCN. The chloride content can be calculated as the total amount of substance (mol) in the solution and the amount of $AgNO_3$ substance that did not react with chloride are known.

The third main type of titrimetric determination is titration of a substituent (substitution titration, indirect titration). In this method, a special reagent is added to the analyte, which reacts with it. One of the reaction products is titrated with a working solution. For example, with iodometric determination of copper, a known excess of KI is added to the analyzed solution. Reaction occurs

2Cu2+ + 4I- → 2CuI + I2.

The evolved iodine is titrated with sodium thiosulfate.

2.5.3 Spectrophotometric analysisrefers to absorption methods, i.e. based on measuring light absorption by a substance.

It includes spectrophotometry, photocolorimetry and visual photometry, which is commonly called colorimetry.

Each substance absorbs emission with specific (characteristic only for it) wavelengths, i.e. the wavelength of the absorbed emission is individual for each substance, and a qualitative analysis of light absorption is based on this.

The basis of quantitative analysis is the Beer–Lambert–Bouguer law:

$A = e\, l\, c$

where $A = -\lg (I / I_0) = -\lg T$ – optical density;

I_0 and I – the intensity of the light beam directed to the absorbing solution and passing through it;

c – concentration of the substance, mol/l;

l – light absorbing layer thickness;

e - molar light absorption coefficient;

T - transmittance.

The following methods are most often used to determine the concentration of an analyte: 1) molar light absorption coefficient; 2) calibration graph; 3) additives; 4) differential photometry; 5) photometric titration.

Method of molar absorption coefficient. When working on this method, the optical density of several standard solutions of A_{ct} is determined, for each solution, $e = A_{ст} /(lc_{ст})$ is calculated and the obtained value of e is averaged. Then optical density of the analyzed solution $A_x$is measured and concentration of c_x is calculate by the formula

$c_x = A_x /(el)$.

A limitation of the method is the obligatory submission of the analyzed system to the Beer–Lambert–Bouguer law, at least in the area of the studied concentrations.

Calibration curve method. A series of dilutions of the standard solution is prepared, their absorption is measured, and a graph is constructed in the coordinates $A_{ст} – C_{ст}$. Then, the absorption of the analyzed solution is measured and its concentration is determined by the curve.

Spike test. This method is used in the analysis of solutions of complex composition, since it allows you to take into account the influence of the "third" components automatically. First, the optical density A_x is determined for the analyzed solution containing the determined component of unknown concentration c_x, and then a known amount of the determined component (c_{ct}) is added to the analyzed solution and the optical density A_{x+st} is measured again.

The optical density A_x of the analyzed solution is equal to

$A_x = e\, l\, c_x$,

and the optical density of the analyzed solution with the addition of standard

$A_{x+ст} = e\, l\, (c_x + c_{ст})$.

The concentration of the analyzed solution is found by the formula

$c_x = c_{ст}\, A_x / (A_{x+ст} – A_x)$.

Differential photometry technique. If in ordinary photometry the intensity of light passing through the analyzed solution of unknown concentration is compared with the intensity of light passing through the solvent, in differential photometry the second light beam does not pass through the solvent, but through a colored solution of known concentration, so-called comparison solution.

The photometric technique can also determine the components of a mixture of two or more substances. These definitions are based on the additivity property of optical density:

$A_{см} = A_1 + A_2 + ...+ A_n$

where $A_{см}$ is the optical density of the mixture; A_1, A_2, A_n - optical densities for various components of the mixture.

Photometric analysis methods are used to control a variety of production processes. These methods can be used to analyze large and low contents, but their especially valuable feature is the ability to determine impurities (up to $10^{-5}...10^{-6}$%). Methods of absorption spectroscopy are used in the chemical, metallurgical, pharmaceutical and other industries, as well as in medicine and agricultural production.

The industry produces instruments for absorption spectroscopy: colorimeters, photometers, photoelectrocolorimeters, spectrophotometers, etc., which use various combinations of illuminators, monochromators and light detectors.

2.5.4 Electrochemical methods of analysisand research are based on the study and use of processes occurring on the surface of the electrode or in the electrode sheath. Any electrical parameter (potential, current, resistance, etc.), related to the concentration of the analyzed solution functionally and amenable to proper measurement, can be an analytical signal.

Direct and indirect electrochemical methods are distinguished. Dependence of the current strength (potential, etc.) on the concentration of the determined component is used in direct methods. Current strength (potential, etc.) is measured in order to find the end point of titration of the component to be determined with a suitable titrant in indirect methods, i.e. use the dependence of the measured parameter on the titrant volume.

For any kind of electrochemical measurements, an electrochemical circuit or an electrochemical cell is required, analyzed solution is a component of it.

Potentiometric methods are based on measuring the potential difference between the indicator electrode and the reference electrode or *the electromotive forces* (EMF) of various circuits, since EMF that is the potential difference that is experimentally measured.

The equilibrium potential of the indicator electrode is associated with the activity and concentration of substances with the *Nernst equation* involved in the electrode process:

$$E = E° + R\,T/(n\,F)\,\ln\,(a_{oxide}\backslash a_{reduced})$$
$$E = E° + RT\,/(nF)\,\ln\,(\text{oxide}\,\gamma_{oxide}/(\text{reduced}\,\gamma_{reduced})),$$

R is the universal gas constant equal to 8.31 J / (mol. K); *T* is the absolute temperature; *F*- Faraday constant (96500 C/mol); *n* is the number of electrons participating in the electrode reaction; a_{oxide}, $a_{reduced}$ are activities of the respectively oxidized and reduced forms of the redox system; [oxide] and [reduced] are their molar concentrations; γ_{oxide}, $\gamma_{reduced}$ are activity coefficients; E° is the standard potential of the redox system.

Substituting T = 298.15 K and the numerical values of the constants in the equation, we obtain:

$$E = E° + (0{,}059\,/\,n)\,\lg\,(a_{oxide}\backslash a_{reduced})$$
$$E = E° + (0{,}059\,/\,n)\,\lg\,(\text{oxide}\,\gamma_{oxide}/(\text{reduced}\,\gamma_{reduced})$$

Methods of direct potentiometry are based on the application of the Nernst equation to find the activity or concentration of a participant in an electrode reaction from an experimentally measured EMF circuit or electrode potential. pH determination method is The most widely used method among direct potentiometric methods, but the creation of reliable ion-selective electrodes has significantly expanded the practical possibilities of direct methods. The pH is also measured by potentiometric titration.

A glass electrode is most often used to determine pH. The main advantages of the glass electrode are ease of use, rapid equilibrium and the ability to determine pH in redox systems. The disadvantages are the fragility of the electrode material and the complexity of the work when switching to strongly alkaline and strongly acidic solutions.

Besides the concentration of hydrogen ions, the direct potentiometric method with ion-selective electrodes can determine the content of several dozens of different ions.

Potentiometric titration is based on determining the equivalence point from potentiometric measurements. Near the equivalence point, a sharp change (jump) in the potential of the indicator electrode occurs. As in other titrimetric methods, potentiometric titration reactions must proceed strictly stoichiometrically and have a high speed and go till the end.

A chain is assembled from the indicator electrode in the analyzed solution and the reference electrode for potentiometric titration. Calomel or silver chloride electrodes are most often used as reference electrodes.

The type of indicator electrode used for potentiometric titration depends on the properties of the titrimetric mixture and its interaction with the electrode. A glass electrode is used in acid-base titration; an inert (platinum) electrode or an electrode reversible with respect to one of the ions contained in the titrimetric mixture is used in redox; a silver electrode is used in sedimentation;a metal electrode reversible to the titratable metal ion is used in complexometric.

To find the equivalence point, a differential curve is often constructed in the coordinates $\Delta E/\Delta V - V$. The maximum of the obtained curve indicates the equivalence point, and the reading along the abscissa corresponding to this maximum gives the volume of titrant spent on titration to the equivalence point. The determination of the equivalence point to the differential curve is much more accurate than the simple dependence *E - V*.

The main advantages of the potentiometric titration method are its high accuracy and the ability to carry out determinations in dilute solutions, in turbid and colored media, and also to determine several substances in one solution without prior separation. The scope of the practical application of potentiometric titration with the use of non-aqueous solvents is significantly expanding. They make it possible to analyze multicomponent systems that cannot be determined in an aqueous solution, to analyze substances that are insoluble or decomposable in

water, etc. Potentiometric titration can easily be automated. The industry produces several types of autotitrators using potentiometric sensors.

The disadvantages of potentiometric titration include the not always quick establishment of the potential after the addition of titrant and a large number of readings during titration.

In potentiometric analysis, the main measuring instruments are various types of potentiometers. They are designed to measure the EMF of the electrode system. Since the EMF depends on the activity of the corresponding ions in the solution, many potentiometers can also directly measure pX , i.e. the negative logarithm of the activity of ion X. Such potentiometers with the corresponding ion-selective electrode are called ionomers. If the potentiometer and electrode system are designed to measure the activity of hydrogen ions only, the device is called a pH meter.

2.5.5 Chromatographic analysis methods

Chromatography is a process based on the multiple repetition of the acts of sorption and desorption of a substance when moving it in a flow of a mobile phase along a stationary sorbent. The separation of complex mixtures by a chromatographic method is based on various adsorbability of the mixture components.

So-called mobile phase (eluent) containing the analyzed sample moves through the stationary phase during chromatography. Typically, the stationary phase is a substance with a developed surface, and the mobile phase is a stream of gas or liquid, filtered through a layer of sorbent. In this case, a multiple repetition of the acts of sorption-desorption occurs, which is a characteristic feature of the chromatographic process and determines the effectiveness of chromatographic separation.

Qualitative chromatographic analysis, i.e. the identification of a substance by its chromatogram can be performed by comparing the chromatographic characteristics, most often the *retained volume* (i.e., the volume of the mobile phase passed through the column from the beginning of the mixture input until this component appears at the column exit), found under certain conditions for the components of the analyzed mixtures and for reference.

Quantitative chromatographic analysis is usually carried out on a chromatograph. The method is based on the measurement of various parameters of the chromatographic peak, depending on the concentration of chromatographic substances — height, width, area and retention volume, or the product of the retention volume by the peak height.

In quantitative gas chromatography, methods of absolute calibration and internal normalization, or scaling, are used. The internal standard method is also used. With *absolute calibration*, the dependence of the height or peak area on the concentration of the substance is experimentally determined and calibration graphs are constructed or the corresponding coefficients are calculated. Next the same characteristics of the peaks in the analyzed mixture are determined, and the concentration of the analyte is found from the calibration curve. This simple and accurate method is fundamental in determining trace amounts.

When using the internal normalization method, the sum of any peak parameters is taken as 100%, for example, the sum of the heights of all peaks or the sum of their areas. Then the ratio of the height of an individual peak to the sum of the heights or the ratio of the area of one peak to the sum of the areas when multiplied by 100 will characterize the mass fraction (%) of the component in the mixture. With this approach, it is necessary that the dependence of the measured parameter on concentration be the same for all components of the mixture.

2.5.6 Types of chromatographic methods

M. Tsvet formulated the law, which he called the law of adsorption substitution:

Substances dissolved in a certain liquid form a certain adsorption series A, B, C, ..., expressing the relative adsorption affinity of its members to the adsorbent. Each of the members of the adsorption series, having a greater adsorption affinity than the next one, displaces it from the compound and, in turn, is replaced by the previous one.

Thus, M. Tsvet considered the difference in adsorbability the main condition for the implementation of the chromatographic process - the process of separation of substances on a column.

In modern chromatography, in addition to molecular adsorption, other physicochemical phenomena are used to separate substances. There are several classifications based on various principles. The following are generally accepted.

According to the state of aggregation of the phases used. According to this classification, chromatography is divided into gas and liquid. Gas includes gas-liquid and gas-adsorption chromatography. Liquid chromatography is divided into liquid-liquid, liquid-adsorption and liquid-gel. The first word in this classification characterizes the state of aggregation of the mobile phase.

According separation mechanisms, i.e. by the nature of the interaction between the sorbent and the sorbate. According to this classification, chromatography is divided into the following types:

1. adsorption chromatography: separation is based on the difference in the adsorbability of the substances to be separated by a solid adsorbent;
2. distribution chromatography: separation is based on the difference in solubility of the substances to be separated in the stationary phase (gas chromatography) and on the difference in the solubility of the substances to be separated in the mobile and stationary liquid phases;
3. ion exchange chromatography: separation is based on the difference in the ability of the substances to be exchanged for ion exchange;
4. permeation chromatography: separation is based on the difference in size or shape of the molecules of the substances to be separated, for example, using molecular sieves (zeolites);
5. sediment chromatography: separation is based on the formation of precipitates of different solubility with a sorbent;
6. adsorption-complexation chromatography: separation is based on the formation of coordination compounds of various strengths in phase or on the surface of the adsorbent.

Very often the separation process proceeds according to several mechanisms.

According to the applied technique:

1. column chromatography: the separation of substances is carried out in special columns;

2. surface chromatography: a - paper - separation of substances is carried out on special paper; b - thin layer - the separation of substances is carried out in a thin layer of sorbent.

Any of the above mentioned separation mechanisms can be used in column and thin-layer chromatography; distribution and ion-exchange mechanisms are most often used in paper chromatography.

According to the method of relative phase, frontal or eluent and displacement chromatography are distinguished.

Frontal method. This is the simplest method of chromatography. Analyzed mixture is continuously passed through an adsorbent column, for example, components A and B in Solv solvent. In the solution flowing from the column, the concentration of each component is determined and a graph is drawing in the coordinates of the concentration of the substance — the volume of the solution passed through the column. This dependence is usually called a *chromatogram* or *output curve*.

Due to the sorption of substances A and B, Solv solvent will flow out of the column first, and then the solvent and the less adsorbed component A, then component B, and thus, after a while, the composition of the solution will not change when passing through the column. The method is used, for example, to purify a solution of impurities if they are sorbed better than the main component, or to isolate the weakliest sorbed substance from a mixture.

Elution development method. When using this method, a portion of the analyzed mixture containing the A and B components in the Solv solvent is introduced into the column, and the column is continuously washed with a carrier gas or Solv solvent. In this case, the components of the analyzed mixture are divided into zones: a well-sorbed substance B occupies the upper part of the column, and a less sorbed component A will occupy the lower part.

Component A first appears, then a pure solvent, and then component B in a gas or solution flowing out of the column.

The higher concentration of the component, the higher the peak and its larger area, which forms the basis of quantitative chromatographic analysis. The development method makes it possible to separate complex mixtures; it is most often used in practice. The disadvantage of this method is the decrease in the concentration of the outgoing solutions due to dilution with a solvent or carrier gas.

Displacement method. In this method, the analyzed mixture of components A and B in the Solv solvent is introduced into the column and washed with a solution of substance D (displacer), which is sorbed better than any of the components of the analyzed mixture.

The concentration of the solution during chromatography does not decrease, in contrast to the development method. A significant drawback of the displacement method is the possible superposition of the zone of one substance on the zone of another, since the zones of the components in this method are not separated by the solvent zone.

Development (eluent) analysis is most often used in chromatography, in this case, the peak in the coordinates of the concentration is observed, volume is called the *chromatographic peak and is characterized by height, width and area*.

In analytical practice, the method of gas-liquid chromatography (GLC) is widely used. This is due to the extreme variety of liquid stationary phases, which facilitates the selection of a phase selective for this analysis. It is

important to choose the stationary liquid phase correctly to ensure column selectivity. This phase should be a good solvent for the components of the mixture (if the solubility is small, the components leave the column very quickly), non-volatile (not to evaporate at the working temperature of the column), chemically inert, should have a small viscosity (otherwise the diffusion process will slow down) and when applied to the carrier to form a uniform film connected with it. The separation ability of the stationary phase for the components of this sample should be maximum.

Carriers of stationary liquid phases. Solid carriers for dispersing a stationary liquid phase in the form of a homogenous thin film should be mechanically strong with a moderate specific surface (about 20 m^2/g), small and identical particle size, and also sufficiently inert so that adsorption on the interface between solid and gaseous phases to be minimal. The weakest adsorption is observed on carriers of silanized chromosorbate, glass granule, and fluoropack (fluorocarbon polymer). In addition, solid carriers should not respond to temperature increase and should be easily wetted by the liquid phase. Silanized diatomite carriers — diamite silica, or kieselguhr are most often used as a solid support in gas chromatography of chelates.

Gas liquid chromatography (GLC) is one of the most advanced methods of multicomponent analysis. Its distinguishing features are expressivity, high accuracy, sensitivity, and the possibility of automation. The method allows to solve many analytical problems. Quantitative GLC analysis can be considered as an independent analytical method, more effective in the separation of substances belonging to the same class.

Liquid-liquid chromatography is close to gas-liquid one. A liquid phase film is also put on a solid support, and a liquid solution is passed through a column filled with such an adsorbent. This type of chromatography is called liquid-liquid distribution chromatography. The liquid deposited on the carrier is called the stationary liquid phase, and the solvent moving through the carrier is called the mobile liquid phase. Liquid chromatography is performed in a column (column version) or on paper (paper chromatography, chromatography on paper).

The separation of a mixture of substances in liquid-liquid chromatography is based on the difference in the distribution coefficients of the substance between immiscible solvents. The distribution coefficient of the substance is equal to:

$$K_{m/s} = c_m / c_s$$

Where c_m and c_s is concentration of the substance in the mobile and stationary phases.

For members of the same homologous series, some regularities in the values of $K_{m/s}$ have been established. For example, the dependence of $K_{m/s}$ in a given homologous series on the number of carbon atoms is known.

The search for immiscible phases providing separation is usually carried out empirically based on experimental data. Ternary systems consisting of two immiscible solvents and a third soluble in both phases are widely used in liquid-liquid chromatography. Such systems make it possible to obtain a set of immiscible phases of various selectivities. An example is a system of immiscible heptane and water, into which ethanol is introduced which dissolves in both solvents.

Although immiscible solvents are selected as the mobile and stationary phases, nevertheless, in some systems there is some mutual solubility. To prevent the processes of mutual dissolution of liquids during chromatography, the mobile phase is pre-saturated with a stationary one. The method of chemical fixing of the stationary phase on the sorbent is also used to maintain a constant phase composition. In this case, the interaction of the solvent with OH-groups on the surface of the carrier is used. Adsorbents with a liquid phase fixed on their surface are produced by industry.

Column efficiency is related to viscosity, diffusion coefficient and other physical properties of liquids. The carrier of the stationary phase must have a sufficiently developed surface, be chemically inert, firmly hold the liquid phase on its surface and not dissolve in the solvents used. Substances of various chemical nature are used ss carriers: hydrophilic carriers — silica gel, cellulose, etc., and hydrophobic — fluoroplast, teflon, and other polymers.

2.6 Principles of molecular diagnostic

2.6.1 Theoretical basics of molecular diagnostic methods

Molecular and biological research methods play an great role in modern medicine and veterinary science, forensics, biology. Thanks to advances in the field of DNA and RNA, a person is able to study the gene of the organism, determine the pathogen, distinguish the nucleic acid in the mixture of acids, etc. This method is the most reliable method for the study of hereditary diseases. The advantage of DNA-diagnosis in comparison with other methods of human molecular genetics is that this method allows to identify and analyse the origin of the disease (gene, its localization, type of damage). This method can detect even minimal violations of the primary DNA structure

(including single-nucleotide substitutions), which can not be investigated by other methods. DNA-diagnosis is a minimally invasive procedure (1-2 ml of blood, buccal epithelium, or even a few cells of the material is enough).

Nowadays, the technology of amplification of DNA fragments, so called polymerase chain reaction (PCR), is actively implementing into the practical system for quality control of agricultural products, as a research tool at the molecular level. The method is based on multiple selective replication of a specific DNA strand using enzymes in simulated conditions (in vitro). In this case, the area that satisfies the given conditions is copied, and only if it is present in the sample under study. Polymerase chain reaction (PCR) raised the diagnosis of infectious diseases to a qualitatively new level. The main provisions of PCR were formulated in 1983 by the American biochemist K. Muillis, and he was awarded the Nobel Prize in chemistryfor this in 1993. It should be noted that the development of the PCR method was preceded by the discovery microorganisms *Thermus aquaticus* in the water of geysers and the extraction of them from the enzyme of DNA-polymerase, which retains its biological activity at high temperatures (72-80 °C).

The discovery of a new method was instantly used and in 1985 Saiki R. K et al. published an article in which the amplification of the genomic sequence of b-globin was described. Since then, the number of publications in which the authors reported the use of PCR in their workbegan to increase in geometric progression. The method has become so popular that it's hard to imagine research in molecular biology without it today. The scope of use of PCR is extremely diverse, and its use to identify pathogens of infectious diseases has begun a new trend which is called "DNA-diagnosis" both in human medicine and in veterinary medicine.

The basic principle of PCR is the exponential increasing of the number of copies of the original DNA strand using enzymes *in vitro*. In this case, only the area that is flanked by primers is copied, and only if it is present in the sample under study.

The main components of the polymerase chain reaction are:

1. Primers are a pair of artificially synthesized oligonucleotides, typically having a size from 15 to 30 ppm, which are complementary to the target DNA strand that is being analyzed. Properly selected primers provide high specificity and sensitivity of the test system.
2. Taq-DNA polymerase is a thermostable enzyme that provides completion of the 3'-end of the opposite DNA chain in accordance with the principle of complementarity.
3. A mixture of deoxynucleotide triphosphates (dNTPs) – deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), Deoxycytidine triphosphate (dCTP), and deoxythymidine triphosphate (dTTP), it is a "building material" used by Taq-DNA-polymerase for the synthesis of the second strand of DNA.
4. Buffer is a mixture of cations and anions in a certain concentration, providing optimal conditions for the reaction, as well as a stable pH. Magnesium chloride ions that are required for the Taq polymerase are also included in the buffer.
5. DNA matrix is mixture of a total nucleic acid prepared for the application into the reaction mixture that may contain a DNA strand that will be a target for subsequent multiple copies (eg, DNA of microorganisms). If the DNA target is absent, a specific product of amplification is not formed.

Each PCR cycle consists of three stages. At the first stage, the double-stranded DNA matrix is denatured at 94° C to form single-stranded DNA. In the second stage, the temperature decreases to 55-65 °C and the addition (hybridization) of the primers to each of the two single-stranded DNAs occurs. At the third stage at 72° C, the elongation (extension) of the DNA chains takes place with the DNA polymerase enzyme, which completes both primers in the direction from the 3'to the 5' end, and in the result of this, two double-stranded DNAs occures. This sequence of processes allows to accumulate a PCR enough product for visualization for 25-40 cycles (Fig. 1).

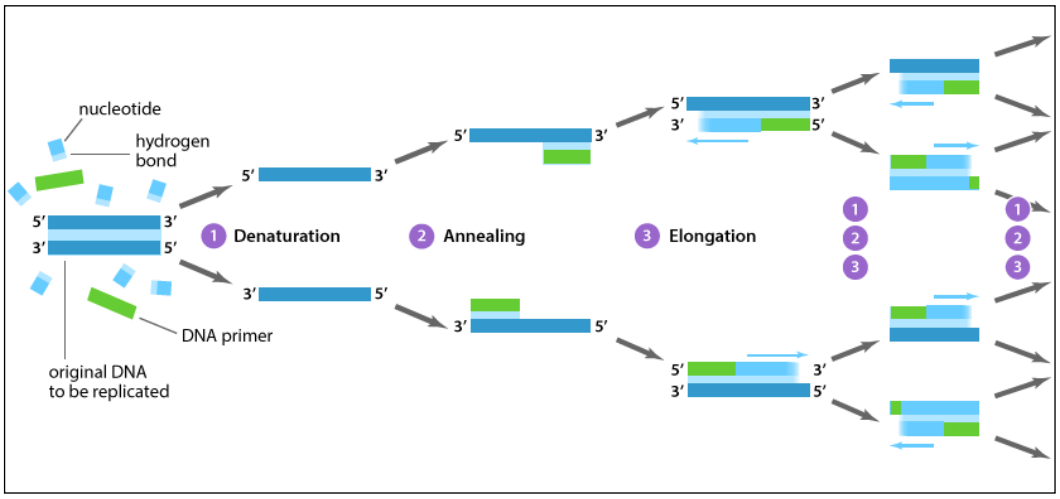


Fig. 1. Principle of polymerase chain reaction.

https://www.abmgood.com/marketing/knowledge_base/polymerase_chain_reaction_introduction.php

Interpretation of the results of the analysis can occur by separating the products of amplification in agarose gel, or on the computer monitor with the help of fluorescence probes.

It should be noted that the process of accumulation of specific products of amplification by geometric progression takes only a certain time, and then its efficiency falls critically. This is due to the so-called "plateau effect".

The term "plateau effect" is used to describe the process of accumulation of amplification products at the last PCR cycles. Depending on the conditions and the number of cycles of the amplification reaction, the following factors influence the period of the "plateau" effect:

- tilization of substrates (dNTPs and primers);
- stability of separate reagents of the reaction medium (dNTPs and enzyme);
- amount of inhibitors, including pyrophosphates and DNA-duplexes;
- formation of primers-dimers;
- incomplete denaturation of the target DNA at high concentration of amplification products.

The lower the initial concentration of the target DNA, the higher the risk of reaction to the plateau. This moment lasts until the number of specific amplification products will be sufficient to be analyzed.

Today there are many modifications of the polymerase chain reaction:

1. *PCR-RFLP (PCR-Restriction Fragment Length Polymorphism*, Initially, PCR is carritd*out, and then restriction of the derived PCR product*
2. *Real*-time PCR. Observation of the reaction in real time, directly measuring the accumulation of PCR product in each cycle. The fundamental feature of the Real-time PCR in contrast to the classic PCR is the ability to quantify the DNA / RNA in the material under study, the absence of the electrophoresis stage, less stringent requirements for the organization of the PCR laboratory, and the automatic recording and interpretation of the results.
3. *RT-PCR (Reverse Transcription PCR).* The starting product is RNA, out of which using enzyme of reverse transcriptase DNA is received. This is convenient, for example, in order to find out expressions of certain genes.
4. *Nested PCR*. It is used to reduce the proportion of by-products of the reaction. Two pairs of primers are used and two consecutive reactions are conduct. DNA strand within the product of the first reaction is amplified using a second pair of primers.
5. *Inverse PCR*. Used in the case if only a small area inside the desired sequence is known. This method is particularly useful when determining the flanking sequences after insertion of DNA into the genome. To perform an inverted PCR, a number of sections of DNA are digested with restriction digestion followed by ligation. As a result, known fragments are formed on both ends of an unknown site, after which it is possible to conduct an ordinary PCR.

6. *Asymmetric PCR*. It is conducted when it is necessary to amplify one of the chains of the original DNA. It is used in some techniques of sequencing and hybridization analysis. PCR is performed according to the classic scenario, except that one of the primers is taken in a large surplus.
7. *Long-range PCR*. It is used to amplify long ranges of DNA (10 kbp and more). Two polymerases are used, one of which is a high-processing factor Taq polymerase (ie, a polymerase capable of synthesizing a long chain of DNA in one pass), and the other is DNA polymerase with 3'-5'-endonuclease activity. The second polymerase is necessary to correct errors made by the first one.
8. *Multiplex PCR* (Multiplex PCR). Several pairs of primers are used and several fragments are amplified simultaneously.

One of the main tasks when using the PCR method to solve a particular task is the correct primer design (in the case of real-time PCR and fluorescence probe). Primers must meet the following requirements.

1. Primers should be specific. Particular attention is paid to the 3'-ends of the primers, since Taq-DNA polymerase begins extract the complementary DNA strand out of them. If their specificity is insufficient, there is a high probability of synthesis of nonspecific amplification products (short or long fragments). It can be seen in the form of heavy or light extra strips on the electrophoreogram. Sometimes it interferes the interpretation of the results of the amplification. In addition, part of the primers and dNTF is spent on the synthesis of nonspecific DNA, which results in a significant loss of sensitivity.

2. Primers should not form dimers and loops, that is stable double chains should not occur in a result of annealing the primers themselves or with each other.

Various online resources, as well as computer programs, are used to work on the construction of primers and nucleotide sequences. The most famous are:

Design of primers and fluorescence probes for polymerase chain reactions https://bitesizebio.com/18992/a-primer-for-designing-degenerate-primers/, http://biotools.umassmed.edu/bioapps/primer3_www.cgi, https://bibiserv.cebitec.uni-bielefeld.de/genefisher2/

Primer and Fluorescent Spectrometry Design for Real Time PCR Primer Express v.2.0http://www.appliedbiosystems.com/support/apptech/

Virtual testing and simulation of a polymerase chain reaction http://engels.genetics.wisc.edu/amplify/

Search and alignment of BLAST nucleotide sequences http://www.ncbi.nih.gov.

For multiple alignment of nucleotide and amino acid sequences are usedhttp://multalin.toulouse.inra.fr/multalin/.

For phylogenetic analysis and plotting of a filogram http://www.phylogeny.fr/version2_cgi/index.cgi/

The most common computer program for analysisof the obtained nucleotide sequences is (Chromas Lite 2.1.1).

2.6.2 Real time PCR

One of the varieties of PCR that is most often used in diagnostic laboratories is real-time PCR (RT PCR). The nucleotide sequence of the complementary DNA strand which is flanked by primers labeled with a fluorescent label (fluorescence probe)is used for this type of PCR in addition to standard components (PCR buffer, magnesium chloride, deoxynucleotide triphosphates, DNA polymerase, direct and reverse primers). The use of fluorescent probes provides continuous monitoring of the fluorescence signal on the computer screen. The advantage of such a modification of PCR is to reduce the risk of contamination, since there is no need for separation of aglow gel amplification products. In addition, using RT-PCR can detect two or more nucleotide sequences in a single test tube (multiplex analysis) and quantify the results of the study.

The amplifier registers the fluorescence values and builds amplification curves on the computerusing the software on the monitor during the real-time PCR. The main criterion for evaluating the results is the determination of the boundary cycle *Ct (cycle treshhold*) which characterizes a certain cycle of a real time polymerase chain reaction, which shows a statistically significant increase in fluorescence compared with the background level (Fig. 2)

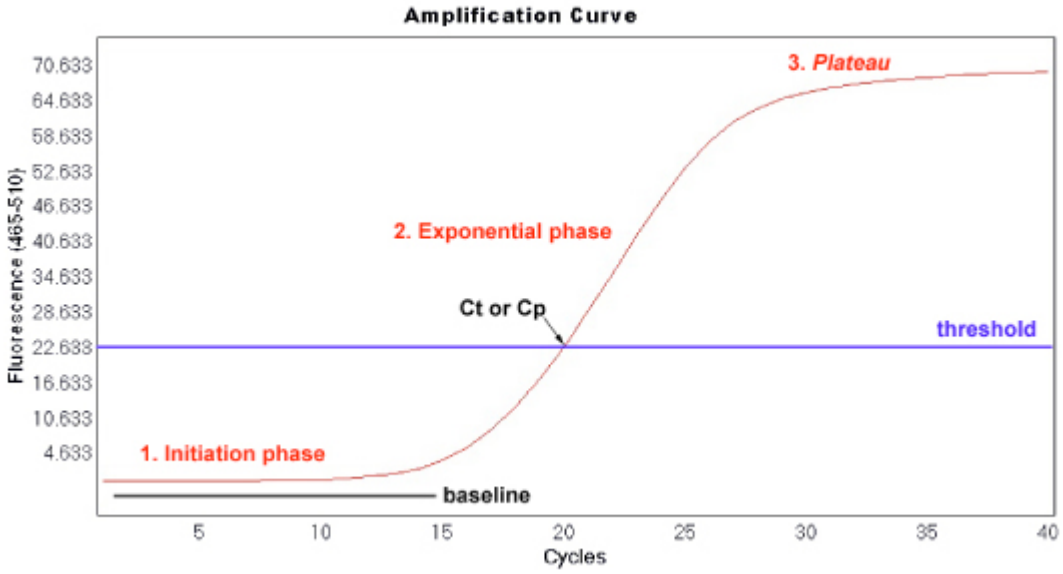


Fig.2 Schematic representation of real-time PCR amplification kinetic curve.

(https://www.highveld.com/pcr/real-time-pcr-quantification-analysis.html).

Technologies which differ by the method of generating fluorescence are used for detection of the amplification products in real time,

The following modifications are most commonlyused:

1. **TaqMan**. The basis of this modification is the use of oligonucleotide probes labeled with fluorescence dye at the 5'-end, and at the 3'-end by a fluorescence dye. Such probes are complementary to the amplifying area. The dampener absorbs the radiation emitted by the fluorescent label, provided that the probe is intact. At the elongation stage, Taq-DNA polymerase synthesizes the complementary DNA strand and begins to split the probe through of 5'-exonuclease activityupon reaching the site of hybridization with the probe. As a result, the fluorescent label is separated from the dampener and its radiation can be detected by the photo-optical system of the device (Fig. 3)

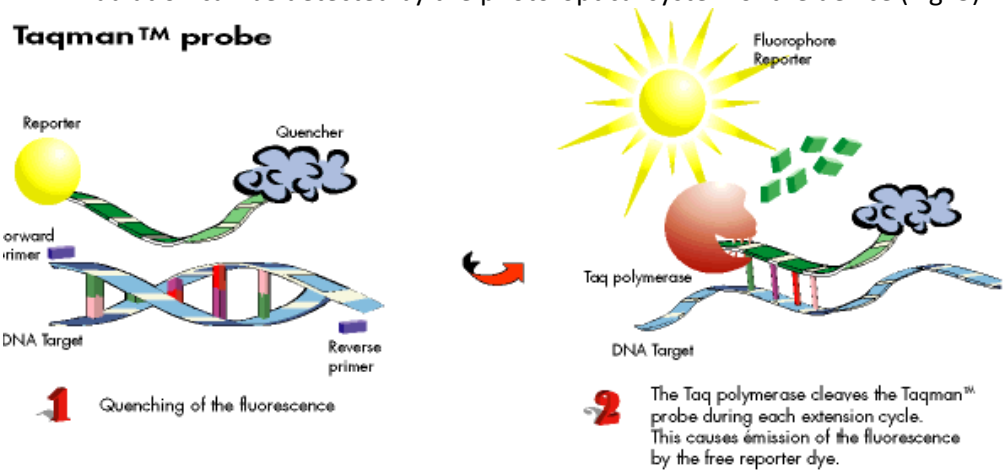


Fig. 3. The concept of TaqMan probes

http://www.biogene.com/ApplicationNotes/Amplification/Methodology/Chemistries.htm

2. **MolecularBeacons (молекулярні маячки)**. Different from TaqMan is that the ends of the samples, which are respectively fluorescent label and fluorescence dummy are complementary to each other.

When the primers are annealed, they are joined together and form a structure in the form of a pin (stem-loop), where the complementarity zone of the probe with the matrix is in the loop. In this case, the dampener absorbs the radiation of the dye. When the sample is hybridized with the matrix, the secondary structure is destroyed, the fluorescent label and the dampener disperse to a distance at which the dampener no longer absorbs the radiation of the dye and the fluorescence signal can be fixed (Fig. 4).

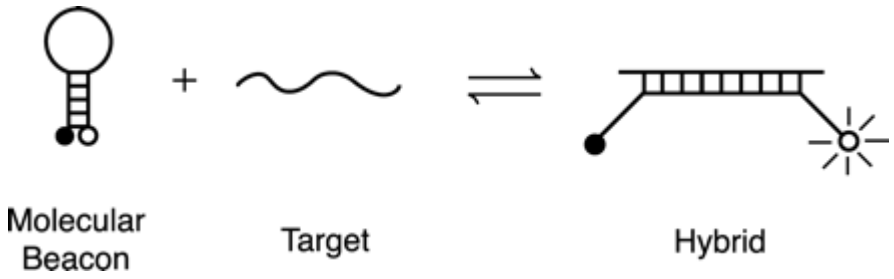


Fig. 4. The concept of MolecularBeacons

https://www.pnas.org/content/96/11/6394

3. **FRET (Fluorescenceresonanceenergytransfer).** In this modification, two probes labeled with fluorescent marking are used. The principle of the method is to transfer the energy from one fluorophore, which is at the 3'end of the first sample, to another which is located at the 5' end of the second sample, in the case when the distance between them is 1-3 nucleotides. During simultaneously binding both probes with DNA, the matrix of radiation from the first fluorophore is transmitted to the second, and its radiation is detected by the device (Figure 5).

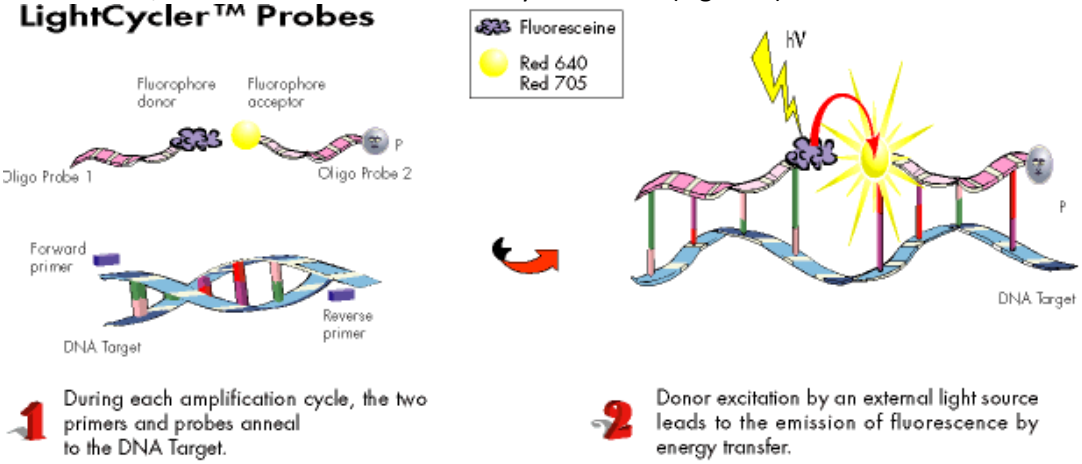


Fig. 5. Concept of FRET

http://www.biogene.com/ApplicationNotes/Analysis/Application/Hybridisation_Probes.htm

Spectral characteristics of the main fluorescent dyes used in the synthesis of probes for real time PCR are presented in Table. 1

Table 1. Spectral characteristics of fluorescent dyes and fluorescence dampeners

Характеристика:	FAM	JOE	ROX	BHQ1
Characteristics, λ_{max}	492 nm	520 nm	580 nm	534 nm
Fluorescence, λ_{max}	520 nm	548 nm	605 nm	-

Quantum output	~ 0,8	0,75	~ 0,8	-

Organization of work in diagnostic PCR laboratories

Requirements for rooms

The proper organization of the PCR laboratory is crucial for obtaining reliable research results. The principle of organization of the laboratory depends on the method of detection of amplification products. There are two fundamentally different ways to obtain the results of the research during PCR. One of them is the division of amplification products in agarose gel, and another is obtaining the results of the research on a computer monitor in the form of amplification curves when performing real time PCR.

In general, separate workshops should be created for each stage of the PCR test.

1. *The room for reception, registration, and initial processing of samples*, which is intended for registration of samples, their temporary storage and preparation for DNA extraction (homogenization). The room should be located in a separate unit and equipped with a laminar cabinet (II-III class of protection depending on the degree of pathogenicity of the objects being studied).

2. The nucleic acid extraction room is designed to extract nucleic acids from the test material which has undergone processing in the previous zone, as well as storage of DNA samples. Samples of nucleic acids and their reagents should be stored in separate refrigerators.

3. The room for reaction mixing is designed to assemble the components of the mixture for PCR (this room may be absent in the case of use of ready-made commercial kits). Such a zone can be arranged using a separate PCR box. However, if there are unoccupied rooms in the laboratory, such a room is desirable.

4. The PCR room is designed to conduct PCR analysis and real-time detection of results. In this room amplifiers for real-time PCR and / or classic PCR are placed.

5. *A room for separating amplification products in agarose gel (electrophoresis)*is for visualization of the results of the study during classical PCR. This area is most dangerous regarding to contamination by products of amplification and obtaining false-positive results of the study. The room for electrophoresis is placed as far away from the main rooms as possible.

All rooms of the PCR laboratory must have a pre-box in which the staff changes the overalls.

General planning of the PCR laboratory, the placement of work rooms in it should ensure the movement of the samples under study in one way.

Accordingto the functional load all rooms must be equipped with a set of necessary equipment, reagents and consumables.

Extraction of nucleic acids must be carried out in PCR-boxes (or in boxes of biological safety, ie. laminar cabinets of the II class of protection). It is recommended to increase the number of boxes at high load.

The floor in the PCR laboratory is covered with a tile which should not be slippery, and the walls and ceilings must be painted with oil paint that is resistant to detergents and desinfectants. Bactericidal lamps at the rate of 2 W/m3 are set in all premises. Light-protective films which are resistant to disinfectants are used to protect the desks from direct sunlight. Laboratory furniture should have covering which is resistant to detergents and desinfectants, and the surface of the tables should not have cracks and seams. It is not recommended to use window-blind because of dust accumulation on them.

The PCR laboratory should be equipped with a system of flow-exhaust ventilation with air supply through filters in accordance with the current sanitary-epidemiological requirements.

The following auxiliary premises should also be available in PCR laboratory:

1) office of the Head of laboratory;

2) staff room;

3) lavatory;

4) aux rooms;

5) a room with an autoclave for disinfection of the test material.

Premises of PCR laboratory should be rodents- and insects-proof, and also be equipped with fire fighting equipment.

Requirements to laboratory equipment

Each room of the PCR laboratory must be provided with an appropriate set of laboratory equipment based on its functional load, as well as depending on the used techniques.

It is not permitted to carry devices, automatic pipettes, etc. over one room to another.

The equipment used in the laboratory must be technically sound, have a technical passport and a manual.

Measuring instruments, including automatic pipettes, thermometers, measuring dishes should be calibrated at appropriate facilities for metrological control.

It is necessary to control the change of tips after each manipulation, use disposable micro-probes with tight-fitting covers, use solid-state thermostats for the extraction of nucleic acids from the biological material to prevent cross-contamination and the emergence of false-positive results.

Sampling and preparation for research

Sampling is carried out in accordance with regulatory documents, methodological recommendations, guidelines or other documents, which specify the basic requirements for the selection of a particular material. However, in most cases such documents provide for selection rules aimed at the formation of a representative sample for the study of a particular indicator. The main requirement in the selection of samples for molecular genetic studies is to avoid their cross-contamination. A worker must select samples in rubber gloves and if it is possible to use disposable instruments (biological material sampling). If reusable instruments are used it should be treated with detergents and carefully dried at the transfer from sample to sample. Samples are collected in clean or plastic dishes, and expendableplastic bags can also be used.

Samplingrequires the homogenization of the sample for better extraction of nucleic acids. Depending on the type of sections, it may be blending on a laboratory mill or blender, pounding with a pestle. Certain samples do not require preliminary preparation (blood, urine, washouts).

Deoxyribonucleic acid (DNA) Extraction

There are many different approaches to DNA extraction. The most commonly used methods are DNA sorption on silicon oxide, DNA extraction using magnetic particles, DNA extraction on columns. CTAB-methodof the DNA extraction is often used to extract DNA from plants.

DNA extraction by sorption on silica oxide by R. Boom.

Equipment and consumables

1. laminar box;
2. Microcentrifuge 13-14 thousand rpm;
3. Centrifuge - Vortex;
4. Solid-state thermostat for samples of "Eppendorf" type of 25-100 ºC;
5. A set of automatic dispensers of variable volume;
6. Multipurpose disposable plastic nozzles with a filter of 200 µl;
7. Multipurpose disposable plastic nozzles with a filter of 10 µl;
8. Multipurpose disposable plastic nozzles with a filter of 1000 µl;
9. Plastic disposal microtubes of the Eppendorf type 1.5 ml;

10. Plastic tripods for microtubes;
11. Container for used plastic;
12. Disposable rubber gloves without talc;
13. Tripods for microtubes 1.5 ml;
14. Reagents for DNA extraction

- Lysis solution (guanidentiocionate, 0.1 M Tris-HCl (pH 6.4), 0.2 M Na-EDTA (pH 8.0), 1% Triton X-100);
- Sorbent of DNA (silicon oxide);
- Rinse solution 1 (6 guanidentiocionate, 0.1 M Tris-HCl (pH 6.4))
- Rinse solution 2 (70% ethanol, 10 mM Tris-HCl, (pH 7.5));
- Buffer for elution TE buffer (10 mM Tris-HCl (pH 7.5), 2 mM Na-EDTA

Work performance

1. The lysis solution and rinse solution 1 (if they were stored at 2-8 ºC) are warmed up at 65 ºC until the crystals completely dissolve).
2. The required number of disposable tubes are installed in the tripod based on one test tube per test sample, then they should be marked.
3. A sorbent tube is shaken on a vortex until it is completely resuspended.
4. 300 µl of the lysis solution, 25 µl of sorbent and 100 µl of the test samples are added to all test tubes, then mix thoroughly the tubes in the vortex and they are warmed up in the thermostat at (65 ± 0.5) °C for 5 minutes.
5. The test tubes are vortexed and kept at room temperature for 5 minutes before the sorbent is completely deposited, and then centrifuged for 30 seconds at $10x10^3$g. Supernatant is carefully removed, so as not to scrub the sediment absorber (it is convenient to use a vacuum pump with a flask trap for the selected liquid).
6. Add 300 µl of the rinse solution 1, resuspend on the vortex and precipitate by centrifugation for 30 seconds at $10x10^3$g in each tube, and the supernatant is carefully removed.
7. Add 500 µl of the rinse solution 2 to each test tube, resuspend it in a vortex and precipitate by centrifugation for 30 seconds at $10x10^3$., Carefully remove the supernatant and repeat the washing procedure with the rinse solution 2.
8. Sorbent precipitate is dried in a thermostat at (65 ± 0,5) ºC for 5-7 minutes until the liquid is completely evaporated.
9. Add 50 µl of elution buffer (TE buffer) to each tube, carefully resuspend and place it in the thermostat at (65 ± 0.5) ºC for 5-6 minutes and jolt in vortex every minute.
10. Precipitate the sorbent on a microcentrifuge for 1 minute at $10x10^3$g.

The supernatant liquid contains purified DNA samples, which are used for further studies.

Ribonucleic acid (RNA) extraction

Different approaches are also used to extract RNA, for example, RNA sorption on silicon oxide, RNA extraction with magnetic particles, phenol-chloroform extraction, etc.

Equipment and consumables

1. laminar box;
2. Microcentrifuge 13-14 thousand rpm;
3. Centrifuge - Vortex;
4. Solid-state thermostat for samples of "Eppendorf" type of 25-100 ºC;
5. A set of automatic dispensers of variable volume;
6. Multipurpose disposable plastic nozzles with a filter of 200 µl;
7. Multipurpose disposable plastic nozzles with a filter of 10 µl;
8. Multipurpose disposable plastic nozzles with a filter of 1000 µl;
9. Plastic disposal microtubes of the Eppendorf type 1.5 ml;
10. Plastic tripods for microtubes;
11. Container for used plastic;
12. Disposable rubber gloves without talc;
13. Tripods for microtubes 1.5 ml;

- Reagents for RNA binding (guanidentiocionate, 0.35 M Tris-HCl (pH 6.4), 0.2 M Na-EDTA (pH 8.0), 3%

Triton X-100, 6% $MgCl_2$);
- Sorbent of DNA (silicon oxide);
- Rinse solution 1 (guanidentiocionate, 0.1 M Tris-HCl (pH 6.4))
- Rinse solution 2 (70% ethanol, 10 mM Tris-HCl, (pH 7.5));
- Buffer for elution TE buffer (10 mM Tris-HCl (pH 7.5), 2 mM Na-EDTA

Work performance

1. The solution for RNA binding and rinse solution 1 (if they were stored at 2-8 ºC) are warmed up at 65 ºC until the crystals completely dissolve).
2. The required number of disposable tubes are installed in the tripod based on one test tube per test sample, then they should be marked.
3. A sorbent tube is shaken on a vortex until it is completely resuspended.
4. Add 300 µl of the solution for RNA binding, 25 µl of the sorbent and 100 µl of samples to all of the test tubes, mix thoroughly the test tubes in the vortex and warm up in the thermostat at (65 ± 0.5) 0C for 5 minutes.
5. The test tubes are vortexed and kept at room temperature for 5 minutes before the sorbent is completely deposited, and then centrifuged for 30 seconds at $10x10^3$g. Supernatant is carefully removed, so as not to scrub the sediment absorber (it is convenient to use a vacuum pump with a flask trap for the selected liquid).
6. Add 300 µl of the rinse solution 1, resuspend on the vortex and precipitate by centrifugation for 30 seconds at $10x10^3$g in each tube, and the supernatant is carefully removed.
7. Add 500 µl of the rinse solution 2 to each test tube, resuspend it in a vortex and precipitate by centrifugation for 30 seconds at $10x10^3$. Carefully remove the supernatant and repeat the washing procedure with the rinse solution 2.
8. Sorbent precipitate is dried in a thermostat at (65 ± 0,5) ºC for 5-7 minutes until the liquid is completely evaporated.
9. Add 50 µl of elution buffer (TE buffer) to each tube, carefully resuspend and place it in the thermostat at (65 ± 0.5) ºC for 5-6 minutes and jolt in vortex every minute.
10. Precipitate the sorbent on a microcentrifuge for 1 minute at $10x10^3$g

The supernatant liquid contains purified RNA samples, which are used for further studies.

Reverse transcription

Reverse transcription is a process for synthesizing cDNAs using RNA as a matrix. This is called “reverse” because transcription occurs in most living organisms in another direction, ie. RNA transcript is synthesized from the DNA molecule. The process of reverse transcription is provided by a special enzyme, so called reverse transcriptase (revertase). With the aid of revertase, the deoxyribonucleoside triphosphates are joined complementarily to the RNA matrix and one DNA chain is synthesized. In this case, a hybrid united molecule of RNA-DNA is formed. Then the enzyme ribonuclease H removes the ribonucleotide chain from the hybrid molecule, and the synthesis of the second DNA chain is carried out complementarily in the presence of the enzyme DNA polymerase on the DNA chain.

Commercial kits are used for reverse transcription.

Real time PCR (on the example of determining 35S promoter in soy)

The 35S promoter is one of the regulators of transgenic insertion expression for creating GM plants and it is used for screening for GMO specimens. As a rule, for the control of DNA allocation for each plant, the detection of the site of a specific endogenous gene is used. As the endogenouscontrol for soya, the gene encoding lectin (lec) is most commonly used.

Material for the study: DNA samples of beans or soya leaves; control DNA samples.

Equipment, reagents, utensils.

1. real-time PCR system (ABi Prism 7 000 (AppliedBiosystems), Rotor-Gene (CorbettResearch), Q-Cycler (Bio-Rad) CFX 96 (Bio-Rad) or similar;

2. PCB-box with UV lamp;

3. Vortex centrifuge;

4. Refrigerator with temperature 2-8 ^{0}C and a freezer with temperature 20^{0}C;

5. A set of automatic dispensers of variable volume;

6. Disposable nozzles with an aerosol barrier;

7. Optical 8-hole strips of 0.2 ml,

8. 96-hole thermoboard for microtubes;

9. Disposable polypropylene tubes with a capacity of 1.5 ml^3 of "Eppendorf" type.

10. A set of reagents for the detection of 35S-promoter and soy lectin:

- PCR mixture (PCR buffer (without $MgCl_2$); solution of $MgCl_2$of molar concentration 2.5 mmol; mixture of deoxyribonucleotide triphosphates (dNTP) of molar concentration of 2.5 mmol each; 5 pmol primers for detecting of the 35S promoter and soybean lectin, 2.5 ppm fluorescent probe for detection of 35S promoter (labeled with FAM fluorescent dye), and soy lectin (labeled with a fluorescent dye JOE), deionized water.

- Taq DNA polymerase;

- Uracil-DNA-glycosylase

Work performance

Before the analysis prepare (or defrost) a PCR mixture. A reaction mixture is prepared for the test specimens at 19.7 µl of the PCR mixture, 0.2 µl of Taq DNA polymerase solution and 0.1 µl of uracil-DNA-glycosylase per sample. The prepared mixture is thoroughly mixed on a vortex centrifuge.

Take the necessary number of tubes of 0.2 ml, add 20.0 µl of the resieved PCR mixture to each hole and 5.0 µl of the examined DNA samples in each hole.

In order to prevent the receipt of false-positive and false-negative results of a real-time PCR test the following controls are used:

1) positive;
2) negative;
3) control without matrix (NTC, no template control);
4) negative selection control (NSC).

5.0 µL of “Positive Control” and “Negative Control” reagents are applied in the holes forrespectively positive and negative control (provided to monitor the correctness of real-time PCR), for NTC control 5.0 µLof deionized water is applied, and for NSC control - 5.0 µL of NSC, which was obtained when DNA has been isolated.

Studies are conducted in accordance with the "Operation manual" respectively to a certain real-time PCR.

The temperature profile of the amplification is given according to the data given in Table. 2

Table 2. - Temperature Protocol of amplification

Stage	Amplification mode		Process	Number of cycles
	Temperature, °C	time, min.		
1	50	2,0	Activation of uracil-DNA-glycosylase	1
2	94	9,0	Activation of polymerase	1
3	95	0,2	Denaturation of DNA	45

	60	1,0	Primers annealing *	
	72	0,3	Elongation of the DNA chain	

*Note: * - The fluorescence data is collected at this stage*

To interpret the results of qualitative determination of GMO content, an analysis of the amplification curves of the samples and controls is performed:

1. A sample is considered to be positive if there is an amplification of fluorescent dyes FAM (35S-promoter) and JOE (lectin soy) (Fig. 6).

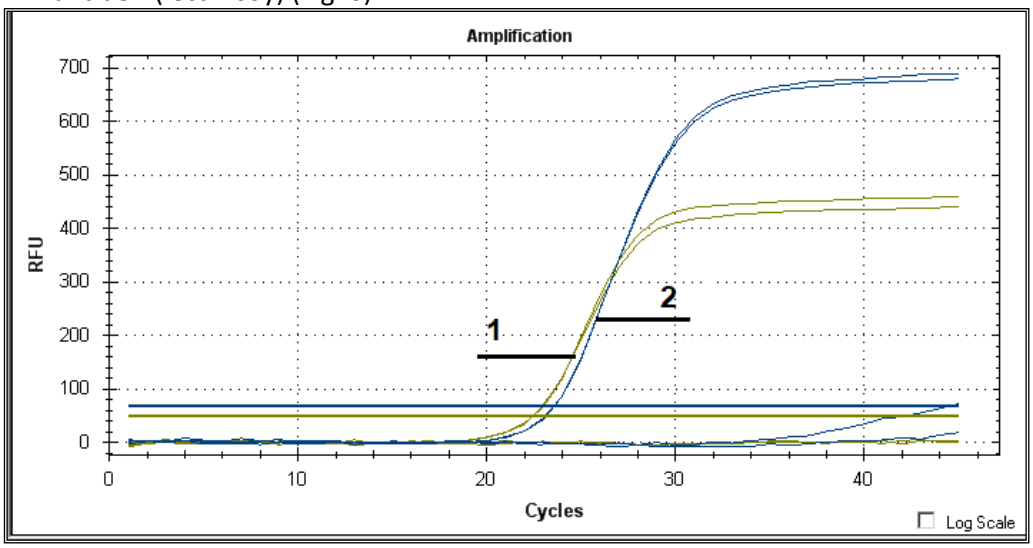


Fig. 6. Example of a soy sample in which the GMO was detected:

1. – curves of soybean lectin amplification, 2. – 35S-promoter amplification curves,

RFU - relative fluorescence units

2. The sample is considered to be negative if there is an amplification of soy lectin (JOE dye) with the value of Ct 23-24 (real-time polymerase chain reaction limiting cycle, which characterizes a certain cycle of PCR, in which the fluorescence signal exceeds a certain base value) and there is no amplification of 35S promoter (Fig. 7).

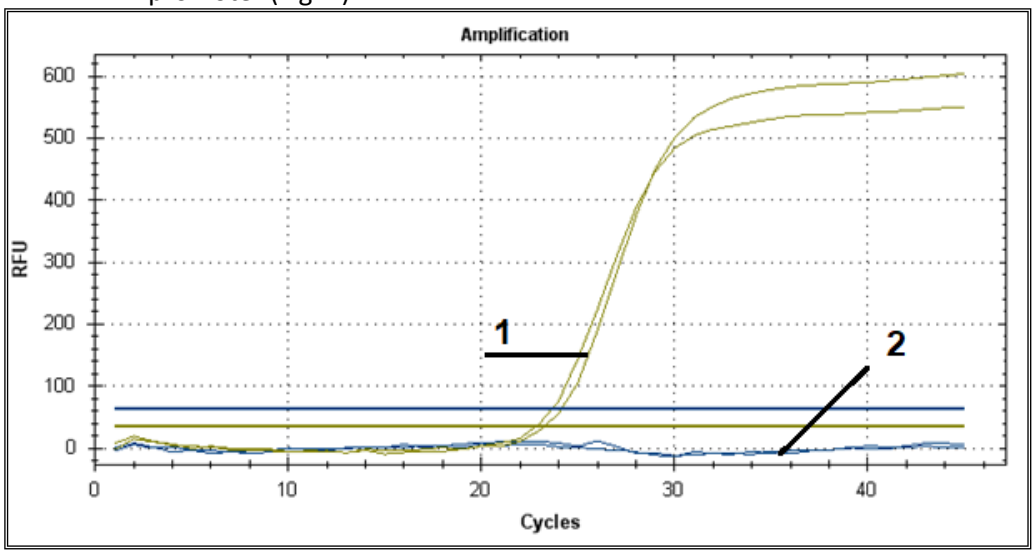


Fig. 7. Example of a soy sample in which GMO was detected:

1. – curves of soybean lectin amplification, 2. – The amplification curves of the 35S-promoter RFU

3. There should be an amplification for FAM (35S promoter) and JOE (soybean lectin) in the positive control sample;
4. There should be an amplification only for JOE (soybean lectin) in the negative control sample;
5. NTC and NSC controls have no amplification for any of the dyes.

The analysis are not be taken into account:

1. If there is no amplification of the JOE dye in the tested sample;
2. If there is no amplification of the FAM and JOE dyes in a positive control
3. If there is amplification amplification of FAM dye in negative control;
4. If there is amplification of FAM or JOE dye in NTC and / or NSC controls;
5. If there are large differences in the value of Ct in parallel measurements (SD ^ 0,05).

Horizontal electrophoresis of products of amplification in agarose gel

Reagents and equipment

1. Camera for horizontal electrophoresis;

2. DC source;

3. A set of automatic dispensers of variable volume;

4. Disposable plastic universal nozzles with a filter of 200 µl;

5. Transyluminator for visualization of separation products;

6. Agarose;

7. Tris-borate-buffer (TBE buffer);

8. Ethidium bromide;

9. Molecular weights marker.

Preparation of agarose gel

For the preparation of the gel, the appropriate weight of agarose, for example, "Top Vision" (Fermentas, Lithuania), is melted in a tris-borate buffer (TBE) in a microwave oven. After complete melting of agarose in the mixture, an intercalating dye of bromide ethidium is added to the final concentration (2 mg / l), mixed well and cooled to 50-60 °C. The gel is poured into a special form (5-6 mm thick) and placed in a comb for the formation of wells. After complete solidification of the gel (30 minutes at room temperature) gently remove the combwithout destroying the wells.

Work performance

Ready gel is transferred to the electrophoresis chamber in such a way that the wells are turned towards the negative electrode (anode). DNA from the wells moves toward the positive electrode (cathode). Pour the prepared TBE buffer so that it covers the gel 4-5 mm above it. Samples with amplification products are mixed with a 6x boot buffer and carefully inserted into the bottom of the well. Simultaneously with the samples, a marker of length of DNA fragments is inserted. Connect the camera to the current source, keeping the polarity. The negatively charged DNA starts to move in the gel from minus to plus. In this case, shorter DNA molecules move faster than long ones.

The rate of movement of DNA in agarose gel is influenced by the concentration of agarose, the voltage of the electric field, temperature, and so on. All molecules of the same size move at the same speed.

Electrophoresis is carried out in a voltage gradient of 10 V/cm until the blue dye (xylenethanol) passes about 1.0 cm from the gel holes. After electrophoresis, the gel is transferred to the transyluminator and the location of the DNA strips under ultraviolet radiation is reviewed. After the electrophoretic separation, the buffer and gel containing the bromide etidium are deactivated.

2.7 Parasitological analyses

2.7.1 Diagnosis of helminthiasis and protozooses in animal feces. Selection of solutions and procedure.

Laboratory analysis of faeces provides extensive information on the presence of protozoa, helminths and their fragments.

Diagnosis of helminthiasis and protozooses is carried out with research or prophylactic purposes, to establish the extent and intensity of invasion, as well as for the differentiation of pathogens. Qualitative (identification of species composition of parasitic helminths) and quantitative research methods are used to evaluate the intensity of invasion (weak, medium, strong), as well as the effectiveness of dehelmintization.

Life-time diagnosticsis based on the study of epizootic data (zonal features of the disease, species composition of the pathogens, breed and age of the animals, season, source of invasion), clinical signs of the disease and the results of laboratory tests. Parasites can be detected in different systems of the body. Biological material for research on the presence of helminths, their fragments, larvae and eggs, as well as cyst protozoa are faeces, urine, duodenal content, bile, sputum, rectal and perianal mucus, blood, muscle tissue. Most often the subject of the study are feces, whereреproductive helminths could be found, eggs, larvae or cysts that are in the gastrointestinal tract and respiratory system as well as ticks that are swallowed by animals. Helminthocoproscopic studies are divided into helminthoscopic (detection of mature helminths or their fragments), helminthoovoscopic (from Lat. Ovum - egg) and helmintholarvoscopic (from Lat. Larva - larva), during which eggs or larvae are found. Most helminths and protozoans are similar in morphological features, they secrete similar eggs (strohalid type), oocysts, and it complicates identification. Fecal examination may also indicate digestive status, such as undigested muscle fibers, fat droplets, starch, and so on.

Accuracy of sampling.

For research, 4 - 10 g of faeces from the rectum or from the floor are taken by hand in a rubber glove. They must be fresh and known which animal they belong to. Feces should be taken with the middle and index fingers in the fingerstallsfrom pigs, calves, sheep, goats. Feces of rabbits (several balls) are obtained by pressing the abdominal wall in the rectum zone. From birds, fur animals, carnivorous animals, wild predators (in zoos), feces are collected from the cage floor (group tests). For individual analysis there should be several grams of faeces to ensure proper identification of the sample from the animal. The feces must be fresh (up to 2 hours) and can also be refrigerated in plastic bags.

The sample should be examined before carrying out the methods. Attention should be paid to its visual appearance; sequence, color, and the presence of blood or mucus which may indicate specific parasitic infections.

Flotation.

Flotation methods are based on the principle of using flotation solutions in which the density is higher than parasites have. These methods are relatively cheap to carry out.

Selection of solutions and methods.

Many different substances can be used to make flotation solutions. The higher the specific gravity of the flotation solution, the larger the variety of helminth eggs and cyst protozoa can be detected. The range of the flotation liquids ranges from 1.18 to 1.3. Flotation methods widely use zinc sulfate salts, saturated sodium chloride, magnesium sulfate, and others.

33% zinc sulfate solution ($ZnSO_4$, specific gravity 1.18):

1. Dissolve 330 g of zinc sulfate with water to a volume of 1000 ml.
2. Check the specific gravity using the areometer.

Saturated solution of sodium chloride (NaCl, specific gravity 1.2) or magnesium sulfate ($MgSO_4$, specific gravity 1.32):

1. Add salt to warm water until it dissolves and the excess settles to the bottom. To guarantee the saturation of the solution, it should be left overnight at room temperature.
2. Check the specific gravity using the areometer.

Sugarsolution (specific gravity 1.2–1.25)

1. Dissolve 454 gofgranulatedsugarwith355ml of water, stir.

2. Aftersugardissolves, add 6mlofformaldehydeformicrobialgrowthprevention. (either 30ml of 10% formalin to water volume of 3300 ml)

3. Check the specific gravity using the areometer.

Mainly the flotation methods are the same.

Fulleborn's method

Add 5 g of feces to the glass and stir with a glass or wooden stick, add a saturated solution of salt (magnesium sulfate, sodium hyposulphite, zinc sulfate). The feces suspension is filtered through a metal sieve and clarified for 40-50 minutes. Light-weight eggs float up. A few drops of the surface layer are transferred with a metal loop to a slide and examined under a microscope.

Modified McMaster Test

1. For ruminants, 4 g of feces from 56 ml of flotation solution should be stirred to obtain a total volume of 60 ml. The test can also be performed with 2g of feces and 28 ml of flotation solution (in the case of a small amount of feces). Stir well and strain through a gauze or strainer. The mixture should not be very saturated.

2. Using a filter pipette, transfer the material to the counting chamber and fill each cell of the McMaster camera. The entire camera should be filled but not just the area under the grid.

3. Wait 5 minutes and examine under a microscope. Eggs and oocysts of different types of parasites should be counted separately. In some cases, eggs can be identified by genus or species (eg, *Strongyloides, Trichuris and Nematodirus*) while others should be considered a category of parasites (coccidia, eggs).

To determine the number of eggs in one gram of faeces, add the number in both chambers and multiply by 200. However, since totally 4 grams of feces were used in the test, the result should be divided by 4.

Centrifugation.

Centrifugation is more effective than standard flotation methods, regardless of the flotation fluid solution:

1. Mix 3–5 g (about 1 teaspoon) of faeces with a small amount of flotation solution in paper or in a plastic glassful. Cat faeces and small pellets of ruminants can be ground with a mortar or soaked in water until soft. Feces should be washed with water if the sample contains a large amount of fat or mucus.

2. Strain the mixture of faeces and flotation solution through a double layer of gauze or strainer.

3. Pour the mixture into a 15-ml test tube and centrifuge for 5 minutes at 500-650 rpm, drain the supernatant, resuspend sedimentwith a flotation solution, and repeat 3 times.

4. After centrifugation, remove the tube from the centrifuge and place in a rack. Add the flotation solution to the meniscus and place the cover glass, wait for 5-10 minutes, place on a slide and examine.

Sedimentation.

Sedimentation methods are used in the diagnosis of helminthiasis, the causative agents of which produce eggs with high density (trematodes, acantocephalus and some other flat helminths and nematodes). They are more labour-consumingthan flotation methods, due to the difficulty of washing the helminth eggs in the sediment of the test mixture.

Sequential washing techniques. A small amount of feces (5-10 g) is stirred in a glass with 10–tuple the amount of water. The mixture is filtered through a metal sieve or gauze and set aside for 5 minutes. Then the top layer of the liquid is drained, add again the same amount of water and precipitate for 5 minutes. It is washed several times until the surface layer of the liquid is transparent. After clarification of the upper layer of liquid in the glass, it is drained and the precipitate is applied to a slide for microscopy. You can add one drop of 0.1% methylene blue, which turns the background blue, but does not stain the fasciol eggs, which will be yellowish-brown.

Centrifugal sedimentation method.

1. Mix 1 g of feces with 10 ml of 10% formalin or water. Pour the mixture into a 15 ml glass centrifuge tube (with lid) up to three quarters.

2. Add ethyl acetate until the tube is almost full.

3. Close the tube and shake approximately 50 times.

4. Centrifuge for 3-5 minutes at 500 rpm.

5. Three layers should be formed after centrifugation: (1) the top layer will contain ethyl acetate, fat and debris; (2) the middle layer will contain formalin or water; 3) the bottom layer of sediment is fine solid particles. Remove the supernatant, leave only the precipitate.

6. Resuspend the precipitate in a few drops of water or formalin, place one or two drops on a slide, cover and examine with a amplification of 10×.

Helmintholarvoscopy

Helmintholarvoscopic methods are used to detect larvae of parasitic helminths in feces (dictiocaulosis), milk (strongyloidosis, neoascarrosis), eye rheum (telaziosis), skin (onchocerciasis). They differentiate in size, structure and shape.

The Bermann technique is used for the diagnosis of dictiocaulosis, protostronilidosis and other nematodes of animals. This method is used for the study of faeces, an apparatus consisting of a funnel, a 10–15 cm rubber tube connected to the upper end of the funnel and a clamp attached to the lower end of the rubber tube are used. The

sample of faeces (10 g) is wrapped with gauze and placed in a funnel. Pre-fill the funnel with warm water (35-38 °C). The apparatus with the sample of faeces of sheep and goats is left at room temperature for 3-5 hours, from cattle for 12 hours, from horses for 8-12 hours. During this time, the larvae of the helminths emerge from the test of the faeces into the water and fall down the rubber tube until it is closed with a clamp. Then the clamp on the tube is weakened and the escaping liquid is collected in a test tube and centrifuged for 2-3 minutes at 1500 rpm Then the top layer of the liquid is drained and the precipitate is transferred to a slide for microscopy. Helminth larvae are mobile and seen well in the liquid.

Weid's technique is used to diagnose pulmonary helminthiasis of small ruminants. A few balls of animal feces are placed on a slide or watch glass and a small amount of water is added at 40 ° C. After 40 minutes faeces are removed and microscopy of the liquid remaining on the glass is carried out. The efficiency of the method does not exceed 70%.

2.7.2 Additional research methods for the identification of specific parasitic invasions

A direct smear is used to detect protozoal trophozoites (giardiasis, trichomonas, amoebas, etc.) or other elements that do not float well or are easily distorted by flotation solutions. The quality of this method is low due to the small amount of material.

Procedure: 1. Mix a small amount of faeces with a drop of saline solution on a slide. 2. Cover with glass and examine for 10 × and 40 × amplification. 3. The main characteristic of trophozoite recognition is movement. You can add a drop of Lugol's solution that paints the internal structures of the cysts. You can use several drops.

2.7.3 Differential diagnosis of pseudoparasites

Faeces samples may contain misleading "pseudoparasites" and "false parasites". Often fragments occur as eggs, oocysts; these include pollen grains, plant hairs, grains, mites, mold spores, and a variety of harmless plant and animal debris. "False parasites" are helminth eggs or cysts of protozoa found in faeces and may belong to another due to coprophagy or predation. One of the best ways to avoid errors in the evaluation of fecal samples is to evaluate the various parasites that commonly infect animals.

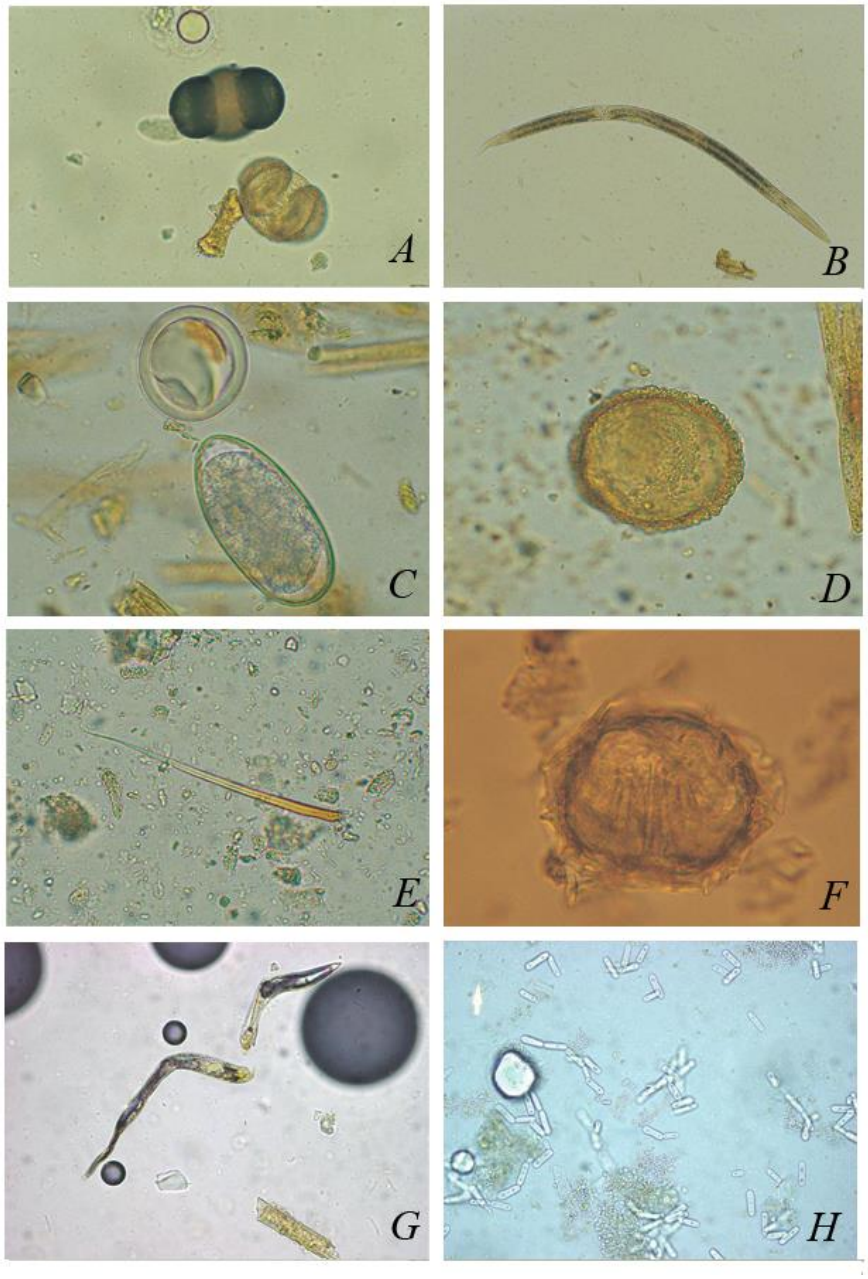
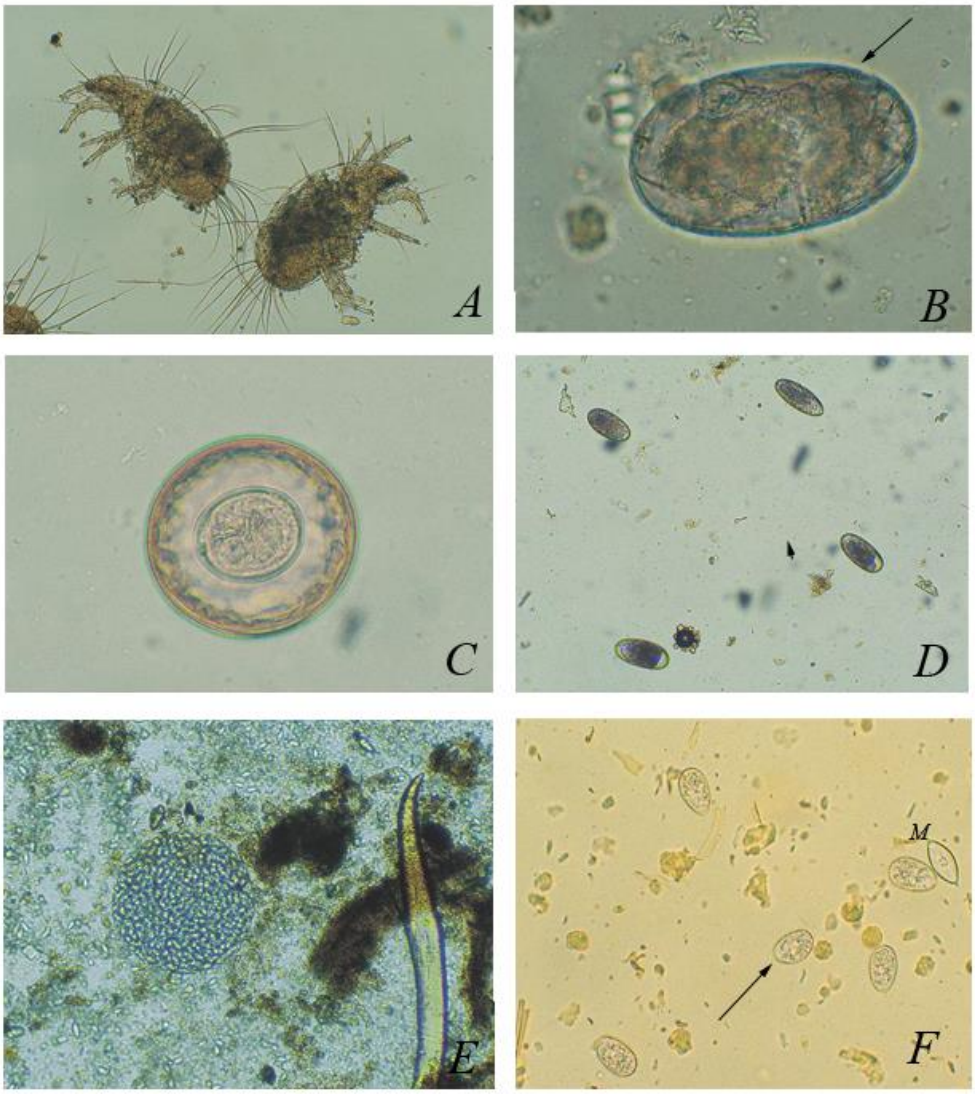


Fig. 1. Examples of pseudoparasites. (A) Pine pollen is a common pseudoparasite found in the faeces samples of many animals (400 ×). (B) Adult free-living nematodes found in faeces collected from the ground. (C) Egg in sheep faeces samples and pseudoparasite (indicated by arrow). Absence of a clear internal structure and breaks in the outer layer is charachteristic for recognition of pseudoparasites. (D) Pollen grains (400 ×). (E) Insect hair from insectivorous bird feces. (F) The artifact in ruminant feces has structures resembling the hooks of a flat helminth embryo, but there is no distinct embryo and the outer layer is poorly defined (40 ×). (G) Vegetable hairs and other fibrous materials that may resemble nematode larvae. They can be present in different shapes and colors, but they can usually be easily differentiated from nematodes because they lack a clear internal structure such as the digestive tract. (H) Saccharomycopsis guttulatus is non-pathogenic yeast in rabbit feces and occasionally observed in dogs (400 ×). (Taken from Zajac A. Veterinary Clinical Parasitology / Anne M. Zajac, Gary A. Conboy. - 8th ed. — American Association of Veterinary Parasitologists : Wiley-Blackwell, 2012 - 19-23 p.)

Fig. 2. Examples of pseudoparasites. (A) Free-living mites that contaminate animal feed. Unlike many parasitic ticks, free-living species do not have specialized structures on their feet (suckers, combs, etc.). (B) Eggs from free-living mites. They are usually very large (> 100 microns). The developed legs of the tick inside the egg (arrow). (C) False parasites: The taeniaegg is found in a calf's faeces sample. Although the configuration of the hooks inside this egg

clearly identifies it as a stick, it is most likely a rodent or bird egg. (D) False parasites spread in dog samples like coprophages. The eggs of ruminants and rabbits look like dog's ones but bigger. (E) False parasites: Monocystis large cyst, the simplest parasite of earthworms found in snake faeces (100 ×). (F) Oocysts of Eimeria spp. (arrow) is a typical false parasite in the feces of dogs. This photo also contains a separate oocyst Monocystis (M) released from a large cyst (400 ×). (Taken from Zajac A. Veterinary clinical parasitology / Anne M. Zajac, Gary A. Conboy. - 8th ed. —American Association of Veterinary Parasitologists: Wiley-Blackwell, 2012. - 19-23 p.)



2.8 Hygiene and security at biological laboratory

2.8.1 Principles of Biosafety in Laboratories

Biosecurity is the practical application of the principles and methods for ensuring biosafety which helps to protect emploees from the workeffect in the laboratory.

Biosafety is providing security measures to reduce the risk of loss, theft or use of microbiological agents or toxins for sabotage which may lead to the inappropriate or criminal use of a particular agent as a biological weapon, as for bioterrorism. Laboratory biosafety describes the principles of isolation, technologies and methods used toprevent the unintentional exposure of pathogens and toxins, or their accidental spread.

The basis for biosecurity practices is the assessment and management of biological risks.

It is possible to obtain information about the microorganisms and used stock culture, their physical location; staff who needs access to a pathogen or toxin by assessing the biological risk which is performed as part of the biosecurity program.

Factors to consider for biological risk evaluation:

1. Pathogenicity of the agent and infectious dose.
2. Potential consequences of infection.

3. Natural ways to transmit infection.

4. Other ways of contamination caused by manipulations in laboratory conditions (parenteral, airborne, with meal).

5. Stability of the agent in the environment.

6. Concentration of the agent and the amount of materials that are supposed to be used in the work.

7. Existenceof the respective "host" of the agent (human or animal).

8. Available information from animal testing, lab reports on infections or clinical reports.9. Planned laboratory activity (ultrasound, centrifugation, etc.).

10. Any genetic manipulation with the body that can extend the range of "host" agents or change its sensitivity to known and effective treatment regimens.

11. Availability of on-site effective preventive and therapeutic interventions.

On the basis of the information revealed during the risk assessment the required level of biosafety of the planned work is determined, appropriate personal protective equipment is selected, a standard course of action is developed, the purpose of which is to ensure the the safest way of conducting of laboratory studies.

Management of biological risks includes:

reducing risk of unintended impact of pathogens and toxins or their accidental leakage (biosecurity), and reducing risk of unauthorized access, loss, theft, unauthorized use, sabotage or deliberate leakage of biological materials to safe, acceptable levels (laboratory biosecurity);

- provision of internal and external guarantees (within the institution, district, government, world community, etc.) regarding the adoption and effective implementation of appropriate measures;

- providing the basis for the continuous increase of awareness of biosafety, laboratory biosecurity, abiding the ethical code of conduct and staff training within the enterprise.

One of the objectives of the biological risk management is to develop a comprehensive culture of laboratory biosecurity and biosafety that allows biosecurity and biosafety to become a part of daily laboratory work, thus improving the overall level of work conditions and the expected level of laboratory management.

2.8.2 HygienicRequirements for Laboratory Rooms. Safety Rules for the Use of the Equipment

For safe conducting of laboratory procedures in the premises it is necessary to ensure: the sufficiency of space; walls, ceilings must be smooth, easy to wash, not permeable to liquids, resistant to reagents and disinfectants, the floor must be not slippery; surface of seats, shelves is waterproof and resistant to disinfectants, acids, alkalis, organic solvents and sufficiently heat-resistant; enough lighting; laboratory furniture is strong and equipped with a free access to cleaning; laboratory equipment and devices are located on a sufficient area; a separate room for safe handling and storage of solvents, radioactive material and reduced gases; separate rooms for outerwear, for meal, rest rooms outside the laboratory area; sank with running water should be placed in each laboratory room, preferably closer to the exit; the doors must have windows, follow the fire safety rules; autoclave and other means of decontamination must be located near the laboratory.

Security systems should include fire and electrical safety, a shower for emergency treatment and eyewash. Properly equipped facilities or areas for first aid must be available.

For designing new premises, it is necessary to consider:

the possibility of creating a system of mechanical ventilation which ensures the fresh air and derivationwasted air without its recirculation. If there is no such system it is necessary to take measures to install well-opened windows and equip them with mosquito nets; system of regulated supply of high-quality water; power supply system, gas supply system, fire safety system.

Along with reliable procedures and practices, the use of equipment that meets the safety requirements will reduce the risks concerned withbiosecurity. When choosing safe laboratory equipment **the following principles should be followed**:

1. It must be designed to restrict or prevent the worker's contact with the infectious agent.

2. It must be made of materials that are liquid-tight, corrosion-resistant and meet the requirements of mechanical resistance.

3. It should not have sharp edges, roughnesses and unfixed parts.

4. It must be designed and installed in such a way as to provide easy handling and maintenance, cleaning, decontamination and control for certification purposes; if it is possible, the use of glassware and other fragile materials shouldbe excluded.

2.8.3 Basics of Transportation and Methods of Working with Biomaterial

Transportation of infectious and potentially hazardous biological materials is strictly regulated by national and international regulations. These provisions outline the correct use of the packaging material as well as other requirements for handling operations. International standard rules are not intended to replace any local or national requirements. However, in cases when thetre is no national requirements international standard rules should be used.

According to the State Sanitary Rules and the norms and hygiene standards "Rules for the placement and safety of work in the laboratories (departments) of the microbiological profile (DSP 9.9.5.-080-02)" any income material entering for the study at the laboratory is considered as potentially dangerous.

Transport safety should ensure the protection of biological materials while driving outside of the controlled access zones in which they are stored befor the arrival to the destination. Transport safety measures are applied to biological materials within an institution and between institutions. The safety of transporting materials within an institution should be provided by necessary documentation, accountability and control over the movement of materials between the areas of restricted access within the facility, as well as internal delivery associated with the processes of transportation and receipt of cargo.

Transport safety measures between institutions should ensure the provision of appropriate licences and communication between objects before, during and after transportation, which may include services of commercial carriers. UN Recommendations on the Transport of Dangerous Goods Model Regulations are basis for the elaborationt of national and international transport regulations and include provisions relating to the safety of the transport of dangerous goodsby different means of transport including infectious substances, (Biorisk management: [Laboratory biosecurity guidance]. - Geneva: WHO, 2006, p. 41).

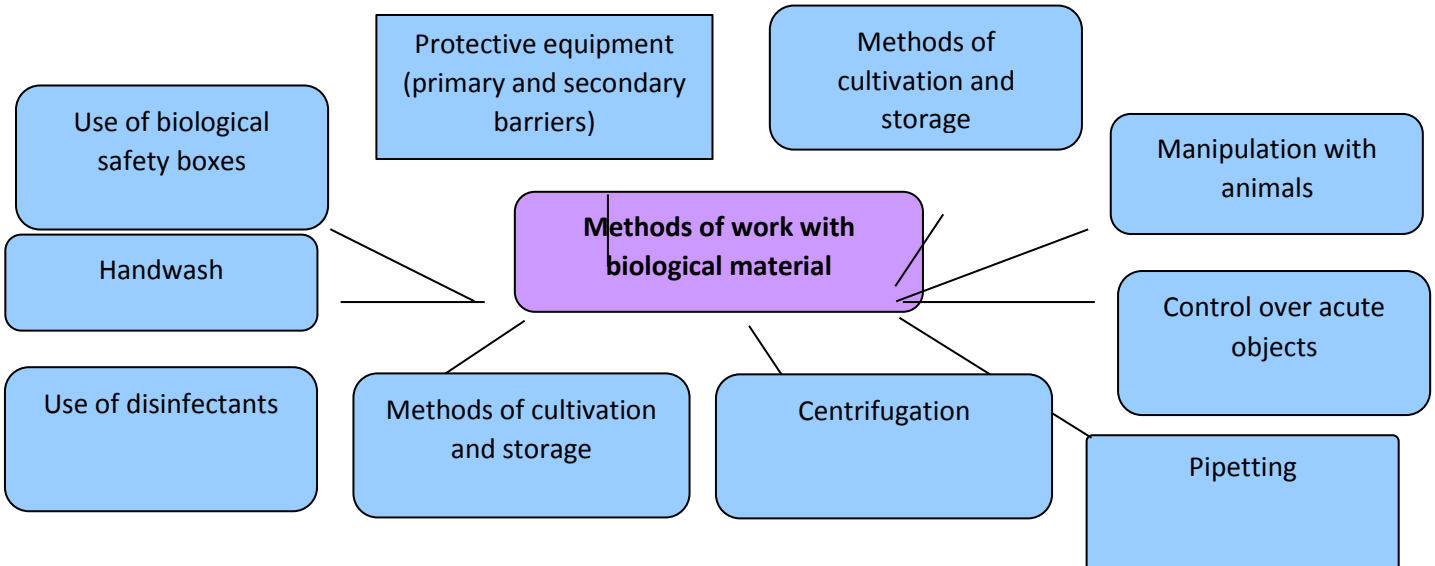
The staff must get necessary training and be aware of the procedures for the prevention of the spread of hazardous materials, proper packaging, marking, registration and transportation of biological materials.

International and national rules for the transport of infectious substances have been introduced to prevent the leakage of these materials during transportation and to protect people, employees, property and the environment from the harmful effects which may be caused by these substances.

One of the important safeguards is the mandatory observance of the special rules for packaging and marking of hazardous biological materials. Protective packaging must withstand hard usageand other effects that occur during transportation, including changes of atmospheric pressure and temperature, as well as vibration and humidity.

Warning meansfor hazardous materials include: transport documents, labeling, marking outside packaging, and other information which is necessary to enable transport workers and members of the rapid response battalionto identify the material correctly and respond quickly to any emergency. In addition, senders and carriers should be familiar with these rules so that they can properly prepare shipments, as well as identify and respond to the risks associated with these materials.

The most important element in preventing the infections is the strict implementation of standard microbiological practices and techniques. People who work with infectious agents or potentially infectious materials should be trained,familiar with potential harm and be highly professional in terms of practical techniques and techniques necessary for the safe handling of such material. The managers or persons responsible for the laboratory supervise the provision or organization of the necessary professional training of the personnel. Each laboratory should elaborate or approve a manual on biological safety. It is necessary to indicate the risks that could be faced and provide practices and procedures elaborated to reduce or eliminate the effects of these factors.



New methods and methodical techniques should be considered by the commission on the compliance with the requirements of biological safety of the institution to and approved by its head before using.

2.8.4 Disinfection and Sterilization

An important and critical aspect of meeting the normatives and standards of biosafety is the correct selection and compliance with the modes of decontamination of materials and waste from laboratories. To implement the biological safety program in the laboratory, it is important to understand the principles of decontamination, cleaning, sterilization and disinfection.

Disinfection is the process of reducing the number of microorganisms (non-bacterial spores) (ISO 15190: 2003).

*Decontamination (decontamination)*is any process for the removal and / or destruction of microorganisms or their reducing to harmless level (ISO 15190: 2003). This term is also used for removal or neutralization of hazardous chemical and radioactive materials.

Sterilization is a process in which all types of microorganisms and spores are destroyed and / or removed.

Disinfectant is a chemical substance or a mixture of chemicals used to kill microorganisms, but not always spores. As a rule, desinfectants are applied to stationary surfaces or objects.

Chemical germicide is a chemical substance or mixture of such substances used for the killing of microorganisms.

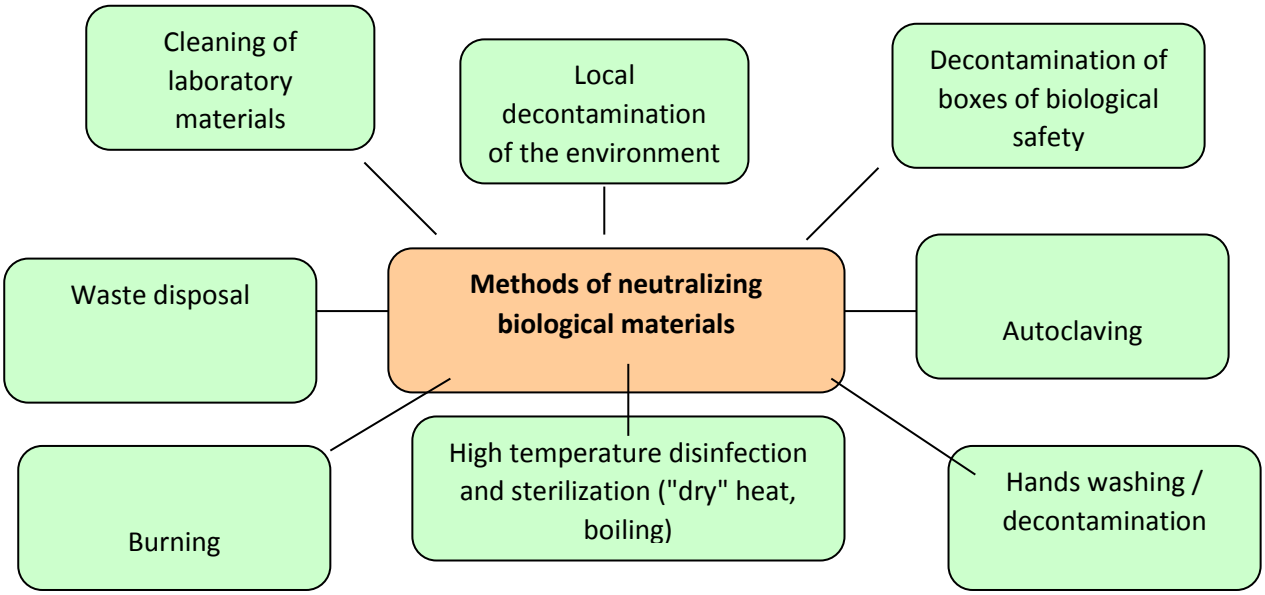
Disinfectants must be effective and safe, noted in the Ukrainian Medicines List, have instructions (guidelines) approved by the Chief State Sanitary Doctor of Ukraine or his deputy and a have certificate of applicability in Ukraine.

The choice of chemical disinfection methods used in the zone should be based on the following criteria: the remedy must be effective to the pathogen with which the work is carried out (the effectiveness of the particular agent should be checked directly at the work place); it must be in the working areapermanently, be stable for storaging and for the influence of external factors; convenient in application. A critical aspect in all countries is the mandatory state registration of desinfectants and the accreditation of its manufacturer. The factors influencing the effectiveness of disinfection include the environmental impact, contact time, concentration and stability of the working solutions, the type of decontaminating surfaces and the presence of organic compounds in the disinfectant.

The biological laboratory must be disinfected very carefully. The main task of disinfection is to reduce the level of microbiological contamination so as to eliminate the possibility of contagion. Resistance of some microorganisms to decontamination is given in Table 1(in descending order).

Spores of bacteria ***Bacillus subtilis, Clostridium sporogenes***
Mycobacteria ***Mycobacterium tuberculosis var. bovis,*** ***Nontuberculous mycobacteria***
Nonenveloped virus ***Poliovirus, Coxackievirus, Rhinovirus***
Fungi ***Trichophyton* spp., *Cryptococcus* spp., *Candida* spp.**
Vegetative bacteria ***Pseudomonas aeruginosa, Staphylococcus aureus,*** ***Salmonella choleraesuis*, Enterococci**
Enveloped virus ***Herpes simplex virus, Cytomegalovirus,*** ***Respiratory syncytial virus,*** ***HBV, HCV, HIV, Hantavirus, Ebola virus***

Table 1 – **Resistance of microorganisms to disinfectants in descending order**



BSL-1-4 laboratories use chemical and temperature methods of decontamination, as well as methods based on their combinations. In laboratories BSL-2-4 all waste is decontaminated, in BSL-1 laboratories only genetically modified derivatives are decontaminated. BSL-1-2 premises use chemical and periodic thermal decontamination, and BSL-3-4 laboratory uses mostly thermal decontamination (periodic and permanent).

2.8.5 Chemical, Fire and Electrical Security. Hygienic Requirements for Lighting, Water Supply, Ventilation, Gas Supply, Noise and Vibration Levels

Violation of the system of isolation of pathogenic microorganisms can lead to emergencies such as fire, chemical, electrical or radiation accidents. For this reason, the biological laboratory needs to maintain a high level of safety in these zones. The elaboration of rules and control over their abiding are under surveillance of national or local authorities, to which laboratory should applyfor assistance.

Laboratories and rooms for animals sometimes become subject of vandalism acts. To except such cases it is necessary to provide reliable protection and fire safety. It is necessary to check all electrical installations and equipment, including grounding-systemsregularly. Automatic circuit breaker with electrical ground fault must be installed in electrical circuit. It is necessary to have emergency lightingwith the indication of a emergency exit, it is desirable to have a backup generator for supply of basic equipment, such as incubators, refrigerators, etc., as well as for ventilation of boxes with animals.

All laboratory rooms should have natural and artificial lighting that meets the requirements. Natural light may be missing in certain rooms (thermal, boxing for sterility studies, photolaborator, etc.). There must be a general switchIn each room of the laboratory. Lamps and fittings should be of a closed type and accessible for wet processing.

The water supply system must be equipped with shut-off devices that prevent counterflow. Vacuum manifolds should be protected by liquid disinfectant traps and HEPA-filters or similar devices. Alternate vacuum pumps should also be protected with appropriate traps and filters.

In laboratories it is necessary to provide the equipment of autonomous inflow and exhaust ventilation with the installation of filters for the fine purification of air emitted from the danger zone (or equipment of these premises, boxes of biological safety). It is necessary to have a reliable and proper gas supply system. The system is obliged to have good maintenance. In production units,levels of noise and vibration must meet current requirements.

2.8.6 Organization of Safe Work and StaffTraining

Each laboratory should provide comprehensive security policy, develop a security guide and auxiliary programs. Director (head) of the institute or laboratory takes responsibility for this,He may impose certain responsibilities on charged with biological safety or other staff. All heads of departments and staff are also responsible for the safety of work in the laboratory. Each employee is responsible for their own safety and security of his colleagues. Employees should do their work in a safe manner and inform the management about any dangerous actions, conditions or incidents. The safe and efficient work of the laboratory is largely depends on support staff, so it is necessary that this personnel has proper safety training.

The heads of laboratories which are responsible for biological safety should develop a safety training program so that the laboratory and support staff are constantly aware of safe working methodss. The effectiveness of training in safety and health, including biological safety, depends on the responsibilities of the administration, motivational factors, the goals and objectives of the organization, proper initial education for new employees, appropriate means of communication . An effective staff training program should contain the following elements (Figure 1).

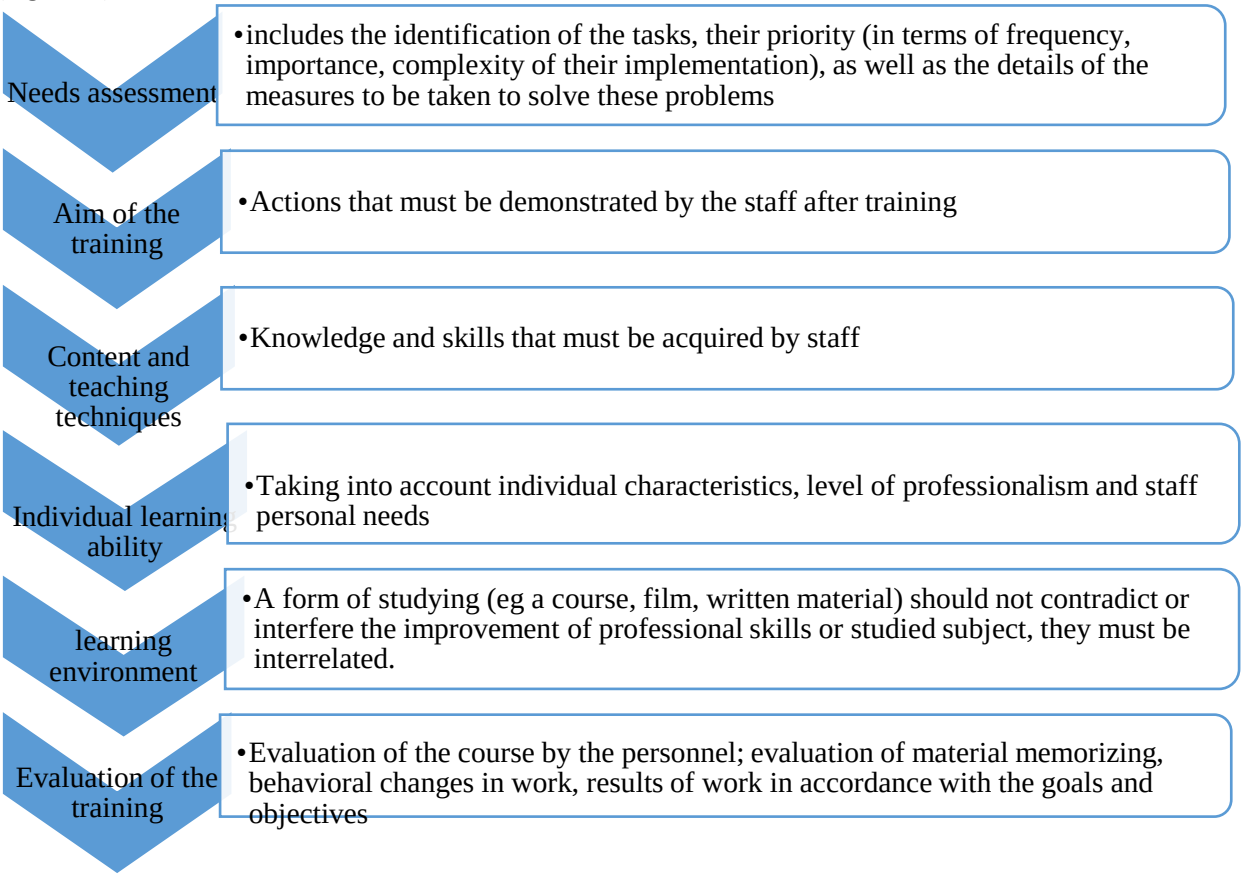


Fig. 1 – **Criteria for staff training program**

The laboratory should have standard operating procedures (SOPs) and maintain records for:
a)definition of requirements for personnel competence;
b)recruitment;
c)staff training;
d)staff control;
e)personnel authorities;
f)monitoring of staff competence.

2.8.7 Risk assessment in a biological laboratory and an algorithm for their management

Workers may be exposed to hazardous and harmful production factors when performing work in laboratories of microbiological profile. (Fig. 2).

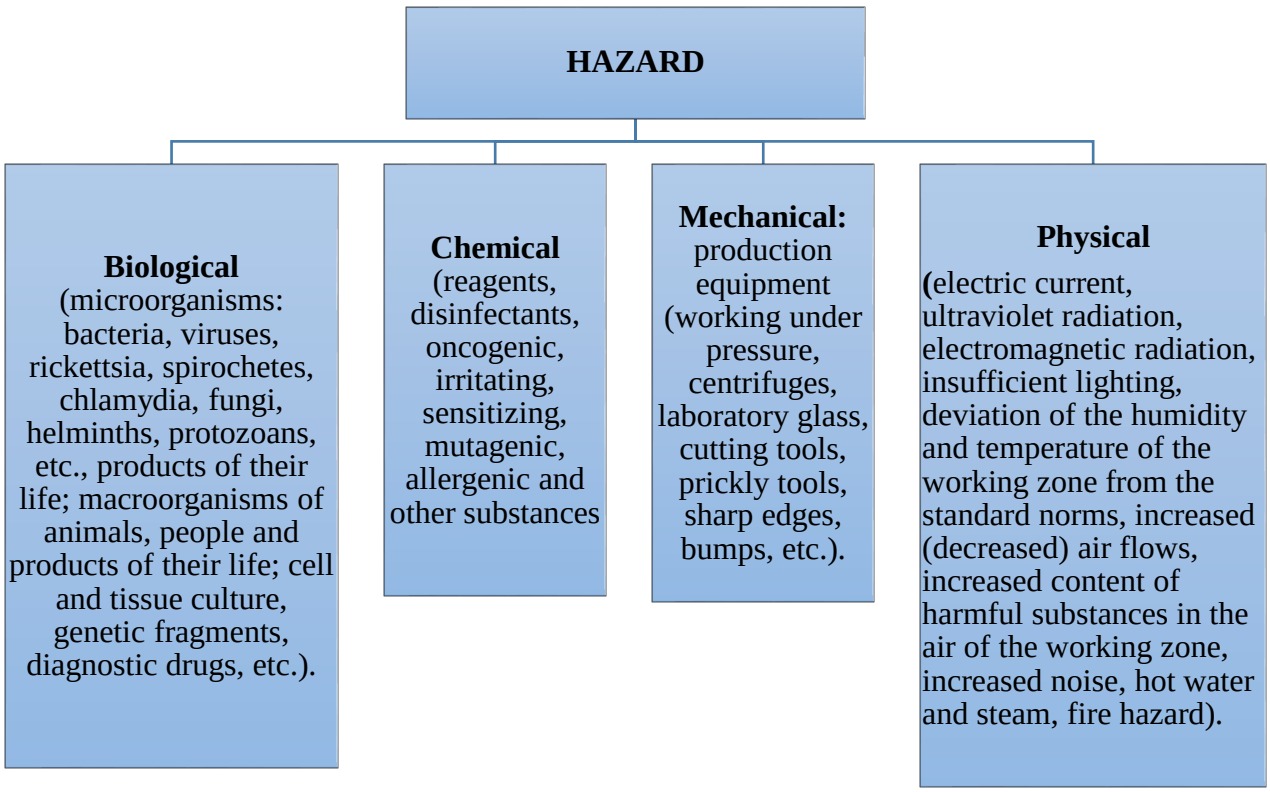


Fig. 2 – **Classification of hazards in the laboratory**

To assess the risks that may occur in a laboratory due to the effects of hazardous factors, it is necessary to carry out a set of procedures (Fig. 3) and develop effective measures to eliminate or minimize risks (Figure 4).

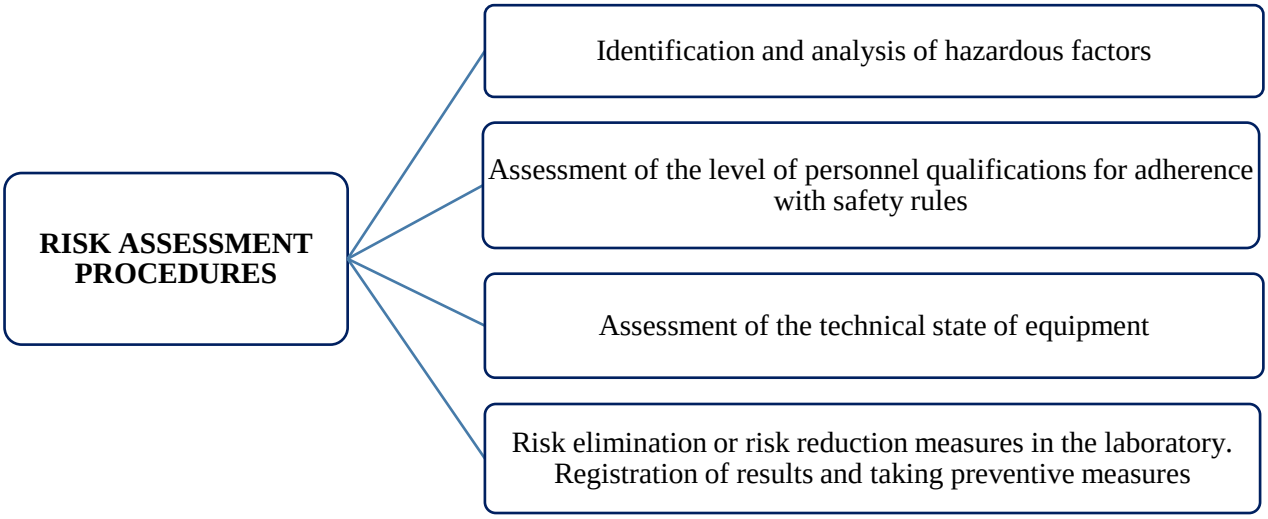


Fig. 3 –**Risk Assessment Procedures**

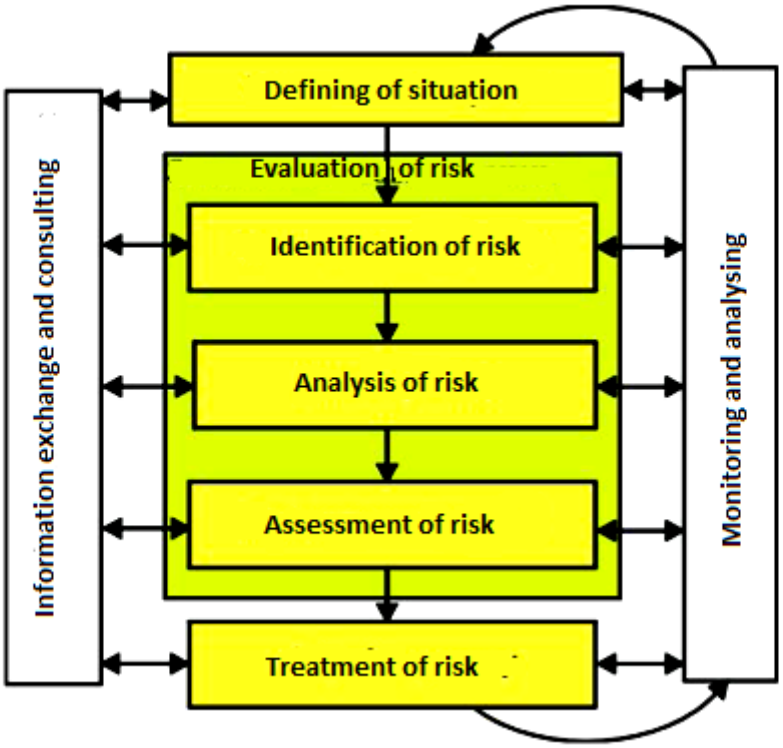


Fig. 4 – **Risk Management Algorithm**

2.8.8 Procedures for eliminating the consequences of accidents and emergencies in laboratories

Injuried (personal or present employees) is obliged to notify the head of the laboratory immediately in case of accidents and emergencies associated with infections, poisoning, injury, burns. If the accident comes amid pouring and / or spilling of infectious material, all people who are in the room stop workingstraightway, hold their breathing, come out of the room to the pre-box, close the door, cover hands with disinfectant or ethyl alcohol; if the face was not protected, in it necessary to treat it with ethanol (70%); protective clothes must be heavily moistened with disinfectant, starting with a scarf or helmet, removing it, immersing it in disinfectant or put it in box for autoclaving. After that, the open parts of the body are wiped with ethanol (70%). A person dresses in change of clothes and treateshis mucous membranes of the eyes, nose and mouth. The mouth and throat are rinsed with ethyl alcohol (70%), drops a solution of protargol (1%)in nose. When a botulinum toxin covers open areas of the skin, it should be washed with plenty of soap and water (flush wateris autoclaved).

If the accident happened without pouring and / or spilling of biological material, tampon with a disinfectant should be put onto the place of contamination with biological material, the head or the person who replaces him must be warned and disinfection of the place of the accidents goes on. After that, the worker leaves the room where the accident occurred, removes and immerses protective clothing in disinfectant. Open skin areasare treated with a disinfectant or ethyl alcohol (70%). If the accident occurred in a proper security box, a worker stops his work, extinguishes alcohol lamp, turns off the equipment (centrifuges, etc. without opening them). Napkins heavely moistured with dsinfectatnts are put onto the place of accident. Bactericidal lamps and emergency alarm are turned on for 30 minutes, and disinfect the safety box. After 2 hours after the disinfection, work in the safety box can be continued. Drawing ventilation during an accident and disinfection must remain turned on.

If the accident involves a trauma or other violation of the integrity of the skin, the work stops, hands are treated with a disinfectant, gloves removed and a wound blood extracts in disinfectant, compressed from a disinfectant or ethanol is out onto a wound for 4-5 minutes. It is necessary to document the situation that has occurred. The laboratory should indicate the possible infectious agent, mechanisms and ways of exposure (through the skin, splashes on mucous membrane or skin, aerosol, etc.), time and place of the accident, used personal protective equipment at the time of the injury, the nature of the assistance to the hurt person (for example, character and duration of flushing and other means, time after processing).

The following protocols must be performed in the biological laboratory:

1. The protocol for pouring and / or spraying a biohazardinside the biosafety cabinet.
2. The protocol for the case of the dispersion of a biohazardoutside the biosafety cabinet.

3. The protocol for the case of biosafety cabinet upset
4. The protocol for the case of damaging or biological contamination of the centrifuge.
5. First-aid protocol

After the elimination of the consequences of accidents and emergencies, the work in the laboratory is restarted with the permission of the head of the laboratory.

Regulatory basis and pedagogical resources:

https://www.who.int/in-vitro-diagnostic/biosafety-guidelines/en/
https://fssuir.sumdu.edu.ua/bitstream/123456789/46037/1/Holubnycha_biobezpeka.pdf
https://www.twirpx.com/file/453879
https://dnaop.com/html/3108/doc-ДСП_9.9.5-080-02
https://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf
https://www.cap-acp.org/Laboratory-Biosafety-Manual.php
https://www.who.int/iris/handle/10665/69390
https://www.cdc.gov/labs/BMBL.html
zakon.rada.gov.ua/laws/show/z0213-19
https://zakon.rada.gov.ua/go/2694-12
https://законодавство.com/...nakaz/nakaz-vid-23072002-280
https://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf
http://www.who.int/csr/.
https://www.iso.org/standard
ISO 31000 Risk management Principles and guidelines
IEC/ISO 31010 (en)Risk management – Risk assessment techniques
ISO Guide 73 Risk management – Vocabulary
Transport of Infectious Substances. Geneva, World Health Organization, 2004, (http://www.who.int/csr/resources/publications/WHO_CDS_CSR_LYO_2004_9/en/).
World Health Organization. Laboratory biosafety manual. Third edition. Geneva, World Health Organization, 2004 / (http://www.who.int/csr/ resources/publications/biosafety/WHO CDS CSR LYO 2004 11/en/).
Recommendations on the Transport of Dangerous Goods, Model Regulations. Fourteenth revised edition. United Nations, New York and Geneva, 2005 (http://www.unece.org/trans/danger/publi/unrec/rev14/ 14files e.html).
13. International Health Regulations. World Health Assembly resolution WHA58.3, May 2005 (http://www.who.int/gb/e/e wha58.html# Resolutions).
FAO Technical Consultation on Biological Risk Management in Food and Agriculture, Bangkok, Thailand, 13-17 Jan 2003 (http://www.fao.org/ ag/agn/food/meetings biosecurity en.stm).
Biorisk management: [Laboratory biosecurity guidance]. – Geneva : WHO, 2006. – 41 p.
ISO 15190:2003 Medical Laboratories – Reguirements for Safety (ISO 15190:2003, Медицинские лаборатории– Требования безопасности)
Стандарти стосовно менеджменту системи якості ISO 9001, 17025, GLP, GMP,
Laboratory Biosecurity Handbook, CRC Press, 2007
CDC/NIH Biosafety in Microbiological and Biomedical Laboratories, 5th edition, 2007
Canada's Laboratory Biosafety Guidelines, 3rd edition, 2004
The International Biorisk Management Standart CWA 15798:2008
www.cen.ue/CENORM/sectors/tecnicalcommittees/workshops/ws31/aps

2.9 Waste management

2.9.1 Types of waste in laboratory practice

Waste is all that you need to get rid of. It is necessary to establish a system of identification and to identify categories for contaminated materials and relevant containers. It is necessary to comply with national and international norms and rules. Categories can be:

1. Non-contaminated (non-infectious) wastes that can be reused or removed along with common "household" wastes.
2. Contaminated/relatively contaminated, including sharp and cutting objects such as needles, scalpels, knives and pieces of glass (in all cases, they must be put in containers with solid walls and lids).
3. Contaminated materials subject to decontamination by autoclaving, which are washed and reused after that.

4. Contaminated materials subjectto autoclaving and removal.
5. Contaminated materials to be burned.

2.9.2 Risks of waste

The dangerous nature of laboratory waste is due to the presence of infectious or toxic agents, as well as the presence of sharp objects. As a result of improper handling of laboratory waste, human diseases, animal diseases, plant diseases, human or animal toxicity, safety hazards, air pollution, soil contamination, the spread of flies, other insects and rodents, the smell and the non-esthetic nature of the landscape may occur.

All people who come into contact with hazardous laboratory waste are at risk from danger, including those working in the laboratory, and other people involved in handling with such waste or are swayed by negligent actions.

Another danger is the hit of chemical reagents into waste, which will be further disposed as household waste. As a result, there is a risk of environmental pollution, a threat to the health of workers involved in recycling in a result of the release of toxins, or the possibility of an explosion, etc.

It is difficult to present in one document all the risks that can be in the laboratory. Therefore, laboratory staff should evaluate all the risks specific to a particular laboratory. It is also important to develop a procedure for workers in case of disposal of chemicals, container damage and other emergencies. It is important to provide means which help to minimize the possible damage. Subsequently, the used materials together with the remnants of the damaged material must be properly isolated and removed.

2.9.3 Accumulation, sorting and temporary storage

All waste which come under utilization by methods that do not differ from the methods of utilization of household waste, except for recycled paper and glass, is called "controlled waste". Items in this category, which include dirty paper, plastic, rubber and wood, should usually be placed in garbage containers that are in each laboratory and will be collected by cleaners. However, each laboratory should also have a container or containers for certain items that are not allowed in usual garbage tanks.

Any sharp objects made of metal or glass should be kept separately, all small powders (preferably inside a bottle or jar), dirty test tubes or other objects contaminated with chemicals (but not syringes or needles) must be stored away.

Waste containers controlled by the laboratory should be regularly emptied and never allowed to be overfilled. Under no circumstances any glass, sharp metals or fine powder may be placed in an ordinary laboratory waste container. Cork should be removed from all bottles for disposal. In addition there should not be any notable chemicalodor from any bottle sorted for recycling.

Glass and waste from glass should be stored in a labeled proof waste container separately from other solid waste for convenienceof recycling. Empty good flasks with reagents can be reused in the laboratory after careful cleaning and removal of old labels.

Sharp wastes such as scalpels, needles from syringes, broken glass, etc. should be stored for disposal in containers with solid walls that will not allow unintentional injury. Containers must be collected by licensed recycling companies.

It is strictly forbidden to pour flammable liquids into a sink. They are poured into adapted containers and then they are taken out for disposal.

Containers should be labeled, for example, with the appropriate inscription and color to prevent incorrect sorting of waste. It is advisable to use internationally recognized inscriptions and signs.

Recycled volatile chemicals should be stored in tanks with proof lids in well-ventilated rooms. However, it is strictly forbidden to store waste in the ventilation hoods where reactions occur.

Cytotoxic waste should be stored separately from other waste in a designated safe place.

Radioactive waste should be stored in containers that prevent ionization. Wastes that must be stored during a radioactive decay should be marked by the type of radionuclide, the date and required storage conditions.

It is forbidden to store used bottles on the floor or in a sink.

Compatible chemicals can be stored together, and incompatible substances should be separated. Also incompatible substances can include burnt and non-flammable substances, as they are further provided with different methods of utilization.

2.9.4 Basic requirements for tools and storage containers

Waste containers should be in easy accessibility but without interfering with the work of employees.

Marking containers for different types of waste should be used. Color coding is often used. In this case, the colors must be contrast. All containers with a biological, chemical or radiation hazard should be marked with appropriate international marks. If labels are used for marking waste, then their background should contrast with the color of the container.

All tanks used for waste should be tight and prevent any negative impact on the environment.

The container for sharp and cutting waste should be with tight walls that make it impossible to damage them. However, it is not recommended to use metal containers made of a material that can corrode.

The capacity of the containers should be appropriate to the amount of waste and be consistent with the frequency of their removal from the laboratory premises. It is not recommended to fill the container more than 3/4 of its volume. In addition, the total weight of the filled container should be taken into account. It should not be too large and hard.

For non-sharp waste you can use strong packages. After filling the package by 3/4 it is recommended to tie the package with a firm plastic tie. For higher reliability, it is recommended to use double packages, or to insert a packet of the appropriate size into a box of corrugator. appropriate markings should be made on the box.

When working with a cellsculture, consider the storage conditions of the waste (for example, Petri dishes) in the laboratory. Microorganisms will quickly multiply at room temperature. Therefore, it is necessary either to create unfavorable conditions for the reproduction of microorganisms or to take into account the planning of the frequency of waste disposal.

The waste container must be resistant to the substances contained therein. It is inadmissible to accumulate the remains of the chemicals that interact with each otherin the same container.

2.9.5 Disposal and transportation of waste.

Disposal of laboratory waste is regulated by various regional, national and international standards, so to develop and implement a program for handling, transporting and disposing of hazardous waste is necessary to learn special literature.

Decontamination of waste and its final disposal are closely linked in laboratories. The basic principle is that infected materials should be decontaminated, autoclaved or destroyed in the laboratory. Before removing from the laboratory any objects or materials that are related to potentially harmful infectious substances, microorganisms or animals, the following basic issues need to be desided:

1. were these objects and materials sterilized or disinfected by means of appropriate established procedures?
2. If not, have these objects or materials been packed in documented processfor immediate destruction on-site or for transportation to another laboratory where there is room for combustion?
3. Is removal of disinfected or sterilized materials/objects connected with additional potential hazard (biological or other) for those who directly carry out the procedure of destruction, or for those who can come in contact with objects or materials outside the laboratory?

All categories of waste that are to be removed must be properly marked. It is important to abide the internationally recognized labeling of hazards that may be in waste. For example, "Biological Danger", "Radioactive Waste", "Fire Accelerant", etc. In addition, it is desirable that the labeling is unchanged for a long period of time.

It is allowed to combine several small containers in a larger one, but they should be supposed fot one category of waste, and a large container should have similar markings.

2.9.6 Documents management

Laboratories should keep clear and easy traceable records on waste management. This documentation is essential for government regulation, planning and tracking, reducing responsibility, facilitating inspections and responding to requests and newsletters. In order to ensure compliance with the requirements, detailed documentation is required. Each laboratory has unique and specific reporting periods and dates of submission, forms of data reporting and storage time of records. Requirements and documentation procedures should be included in the waste management plan of the laboratory.

These requirements include:

- Documentation on the characteristics of laboratory facilities and territory.
- Description of waste types and methods of handling with them.
- Dates of opening/closing containers for waste storing.
- Documents confirming the removal of waste.

• Results of the verification of the effectiveness of measures to minimize waste.

Chapter 3. The sampling

3.1 Sampling (including free-flowing and packed samples), transport, storage, handling and recycling

In order to determine or confirm the cause of the disease or death of animals, in the case of suspicion of an infectious / invasive disease or poisoning, the specialist (veterinary medicine doctor) is obliged to correctly select the relevant pathological / biological material and send it to an accredited (state or authorized) laboratory of veterinary medicine for conducting relevant research.

In addition, samples of feed, water, soil, air for conducting various studies, as well as blood samples and other material from animals for bacteriological / virological, immunological, histological, molecular, biochemical and chemical-toxicological analyzes may be sent to the laboratory.

This section describes the organization, selection rules and sending samples, preservation methods and the timing of delivery to the laboratory of pathological / biological material for life, as well as posthumous diagnosis of infectious animal diseases, the procedure for processing supporting papers to the selected samples.

Describe the requirements for packaging, marking, documentation for infectious materials of various categories and rules for the transportation of such materials. and rules for the transportation of such materials.

This part of the manual describes which specific pathological / biological material is selected for specific bacterial and viral diseases of animals, which are systematized for each individual species.

This part is intended for specialists of veterinary medicine and specialists of diagnostic laboratories.

INTRODUCTION

Considerable effort and resources are spent on optimisation of methodology and in the consideration for minimalising the measurement uncertainty of food analyses. However, how the samples are withdrawn and whether they are representative for the purpose are paid considerably less attention. Frequently, too few units are selected, or they are selected in such a manner that they are not representative of the lot under scrutiny. The consequences may be unreliable results and wasted time and effort, which may ultimately lead to wrong administrative decisions. Wrong results will also be obtained if test samples are incorrectly labelled, inappropriately stored or pre-treated in a manner not conforming to rules or regulations. The sample size should be appropriate to the purpose – either more or less samples would be a waste of money. It is therefore essential to give sampling procedures careful consideration when designing a project for surveillance, monitoring or inspection.

This guide is intended for everyone concerned with the analysis of food and decision-making based on such analyses.

There are many aspects to consider when designing a sampling procedure. In some foodstuffs, the parameters to be examined are evenly distributed (homogeneously) throughout the matrix, but frequently the compounds/microorganisms are unevenly (heterogeneously) distributed. It is, of course, also important to consider health hazards associated with the parameter, as well as the significance of the foodstuffs the parameter may be associated with, when determining the sampling plan.

Ideally, a simple set of rules should be applied to all sampling of foodstuffs. However, that is not possible, and would be just as inappropriate as having one single analytical method to analyse all types of compounds. Many sampling methods are available for certain foods, and should be applied where appropriate. Similarly, where sampling regulations already exist, they should be adhered to, in order that the results are legally valid.

DEFINITIONS

Bulk: Material that does not consist of discrete, identifiable, constant units, but rather arbitrary, irregular units or material that consists of small particles or units that would be impractical to sample individually.

Composite (Aggregate) sample: A sample consisting of portions from each unit, taken in proportion to the quantity of product in each unit selected. (ISO 7002-1986). The combined total of all incremental samples taken from the lot. Note: Equal portions, the size of which should be specified in advance, may also be taken from each unit.

Consignment: A quantity of some commodity, delivered at one time and covered by one set of documents. The consignment may consist of one or more lots, or parts of lots. (ISO 70021986)

Counter sample/replicate sample: A sample taken from the same lot, at the same time and in the same manner as the sample used for enforcement purposes, which the counter sample should be as similar as possible. The counter sample must be sealed and may be used by the company to verify the result of the official analyses.

Increment: A quantity of matter taken at one time from a larger body of material (ISO 70021986)/ An individual portion of material collected by a single operation of a sampling device, from parts of a lot separated in time or space. Increments may either be tested individually or combined (composite) and tested as a unit.

Inspection: Routinely random sampling to examine whether or not the sample satisfies given specifications. The results of these samples determine whether there is a need for further action – (tighten inspection). (SNT report No 5. 1995)

Item / unit: An actual or conventional object (a defined quantity of material) on which a set of observations may be made. (ISO 7002-1986)

Laboratory sample /Final sample: A sample prepared for sending to the laboratory and intended for inspection or testing. (ISO 7002-1986)

Lot; batch: An identified quantity of some commodity manufactured or produced under conditions that are presumed uniform. (ISO 7002-1986)

Monitoring: Repeated observation or repeated measurement, carried out on samples representative of individual foods or the diet in a country or a given area within a country. (WHO, 1979) Intended collecting of information carried out repeatedly on a specified object or for a specific purpose, over a longer period of time.

Primary sampling: The sampling steps that lead to the laboratory sample; i.e. sampling performed "in the field".

Sample (general term): One or more items (or a portion of material) selected in some manner from a population (or from a larger quantity of material). It is intended to provide information representative of the population, and, possibly, to serve as a basis for a decision on the population or the process producing it. (ISO 7002-1986)

Sampling plan: The predetermined procedure, enabling the sampling personnel to choose, draw, or separate samples from a lot, in order to obtain the information needed, such that a decision on the lot may be taken.

Secondary sampling: Reducing / withdrawal of composite (aggregate) sample into test samples.

Surveillance: Ascertaining the condition of foods at a certain point in time without repeated analyses, i.e. without the objective of finding any changes in the levels of substances in the foods. (WHO, 1979) Intended collecting of information carried out on a specified object/purpose in a given period of time.

Test sample: A sample prepared from the laboratory sample, according to the procedure specified in the test method and from which test portions will be taken. (ISO 7002-1986)

Tighten Inspection: To be performed when there is reason to believe that the product is not in conformity with given specifications. If test results from a previous inspection of the same or a comparable lot exceed legal limit of a certain component, if there is reason to believe that the lot is contaminated or there is reasonable suspicion that the lot of interest does not comply with the specification, more increments need to be collected than in a normal inspection.

AIM OF SAMPLING

Prior to sampling, its aim should be defined: what is to be tested, and the intended purpose for the results. The needs and expectations of the stakeholders and other potential users concerning the test results should be carefully considered.

It is also prudent to consider whether analysis, and thereby sampling, is in fact warranted. For instance, if a lot has been inappropriately stored and further action is precluded as a result of the incorrect treatment, sampling is not necessary. If improper stored samples are withdrawn, the controller would be in a dilemma if the obtained results of the analyses were satisfactory.

In an inspection carried out by the authorities, the sample may be a part of an ongoing monitoring project, surveillance or a tighten- or normal inspection. Foodstuffs may also be sampled, as part of a research project or in a manufacturer's own quality control. Sampling may include samples (spot checks) of final products, semi-manufactured products, raw materials and packaging materials, as well as sampling in the course of hygiene control and from the production line. When sampling, it must be ensured that the sample is as representative as possible of the material to be examined, and that the final sample size is sufficient for the analyses requested.

Sampling may be either selective or objective. The difference can be illustrated by an analogy to the speed of traffic. When performing a selective speed check, only the drivers going too fast (or excessively slowly) are selected. When performing an objective speed check, roadusers representative of the traffic are selected, in order to compare the mean and variation at different times or places.

3.1 Objective sampling The aim of objective (non-biased) sampling (random sampling/inspection, surveillance, monitoring,) is to obtain a representative random sample from the lot or the geographical area in question. Each portion/increment of the lot should have the same probability of being sampled.

Objective sampling is used in the surveillance and monitoring of foodstuffs. When monitoring, the same type of foodstuff is examined for the same parameters, repeatedly, over month/years, to examine if the parameters vary and to determine conformities/nonconformities. It is vital that the sample is selected at random to, as far as possible, ensure that it is representative of the lot.

3.2 Selective sampling In selective sampling, samples are normally collected to either illustrate or document unsatisfactory conditions or suspected adulteration of a product. The sampling is deliberately biased, and is directed at the particular products or manufacturers, often in response to information obtained previously by the objective sampling. In so-called tighten inspections, more samples should be taken, than in objective sampling of non-suspect lots, increasing the probability of proving the product's inconsistency with the legislation. This type of sampling is not necessarily representative of the entire lot, as the samples may not be randomly selected, and consequently the results cannot be considered statistically. On the other hand, resources may be best utilized in selective sampling. Consumer complaints, such as contamination by insects, and the incidence of vermin such as rats in a stock/warehouse, will normally result in selective sampling. Samples will be taken at the locations where it is most likely to prove contamination. By analogy, temperature inspections are carried out at the locations where conditions are expected to be most critical.

3.3 Switching rules Objective and selective sampling can supplement each other. If surveillance reinforces suspicions of problems with certain foodstuffs, from a certain origin or company, a selective sampling should then be conducted, focusing on these products. Changing from one sampling plan to another is referred to as switching rules. If the analysis proves defective or inconsistent when using objective sampling, it is possible to switch to a selective sampling plan, focusing on the problem demonstrated. When the results indicate that adherence to this more stringent plan is no longer necessary then a return to monitoring using an objective sampling plan can be made.

PROJECT DESCRIPTION INCLUDING SAMPLING PROCEDURE

When planning a project, a description including the following topics should be given:

- Why sampling – the aim
- Which analytes/parameters are to be determined
- Which matrices should be sampled, what kind of foodstuffs
- Where to perform the sampling – location
- How to sample - equipment and techniques, sampling personnel
- How large items/ aggregates to be sampled – type of sampling plans
- How to label the samples
- What kind of information should be recorded during sampling – sampling protocol
- How should the samples be transported or shipped – any pre-treatment necessary
- Who shall perform the analysis of the samples (which laboratory)
- How to store and pre-treat the samples at the laboratory
- Which analytical methods are to be used
- How are the analytical results to be reported and used, and to whom
- How to evaluate and follow up on the results

THE CHARACTER OF THE PARAMETER AND MATRIX TO BE EXAMINED

The effort and resources utilized should reflect the health hazard-related properties of the parameter to be determined the dietary importance of the foodstuff consequences for individual groups with serious intolerance against certain foodstuffs the intended purpose of the product (direct consumption, raw material, intermediates, process additive etc.) available methods of analysis; level or precision required legal requirements what compositional information is needed; the mean, extremes or variability of composition

It is important that the physical and chemical properties of the analyte are known. If the analyte metabolises or undergoes physical or chemical changes, this should be considered when choosing the sampling device and equipment, and in considering prospective prepreparation and storage. It is also essential to know whether the lot of interest consists of single items or bulk.

Success in obtaining representative samples of food is directly dependent on the physical characteristics of the product (e.g. liquid, powder, coarse lumps, gas), and the type of potential contamination. The uncertainty in the sampling is directly proportional to the variation among the particles of the sample. For a fixed sample size, the smaller the size of the individual components, the smaller the sampling error. It is easier to obtain representative samples of foods that are either liquid, e.g. water, milk or beer, or pastes or powders, than foods made up of larger, irregularly-shaped components, e.g. grain, whole nuts, fruit, mixed food/feeds etc. This does not necessarily apply to sampling for examination for genetically modified organisms, as DNA can be fragmented too much if the particle sizes are too small. For a complete and exhaustive theory of sampling and sampling error the reader is referred to the works of Dr. Pierre Gy; Sampling of Particulate Material, Theory and Practice, Elsevier, Amsterdam, 1992. The composition and complexity of the food matrix has a significant influence on obtaining representative samples, particularly at extremely low concentrations and/or when the contamination is not homogeneously distributed throughout the lot. In such cases more increments should be taken. Gy and other authors elaborate on that statement.

Hazardous compounds such as mycotoxins and pathogens are often unevenly distributed in foods. When sampling heterogeneous material, the number of increments taken should be increased, in order to obtain a representative sample. When investigating outbreaks of food poisoning, it is strongly recommended to sample a large number of increments, in order to get large aggregate/composite samples of all suspected foods. However, it may be necessary to accept smaller than desired samples, due to the difficulties in obtaining sufficient material (most of it is already eaten), excessive costs or the desired sample size being too large to handle. Before sampling, it is always advisable to check with the laboratory, in the event of any special requirements with respect to sample size and handling of the sample.

For sensory analysis, it is to some extent other considerations to be made. To analyse potentially harmful products have to be excluded for obvious reasons when it comes to tasting, but to test for the appearance and odor may in some situations be appropriate.

It is rarely appropriate to make sensory test samples homogeneous by mixing or grinding. The samples are either homogeneous (all kinds of liquids) or they are heterogeneous samples and must be analysed as such. Thus, it is important to note that it is taken for granted that the test samples are representative of the laboratory samples and that the laboratory samples are representative of the relevant lot.

WHERE TO PERFORM THE SAMPLING - LOCATION

The decision on where to sample depends on the purpose of the project. If the purpose is to check critical points (in a warehouse, or a particular production step) the critical points should be defined. For instance, at a refrigerated storage facility or in a refrigerator van, the samples should be selected from the items closest to the doors (at both top and bottom), approximately in the middle of the store (at both top and bottom) and close to the air intake to the refrigeration unit. Sometimes it is necessary to unload the entire van, as there may be some speculation in the fact that only certain parts of the lot may be checked. If the merchandise is stored at room temperature instead of +4ºC, there is no need to sample and analyse before taking action. Spots on the floor, indicating thawing/melting are typical signs.

If the purpose of the surveillance is related to consumer's intake, the sampling should be conducted at the manufacturer, wholesaler/importer or retailer. Samples for enforcement purposes should be taken as close as possible to both origin of the problem and product, i.e. during manufacture or at import, in order that evaluation of the results and following-up shall be as efficient as possible.

EQUIPMENT

Materials in the sampling equipment (including tools, containers and closures) should not cause any change in the sample, which might affect the results of the examination. All surfaces of sampling equipment and sample containers should be clean and dry, smooth and free from crevices, and corners should preferably be rounded. In addition, when sampling for microbiological examination, all equipment used should be sterile. Equipment to be used is sometimes specified in the regulations, and in such cases, should be used. Automatic or semiautomatic sampling can also be used. Such equipment shall undergo appropriate tests prior to being used and at regular intervals during use.

- ✓ Sampling tools: Tools e.g. shovels (rather than spoons), knives, pipettes, drills and probes should be appropriate for their intended use. It should be verified that the tools do not discriminate between different portions of the lot (e.g. too small holes/slits can discriminate against larger particles/bites) Specific sampling probes, "thieves" and augers are available. "Thieves" are spear-like implants used for sampling of textile sacks containing coffee, cereals, etc,
- ✓ For microbiological examinations Sterile tools should be used for opening sealed packages and for sampling. In exceptional cases, the tools used by the retailer/shopkeeper may be used, in which case this should be noted in the sampling report. The tools should be made from a material, easy to clean and sterilize. It is important to be prepared for sampling, by having the necessary tools available. The tools should be touched as little as possible before use and should be protected against contamination until the sampling is carried out. Sterile disposable tools and containers should preferably be used. For microbiological analysis, repted secondary sampling is not favourable as it increases the risk for contamination.

In microbiological sampling, it is important to measure the temperature. Do not measure the temperature on the sample to be withdrawn as the measurement can contaminate the sample. Calibrated thermometers capable of measuring temperatures from -300C to 1000C, with an accuracy of ±10C, should be used. The calibration must be traceable to reference thermometers.

- ✓ For sensory examinations Samples for sensory analyses are usually not obtained from large lots where the above examples of sampling devises are appropriate. More common are natural entities, either produced (such as sausages, cans of peas and bottles of wine) or "biological units": such as ham, apples, fish, and berries. A" thief" or probe is unlikely to be particularly suitable for such samples. For sensory analyses, the sampling procedure will be e related to how the units are selected for analyses rather than on the tools to be used.
- ✓ Sample containers. The shape and capacity of the container should be appropriate for the product being sampled. Sampling containers and closures should be of materials and construction, which will adequately protect the sample. Note! Use sterile containers for sampling for microbiological examinations. Sample containers and closures should be of glass, suitable metals or plastics. Disposable plastic containers, sterile plastic jars, containers made from plastic laminates including aluminium foil or plastic bags, with appropriate means of closure, may be used. Any stopper or liner used should be insoluble, non-absorbant and greaseproof, and should not influence the odour, flavour, properties or composition of the sample. The container should preferable be opaque. If transparent or translucent, the container with contents should be stored in a dark place.

If the sample is representing a product that can be purchased in a retail store, it may be relevant to use the ordinary tool that handles the sample in the store and the product packaging, such as cake boxes or bags at the bakery.

It is important to communicate with the laboratory regarding necessary precautions, containers, pre-treatment and size of the laboratory sample. Container size should be appropriate for the purpose. Containers should have secure closures enabling them to be tightly sealed.

SAMPLING TECHNIQUE

Sampling regulations and procedures described in methods should be followed when applicable.

Precautions must be taken to avoid contamination of the samples, or any other changes adversely affecting the results of the examinations. When sampling, it should be ensured that the sample is as representative as possible of the lot/target of interest, and that the sample size is sufficient. As far as possible, increments should be of approximately similar weight and be taken at varying sites in the lot. It has been shown that selecting and mixing many small incremental samples into a composite, rather than mixing a small number of increments of larger units/items obtains a more correct estimate of the mean value of the lot. Each lot to be examined should be sampled separately. When several samples from containers or tanks are taken simultaneously, for different tests, the sample for microbiological examination should be taken first, and the sample container closed immediately after sampling. Remember to read and note the temperature at the sampling location.

- ✓ Sampling personnel. Sampling should be carried out by a skilled and suitably trained operator. An authorised sampling officer should carry out official sampling. In monitoring programs, over a longer period of time, all operators should be given the same training and instructions, in order to obtain comparable samples. In complex situations, test runs prior to the sampling are helpful in ensuring understanding of protocols and in identifying potential problems and pitfalls.
- ✓ Sampling of solids from bulk. In order to obtain representative increments, "thieves", probes or augers should be used for sampling solids, cereals, powder or pastes from a bulk, rather than a shovel. When a shovel is

used, only the top layer is sampled. A more representative sample is obtained when using a probe. However, the aim of the sampling determines which technique is to be used.

When using a shovel, the probability of obtaining particles from the lower layer is small. Consequently the increments would not be representative of the lot. There is a possibility that poorer quality goods may be found in the lower layer.

When sampling liquids, slurries and pastes, the material should be thoroughly mixed prior to sampling, by either manual or mechanical means. For powder, a mechanical method of sampling is strongly recommended. The sample should be taken immediately after mixing, and if possible, while the product is still agitated.

✓ Sampling from flowing streams. When controlling certain processes, in quality control of raw materials or the final product, or in inspection of cereals, it may be practical to sample from a flowing stream. To make the sampling as random as possible, it is important to sample from the entire stream for a fraction of time (A) rather than sampling from one particular fraction of the stream during the whole of the time (B). By sampling from a particular fraction there is a probability that certain particle sixes would be discriminated.

✓ Sampling of items. When sampling products in retail packages meant for consumers, unopened packages should be sampled, to avoid contamination from the environment or other persons. The samples should be taken as randomly as possible, according to the needs of the test.

✓ Preparation of the composite sample All the increments obtained are collected and immediately placed in an appropriate container. The capacity of the sample containers used should be such that they are almost completely filled by the sample, thus allowing proper mixing of the contents, but avoiding churning during transport. The increments are mixed thoroughly with a shovel immediately after being collected.

For microbiological examinations, composite samples of non-pre-packaged food, or samples from larger packages, should not weigh less than 200 g. The total amount of increments of pre-packaged foods should generally not weigh less than 100 g. For chemical analysis, the size of the increments and aggregate samples depend on the parameter of interest, as well as its distribution in the matrix and the heterogeneity of the matrix.

For sensory analysis, the size of the samples depends on the product. For products that can be cut and mixed (carrots/carrot cubes, sausages/sausages slides, fish/fish filets etc.), the purpose of the analysis determines whether to taste all units or a collection of entities. All entities are selected if the aim is to get information on the variation between units. The second option, usually resulting in a simpler statistical model, should be considered if the purpose is to even out the physical variation.

NUMBER OF SAMPLES TAKEN

The number of items/increments to be sampled, i.e. the sampling plan chosen, is determined by:

- the aim of the sampling (HACCP and critical control points based on risk analysis)
- the accepted confidence interval and acceptable uncertainty
- the characteristics (homogeneity) of the lot
- the characteristics (health-damaging, distribution, etc) of the analyte
- economy

For certain analytes/parameters in foodstuffs, there are regulations, instructions and directives on the sampling method and number of samples, and these should be used where applicable. More often than not, too few samples are collected, but for economic reasons it is also important to note that more samples should not be taken than necessary.

Sampling plans specify the numbers of samples to be taken. They are statements of criteria of acceptance applied to lots, based on relevant analyses of required numbers of samples. Stricter sampling plans should be used when examining for health-related properties (e.g. in assessment of microbiological spoilage, microbial hazards, toxins and carcinogenic compounds) than when examining for compositional characteristics and commodity defects. Sampling plans are described in the enclosed annex.

SEALING AND LABELLING THE SAMPLES

Samples or sample containers should be clearly and permanently labelled, immediately before or after taking a sample, to ensure reliable identification. Labelling should be done in accordance with the sampling protocol.

If samples are to be transported to another laboratory, the transport material should be clearly labelled with the name, address and telephone number of the recipient. If the transport material is to be returned, the name of the sender should be written on the label. Other markings on the transport material may be 'fragile', 'store cold', 'store frozen', etc.

Upon online registration of samples, it is important that the sample is marked so that it is traceable to the information in the electronic system.

If possible, samples of pre-packaged foods should be delivered to the laboratory in sealed, original packaging. In the case of large amounts of food, which are non-pre-packaged, or of packages too large for delivery to the laboratory, samples should be transferred to containers, using equipment ensuring that the sample is not microbiologically and/or chemically contaminated. If the sample container must be sealed officially, it should be done in the presence of representatives of all concerned parties. The seal may consist of strong tape or similar material, making falsification impossible without damaging the seal. The date, sample number and the name or initials of the sampling personnel, and possibly a stamp or similar, are noted on the seal. If several samples are required, all should be treated in the same manner. Preparation of the laboratory sample, reducing of the sample, grinding and homogenising should be carried out in suitable (protected) places.

SAMPLING REPORT

The completed sampling report should contain the information listed below, which ensures unequivocal identification of the sample and gives information about the characteristics of the sample:

- Place and site of sampling (e.g. producer, wholesale/retail dealer, transport vehicle, consumer and room x, refrigerator y, counter z etc.).
- Lot number (identification) /farm code (+ date of production, date of packing, expiry date and possibly production number).
- Date and time of sampling.
- Name and nature (composition) of the sample.
- Parameters to be analysed.
- Clear labelling, in accordance with the sample, e.g. a laboratory code.
- Manufacturer's name, possibly registry or authorization code.
- For perishable goods: the temperature of the sample or of the storage room at the time of sampling.
- Information on the sampled food, e.g. where it is sampled (on the outside, in the middle), preparation, handling, storage, time of delivery.
- Information about the transport conditions; time of the shipment, temperature etc.
- Information on counter samples and sealing. Additional information describing the sampled product or other information likely to be of assistance to the laboratory or in evaluating results should also be included.
- Reason for sampling, e.g. routine check, complaint, suspected food poisoning, monitoring quality check, sensory change.
- Method of sampling, if the method differs from the one used as routine/the prescribed method or if there are any comments on the sampling.
- Name, address, e-mail and telephone number of the sampling personnel/officer.
- Name, address, e-mail and telephone number of the laboratory.

When food poisoning is suspected, the sampling report should be supplemented with information on the course of the disease (incubation time and symptoms) and other relevant information.

The sampling report should be signed and the number of copies required made. In some cases, it may be necessary for both sampling personnel/officer and a representative of the food retailer to sign the sampling report. Procedures in such situations should be described in internal guidelines for sampling. Normally, a company is given the opportunity to obtain a counter/replicate sample, when their products are sampled for enforcement, trade and referee purposes. This sample should be taken at the same place, time, in the same manner, etc. and resemble the official sample as closely as possible. One of the samples is sent for analysis. If there is any doubt about the result of the analysis of the first sample, the second sample (the control sample) is analysed.

CONDITIONS FOR TRANSPORT OR SHIPPING OF SAMPLES

When handling, storing and transporting samples, no major changes should occur before the samples are examined. All necessary precautions should be used to avoid contamination or changes in composition of the samples during transportation or storage. The sample should be dispatched to the laboratory as soon as possible

after sampling. The time of delivery of samples to a laboratory should be agreed upon in advance, with the delivery agents. The laboratory should also be informed on the number and type of samples to be examined.

Perishable, unpacked goods should be analysed as soon as possible after sampling. It is therefore essential that such samples are transported to the laboratory as quickly as possible and maintained at the prescribed temperature. It should be noted that certain particularly perishable goods, e.g. fresh fish and shellfish, cannot be transported for very long before they spoil. When transporting such foods, it is important to minimize the transport time and to ensure that the temperature of the sample is as low as possible, without freezing.

Fresh fish and shellfish should be packed in crushed ice or snow, and be transported in insulated and watertight containers. Other containers are acceptable, if the samples are kept below 2°C during the entire transport.

For microbiological analyses, the analysis shall start within 24 hours after sampling, including transportation. For some parameters in water, a shorter time interval between the sampling and the analysis might be required.

During sampling, frozen samples should be stored in a pre-chilled container and placed in a freezer immediately after sampling. These should then be transported to the laboratory in isolated boxes. If the duration of transport is such that there is a risk of the samples thawing, dry ice (carbon dioxide snow) or possibly cooling elements should be used.

When transporting dry products or preserves, cooling is not usually needed. However, the temperature should not exceed 25oC and the samples must be protected from high humidity.

STORAGE AND PRE-TREATMENT OF THE SAMPLES AT THE LABORATORY

In the course of sampling and preparation of laboratory samples, precautions should be taken to avoid any changes, which might affect the contents, adversely affect the analytical determination or make the samples unrepresentative.

✓ Registering samples at the laboratory. On arrival at the laboratory, the following should be checked: the samples are accompanied by a sampling report (delivery note), containing necessary information the sample labelling agrees with the report; if not, the samples should be discarded the samples have been correctly transported (if necessary, control the temperature) the sample containers are intact.

The laboratory must have a written standard procedure for checking and rejecting samples.

If samples arrive at the laboratory after the specified period of time from sampling has elapsed, and it is too late for them to be examined (applies to microbiological analysis in particular), or if the sample temperature is too high, or if samples (package or wrapping) are damaged or contaminated during transport, rejection of the samples should be considered. However, if such samples are analysed, the noted deficiencies should always be recorded and mentioned in the analysis report. Samples of foods suspected of having caused disease should always be examined, even if deficiencies during transport have been noted. If the purpose of the analysis is something else than quality control, such as contracted analysis in connection with research and development, it is of no interest to analyse damaged or atypical samples. Ethical reasons also indicate that sensory analysis, where tasting is included, should not be conducted on potentially poisoned samples.

✓ Preparation of the laboratory samples After the primary sampling, the laboratory receives either a composite sample or a single increment, and if the sampling has been carried out correctly, the sample represents the lot. The sample can consist of a few grams up to many kilos. For small sample quantities, the laboratory might analyse the whole sample, but in most cases, the laboratory will have to reduce the sample size. Often only a few milligrams or grams are needed for the analysis. Usually, the laboratory will provide the analytical results with a measurement uncertainty with a 95% confidence interval, also because it is required by the accreditation bodies. The measurement should include the uncertainty that comes from the reduction of the laboratory sample /the withdrawal of the increments (the secondary sampling). This contribution to the uncertainty might often be larger than the contribution from the analysis. A laboratory should always strive to ensure that the uncertainty of the results is as low as possible, and should therefore have well described and appropriate procedures and equipment for sample reduction and withdrawal of subsamples. Many sample types (cereals, dry food and powder) may advantageously be reduced using commercial sampling divider device

By use of such divider, a sample can be divided into a number of portions with a very uniform composition, which might also be documented statistically. If it is not possible to divide the samples using the equipment, due to too large particles, it should be considered to grind up samples before dividing.

The quartering method for reducing sample size is commonly used. The entire composite sample is homogenized (if possible) and then divided into 4 pieces/parts. The opposing quarters are then combined and divided into 4 again, until the desired size of laboratory sample is obtained,

After reduction of the laboratory sample, the sample volume is still usually more than ten times the volume required for the analysis. The reduced sample is often heterogeneous even though it appears to be homogenous. Thus the sample needs to be grind or for instance suspended in slurry prior to withdrawal of one or several samples, which are then analysed individually or as a composite sample.

For solid and liquid food samples, special procedures are required for dividing and withdrawal of subsamples. Often, industry specific methods are available for this purpose and are described in standards (e.g. dairy products, ISO/IDF standards). Hard cheeses have a very heterogeneous composition from the surface to the centre. Therefore, geometrically representative samples should be withdrawn, and the cheese sample should then be grated before analysis for example of analysis of fat and protein.

The opposing quarters are to be removed

Analyse of fish raw materials is another complex area. Analysis of fish fillets for the determination of for instance fat and water are carried out usually after homogenisation in a food processor. When minced fish meat looks uniform, subsamples are withdrawn for analysis. Tests have shown that the minced meat is heterogeneous even after homogenisation in a food processor, and that the combined measurement uncertainty may be influences statistically by this [cf. Laboratory sampling for chemical analysis].

The sampling handling can have a direct influence of the results, and it may therefore be necessary for extraordinary measures to protect the sample. Many vitamins degrade unless the sample is protected from light with UC filters on windows and special light fixtures. Milling processes can remove water from the samples, and for instance in feed analysis a correction for the water loss is included in the final analytical result.

Optimisation of subsampling can be based on a record of the analytical uncertainty divided on types of samples. If the laboratory registers the results of duplicate determinations for the different matrices, a data analysis might show which matrices that provides the highest spread/variation. The laboratory can then work for a targeted improvement. Often, the result is that a visual assessment gives a false sense of security; samples that appear homogenous may be highly heterogeneous. Especially for some analytes such as vitamins and minerals this can be a challenge.

For sensory assessments of products, the nature of the product determines how the samples will be served. The statistical model is also important; if the statistical model is established, it is conceivable that a particular program needs to be followed, in other cases it may be practical wishes or limitations that determines. When assessing carrots for instance, there several options: 1) Each assessor is served one or more carrots a. The assessor gives a mean evaluation if several carrots are served simultaneously b. The assessor gives evaluates each carrot separately 2) All the carrots from a sample (representing specie, growing location, storage condition or other factors) are cut into cubes. Each assessor gets the number of serving samples (typically 50 - 100g) or sensory replicates required by the statically model. 3) A carrot is divided into as many parts as there are assessors in the panel. A possible disadvantage is that the taste of a carrot depends on whether if the part is close to the top or close to the tip. a. The assessors get randomly assigned parts of the carrot b. Each assessor gets always the same part of the carrot

Option 3 may seem somewhat abnormal, partly because it assumes that the individual carrots are large and/or there very few assessors. However, 3b is often used on large fillets and on similar samples. Practical considerations when it comes to encoding and serving also play a role: usually it is not possible to prepare the sample in advance and analyses them, i.e. serve it to the assessors when it is appropriate for the laboratory managers. In particular in the preparation (usually heating) of the samples may present prone practical challenges.

✓ Storage of samples prior to and following examination. When samples are stored at the laboratory, they must be protected from changes caused by chemical, physical or mechanical factors.

The laboratory should have sufficient cold storage facilities to keep samples/sample remainders, but these should not be stored together with microbiological media, reagents etc. When storing samples in a refrigerator or freezer prior to, or after transport, the temperature of the refrigerator should be between 1°C and 5°C, and that of the freezer -15oC or colder.

Samples to be stored at the laboratory for a specific period of time prior to examination, e.g. in connection with stability studies, should be stored in accordance with the storage instructions on the packaging. The temperatures of such storage facilities should be controlled to the same accuracy as other facilities.

Samples of dried or preserved foods may be stored at room temperature.

Remainders of a sample should be stored in the same way as the original sample (the replicates), for a given time of period, in accordance to the laboratory quality assurance system. Storage conditions must be assessed for each individual sample. Samples of perishable foods should be refrigerated/frozen.

✓ Pre-treatment of samples for microbiological examination. Pre-treatment of samples for microbiological examinations should be performed according to NMKL 91, 6. Ed., 2010: Preparation of the test sample and initial suspension of food and animal feeding stuffs for quantitative microbiological examination. For products forming clumps or a solid gel, it may be necessary to have a higher dilution than 1:10, which is the most common dilution.

Frozen foods should be thawed at a maximum temperature of +4oC within 18 hours. Smaller, easily-thawed samples may be placed in a thermostatic cabinet at a maximum temperature of 37oC, for up to 15 minutes.

Various types of equipment may be used for homogenisation. A homogeniser of the Stomacher type is preferable, since it is easier to use than other equivalent equipment. It is not, however, suitable for homogenising foods containing hard particles that may penetrate the plastic bags. Sterile and non-sterile plastic bags, with and without filters, are commercially available, for use in Stomacher homogenisers. Each case should be individually evaluated. The homogenisation time should be fixed, and should not be allowed to vary from sample to sample; thirty seconds is suitable for most foods. It is advisable to use an electronic timer that stops homogenisation after the set time.

INTERPRETATION OF ANALYTICAL RESULTS

Uncertainties related to analytical results are always present. The uncertainty of the analysis may be included in the result, but the uncertainty in connection with the sampling is seldom referred to. This discrepancy exists, in spite of the fact that the uncertainty related to the sampling is probably much greater compared to the uncertainty obtained on analysis. However, estimation of the uncertainty of sampling is more comprehensive and complicated.

Within microbiology, the recipient of the results is the one who most often consider the results and not any external laboratory unless this is agreed upon. Many microbiological measurements are part of surveillance (e.g. quality control) and where there are often not specified limit. The EU Regulation on microbiological criteria (EC 2073/2005) provides specified limits for several pathogens and for a few hygiene and indicator organisms.

The measurement uncertainty (MU) from sampling will generally be larger than the uncertainty related to the analysis. In addition to MU from analysis and sampling, the heterogeneity and systematic error from the sampling will contribute to the total MU. The figure below illustrates the factors that contribute to the uncertainty in decisions.

3.2 Matrices and applied technologies

3.2.1 The agri-food

3.2.1.1 Meat products

Scope

In this document are described sampling methods, storage and transportation , which applies to meat and meat products, including meat and poultry products.

Sampling methods. General requirements

Sampling is performed by a specialist authorized by interested parties and properly trained in the relevant field. He must act independently and not allow the intervention of a third party, under his responsibility may use the help of others. The sampling specialist and his assistants should take appropriate measures to prevent contamination of the supply or batch and the samples taken (for example, wash hands before sampling).

An accompanying document (for example, a report or protocol, or an act) signed by a sampling specialist and representatives of interested parties, if present, should be attached to samples sent to the laboratory.

The accompanying document should contain the following basic information:

- the name and address of the sampling specialist;
- the names and addresses of representatives of interested parties (when they are present);
- location, date and time of sampling;
- type and source (origin) of the supply or batch;
- the number and number of units of products that make up the supply or batch;
- marking (designation) and the number of the lot;

- identification of used railcars, trucks or vessel;
- name of the point of shipment;
- name of the destination of the goods;
- the date of arrival of the delivery or the batch;
- name and address of the seller (manufacturer);
- name and address of the buyer;
- number and date of the invoice or contract;
- sampling method;
- the number of samples taken from each batch;
- designation (name) of the samples taken;
- number and labeling of the batch (s) from which samples were taken;
- the mass of individual single samples;
- the name of the organizations (for example, laboratory, center) where the selected samples are sent.

The accompanying document should also indicate all factors that may affect sampling, such as packaging conditions and environmental conditions (temperature and humidity), temperature of the product and certain types of samples, methods for sterilizing instruments and containers used for sampling, as well as Any other special information relating to the materials from which samples are taken.

To the extent possible, representatives of interested parties should be provided with a sample.

Each sample sent to the laboratory must be isolated (sealed) and labeled. Sealing should be carried out in such a way that access to the content or label is open only when the seal is destroyed.

Labels should be of a quality and size appropriate for their intended use (for example, a slightly colored, greaseproof, waterproof plate with a reinforced hole). The marking must be indelible and non-erasable and contain information necessary for the identification of single samples:

- type and source (origin) of the supply or batch (s);
- the number and number of units of products that make up the supply or batch (s);
- location, date of sampling;
- name of the seller (manufacturer) and buyer;
- the number and labeling of the batch (s) from which single samples were taken;
- ambient air temperature at the time of sampling .

Requirements for equipment and containers used for sampling.

General requirements

Materials used in the manufacture of containers that are in direct contact with samples should be water- and grease-resistant, insoluble and non-absorbent.

The capacity and shape of the container for sampling should correspond to the size of a single sample to be taken and be securely closed, for example, when using bottles, cans with rubber, plastic stoppers, cork stoppers, metal or plastic screw caps. Before sealing, the cap must be covered with a thin film of inert material. Threaded caps must have an inert material sealing gasket.

Materials and equipment should not affect the results of the research . If necessary, exposure to light and / or oxygen should be minimized.

Requirements for equipment and packaging used for sampling for chemical analysis

Sampling equipment and containers should be dry and clean, and should not affect the chemical composition of the product.

Requirements for equipment and packaging used for sampling intended for sensory (organoleptic) analysis

Sampling equipment and containers should be dry and clean and should not impart any taste or smell to the product.

Requirements for equipment and containers used for sampling intended for microbiological analysis and for other purposes (for example, for biological, parasitological, serological, histological, toxicological tests or for determination the persistence of products by temperature control method)

Sampling equipment and containers should be clean, sterile and should not affect the microflora of food.

If necessary, equipment and sampling containers are sterilized in one of the following ways:

1. wet sterilization - at least 20 minutes at a temperature not lower than 121 ° C;
2. dry sterilization - not less than 1 hour at a temperature not lower than 170 ° C in a drying cabinet with forced air circulation to maintain an appropriate temperature throughout the cabinet or hot air in the sterilizer without forced air circulation at a temperature of from 180 to 185 ° C for 15 minutes or at a temperature of from 160 to 165 ° C for 120 minutes.

Handling of tools is allowed by immersion in ethyl alcohol with subsequent flaming.

If it is impossible to use these methods, as well as if the equipment or packaging should be used immediately after sterilization, one of the following methods can be applied:

1. exposure to steam for 1 h at 100 ° C;
2. immersion in 96% ethanol followed by flaming until ethanol is completely burned;
3. treatment of all working surfaces with a flame of hydrocarbon gas (propane or butane);

Number of samples taken

The number of samples to be taken in order to obtain a representative primary sample of the delivery or batch (s) must comply with the standard methods of sampling for a specific type of product specified in the contract or other agreement between the parties concerned.

For various types of studies (chemical, microbiological, physical or sensory analysis), sampling is carried out separately for each type.

Sampling methods

Classification of meat and meat products for sampling

To determine the sampling method, meat and meat products are classified by type:

A- delivery or batch of meat and meat products, produced in the form of single products or individual packages of products of any mass (for example, sausages, sausages; Half fabricated products, minced meat, packed under vacuum; sausage, sliced; canned boiled ham) or in the form of meat pieces, or carcasses (parts of carcasses) not exceeding 2 kg in mass;

B - carcasses, parts of carcasses, meat, salted, dried or other preservation methods, in chunks exceeding 2 kg in weight (for example, chopped bacon, bacon half, fresh or frozen meat cut, fresh or frozen boned meat, beef carcass or quarter, pork half carcass, lamb carcass, poultry carcass, venison), and meat obtained by the method of separation or dehydrated meat.

Depending on the mass and trade quality of products, it may be necessary to take secondary samples using only a portion (s) of each primary sample, taking into account the types of studies for which they are taken.

Sampling of meat or meat products of type A

As the primary sample take a part or a whole piece of the product. In accordance with the standard sampling methods for the specific type of product, the required number of primary samples is taken from each batch.

Sampling of meat and meat products of type B specified

In accordance with the standard methods of sampling for a specific type of product, the required number of primary samples is taken from each batch and packaged either for further selection of secondary samples for destructive control in the laboratory (for example, for chemical or microbiological research) or for non-destructive testing (for example, visual inspection, organoleptic analysis, microbiological research using a tampon).

No single sample taken from a carcass or other large piece of meat can be representative of the product as a whole, however, it is almost impossible to conduct research on a whole carcass or large piece of meat. Therefore, for taking primary or secondary samples, depending on their purpose, one of the following sampling methods should be selected.

Sampling is generally carried out in the following ways:

a) single samples from the surface (for example, to detect bacteria of the Escherichia coli group or Salmonellae) are selected by wiping the entire surface of the product (or selected areas) with large wet tampons or (for quantitative microbiological studies) by marking using the template (stencil) of the areas from which then the sample is cut or, in the case of frozen meat, scraped from the surface;

b) from a primary sample with a mass of 500 to 1000 g taken for chemical or microbiological examination, a secondary sample is taken from the side of the surface of the fresh section, causing minimal damage to the tissue;

c) a muscle sample for microbiological examination (for example, to determine the causes of bone spoilage - bone tan) is taken from the affected part of the carcass using a stainless steel tool for dissecting the muscle, and from frozen meat using a grater;

d) single samples of fat (for example, to determine the content of fat-soluble substances, such as pesticides) are taken, if possible, from animal renal fat or internal fowl fat;

e) single samples of separating juice (for example, from frozen meat packaged under vacuum) are taken aseptically using sterile syringes and / or flasks and cans through foil or after opening the package. If meat is returned to the batch, it must be done after unpacking under vacuum.

Temperature

If possible, the temperature of each selected batch should be recorded.

Sample packaging

Meat or meat products of type A

If individual single samples are in an airtight container, no additional packaging is required. For other types of products it is necessary to place each sample in the appropriate container, carefully close, isolate and label.

Meat or meat products of type B

Each single sample is packaged in a bag of suitable polymeric material, carefully closed, isolated and labeled

Microbiological tampons are placed in sterile containers, and samples of separating juice are placed in sterile flasks or bottles.

Note - If it is possible to pack different single samples together in one or several containers, then there is no need to isolate and label each single sample when fulfilling the requirements for isolating and labeling these containers.

Transportation and storage of selected samples

Selected samples are sent to the laboratory immediately after sampling, and the sample temperature should correspond to the storage temperature of the product; in the case of refrigerated foods, samples are transported:

a) at a temperature of from 0 to 2 ° C, if the study will be conducted within 24 hours;

b) at a temperature not higher than minus 24 ° C, if the study will be conducted after more than 24 hours; samples for physical or sensory (organoleptic) analysis, in general, should not be frozen.

When transporting it is necessary to take precautionary measures against exposure to direct sunlight on selected samples. Samples must be delivered to the laboratory in an intact condition, without compromising the integrity of the packaging and isolation (seals).

3.2.1.2 Seafood and fish

Organization of work of industrial laboratory

The leading role in providing of high quality production by enterprises belongs to the technical control departments and industrial laboratories.

The work of QCD and industrial laboratories for improving the quality of products should be carried out simultaneously in several ways.

The main area includes control and analytical functions of the industrial laboratory. QCD and the industrial laboratory controls the production in the following order:

1) Control the quality of raw materials;
2) Control the accuracy of the technological process in accordance with technological instructions in order to improve product quality and reduce production losses;
3) identify the causes of shortage and decrease of the fish products quality and develop measures for their elimination;
4) check the quality of auxiliary materials to prevent the use in the production of bad materials, which can cause a decrease in the quality of products or increase losses. Check the quality of tare and packing

materials in order to ensure the preservation of the goods during transportation and storage in the trading network;

5) check the consumption of raw materials per unit of production, the consumption of materials and packaging in order to reduce production losses and reduce the cost of production;
6) control the quality of the finished product in order to ensure the production of high quality products. All types of products produced at the enterprise can be delivered to the consumer only after quality acceptance of QCD or laboratory.

Terms and definitions

A **batch** is the quantity of goods of uniform quality and name, produced within a certain time interval in the same conditions, intended for import / export, drawn up by one document certifying the quality and by one customs declaration.

Sampling is the number of items of goods to be selected from the total number of items for inspection.

Sample volume is the quantity of goods selected from each batch.

Specimenis a separate unit of the object (product) under study.

A sample is the quantity of a non-piece item or part of a specimen selected from the batch being examined, identical to the composition and characteristics of the batch.

Split sample is a sample selected from the productionin one step. It characterizes the quality of the goods in a facility or container, or at a particular level of a tank or vehicle.

Consolidated sampleis a sample composed of mixed split samples, selected in the appropriate order and combined in the specified ratio, characterized by the average values of the characteristics of the goods.

Analytical sampleis a part of a consolidated sample used for laboratory testing.

Acontrol sample is a portion of a consolidated sample stored in the laboratory for two months and used for arbitration studies.

An arbitrage sample is the part of a fasten up consolidated sample that is stored by the person who sent the sample for research, or by the research organization. It is used for arbitration proceedings in case of disagreement or appeal against the decision made by the research organization.

Sampling procedure

Live, asleep, chilled or salted fish comes in large batches in receiving and transport vessels to the processing plant. It is already sorted in breeds, sizes and freshness. However, there are times when on-site fishing is not possible and fish comes to the plant mixed in breeds and sizes, and sometimes in quality.

Information about the time (date), place, tools and character of the fishing, as well as the terms and conditions of transportation plays very important role in the quality of raw fish. This information can make it clear when recognizing the causes of defects in the raw material.

Live fish deliveredin the slots to the fish factoryis in no doubt about freshness. To determine other features (size, fatness, condition of the investment) the sample is taken from living fish in such way: individual specimens of fish are hooked by a dip net at different places, then they are subjected to visual analysis. If there is dormant fish it is separated from the living one.

The sampling of fresh, chilled or salted fish delivered to the fish factory by fishing, receiving or transport vessels is more complex process. The size of the original sample for determining the quality of the fish depends on the quantity of fish on the vessel, as well as on the degree of its uniformity, however, it should not exceed 1-2c.

Sampling is complicated by the possible heterogeneity of fish in quality in different layers of the same chest or compartment. Therefore, If the situation makes it possible, after a preliminary visual analysis of all fish and certain specimens, the selection of the original sample is made in three steps: at the beginning of unloading the chest or compartment, after unloading half of all fish, and finally, from the last quarter. For better characterization of fish quality, each unloading is made up of several private unloadings. The unloadings are joined together, thus forming the original sample for the whole batch.

After determining the total weight of the original sample, the fish are dissected by breed, size and quality into separate tare stretchers.

Even greater difficulties are encountered in the selection of the sample for laboratory testing, since, having a minimum weight, at the same time it must sufficiently characterize the batch of fish with organoleptic prisms and is superfluous with the apparent freshness or deterioration of the fish.

In the selection of laboratory samples, the following two cases may be presented.

1. The original sample is externally homogeneous in freshness, and thus any single specimen of fish will sufficiently characterize the whole batch. However, it is recommended to take at least three to five

specimens, depending on the size of the fish. Small fish, such as sprat, tusks, anchovies, salacas, etc., are selected by weight in an amount of not less than 500 g. The selected specimens are immediately submitted for research, and the test results are referred to the batch as a whole.

2. The original sample is a mixture of fish of different freshness. In this case, you need to sort the original sample by external features. After weighing from each part of the original sample, consisting of a homogeneousfish the average sample is taken for laboratory research in the same way as for a homogeneous batch. Knowing the weight ratios of the certain categories in the original sample, the results of the study can be attributed to a very specific weight part of this batch. The Astrakhan branch of VNIRO (Russian Federal Research Institute of Fisheries and Oceanography, 2) recommends the following practical methods of fish sampling which is salted delivered to the plant.
3. To determine the quality of fish from each chest of the transport vessel make 25-30 unloadings. The weight of each unloading should be: a) for the herring not disassembled - about 5 kg, b) for the small herring, Caspian shads, vobla – from 2.5 to 3.0 kg. The selected initial sample (the sum of all recesses) is sorted by external characteristics and the result obtained is attributed to the whole pair. In the case where no determination of grade is required, but only the detection of average salinity of fish and batch, the following method of sampling from boxes of transport vessels is used.

150-200 specimens of fish of predominant size are selected from different places in each chest. Selected fish are sorted by consistency into three categories: a) hard, b) medium and c) soft. In proportion to its weight,from each category a correspondingamount of fish is selected for the compilation of the average sample for chemical research.

The main issues of expertise

Chemical analysis gives successful practical results only if the sample for study has a composition typical of the whole batch.

The number of samples taken for analysis shall be determined in advance, in accordance with the requirements of the relevant regulatory documents. The sampling officer must take at least the minimum number of goods required for laboratory testing. In the case of a tightly closed and sealedcontainer containing packages for retail sales, the smallest package can usually be considered as an appropriate sample for research.

The sample under study should characterize the typical properties of the goods of the whole batch, so the method of its obtaining is important. Most of the unliquid materials and some liquid materials can be heterogeneous, so one has to work hard to obtain an appropriate sample from a very large batch. In cases where the bratch from which the sample is taken is large, small portions of the sample should be taken from different places of the bratch so that they can be combined into one easily processed sample having the average composition as the whole bratch. If the sampling officer does not have the technical ability to mix portions of the sample or cannot determine whether these portions are homogeneous or not, he must send each portion of the sample separately.

Liquidsamples stored in metal jars or barrels should be taken after shaking, stirring or shaking sufficiently, as they are not always homogeneous. Powder, particles or sludge specimens packed in tare should be collected from a portion that does not come in direct contact with the air. Typically, these samples must be taken from more than two separate containers. However, this rule does not apply to homogeneous goods, such as canned goods or bottles.

Samples such as petroleum products or molasses stored in a tank or cisterna should be taken from each of the three layers (top, middle and bottom) after the tank or cisterna is filled and stabilize.

To prevent or minimize contamination, you should take care about the application of sampling methods, sampling devices and sample containers (for example, polyethylene or polypropylene tare with double bottle hood, one of which is screw bottle hood to avoid loss or contamination of content). Rapid sampling should be used for samples sensitive to the atmosphere (moisture, carbon dioxide, etc.). Always use a clean and dry sampler and container. In particular, dark containers are used for samples sensitive to sunlight.

After the sample has been taken, strict precautions should be taken to avoid swapping or tampering with the samples. Such measures may include sealing action the sample container or sealing it with an official seal. Each sample should be labeled immediately, indicating the name, number, and date of sampling in order to exclude completely the possibility of confusion in the determination of the samples and the results of their examination.

Labels shall be fixed to the samples in such a way that they will not be torn off or damaged when the specimen is opened for examination and for peeling off the seal. Because during the expertise the identification label must remain intact.

Careful handling of flammable, explosive, toxic, corrosive or poisonous specimens should be handled, and packaging with such specimens must have a special marking such as "Dangerous".

In the case of perishable products, the sample should be clearly marked as a "perishable product" and transported at a temperature of 0 to 5 °C and not later than 2 hours forwarded to expert customs offices, in the case of those products, at which stipulates special conditions for transportation in the regulatory and technical documentation.

Table 1 – Methods of sampling and size of samples of fish, marine mammals, marine invertebrates and products of their processing

Table 1

Product name	**Vehicle, container**	**Sampling device**	**Size of Split sample**	**Quantity of Consolidated sample**
Raw product (fish and invertebrates), live, chilled, frozen, minced fish, salted, spicy, marinated, stockfish,sun-burned fish dried and smoked fish, salty balyk semi-finished products, stockfish and smoked balyk products, pastes, condensed fish solubles, concentrates, viziga, food and feed waste	Tanks, barrels, blocks, boxes	A net for live fish, stainless knife	From the different places of the transport container, three splitsamples are taken (one specimen or part of one specimen, block of fish, fillet, fish sausage; several specimens or a handful of small fish (sparling, sprat) or part of the product). From frozen blocks separate two diagonally opposite pieces which are up to 0,1 kg each, and from the middle - the strip up to 0,2 kg. 1 or 2 units of consumer packaging is selected from each transport container.	1,0 / 1,0 kg but not less than 1 unit / 1 unit. for retail
Frozen: meat, peritoneum and other products (including liver) from marine mammals, fish liver	Blocks, pieces	stainless knife	three split samples of not more than 0.3 kg each are drawn from different places of the block or piece after defrosting	1,0 / 1,0 kg but not less than 1 unit / 1 unit. for retail
Fat of fish and marine mammals, spermacetate oil	Barrels, cans, cylinders or drums, glass bottles	Siphon, glass tube or tubular sampler, sampler tap, zone sampler	one sample not more than 2.0 dm^3 is taken from barrels, cans, cylinders and glass bottles after thorough mixing. the sampling shall be carried out continuously and evenly throughout the filling or unloading	

			period of each railway and oil tank truck. The jet volume is adjusted so that the consolidated sample is 0.02% of the volume of the railway cisterna and 0.07% of the oil tank truck. From the tanks of vessels and stationary tanks, samples are taken layer by layer after each 2 m. From the lower layer - at a distance of 0.5 m from the bottom, from the upper - 0.2 m from the surface of the fat. Ifthe lower layer of the fat is heterogeneous thesampleistaken every 0.5 m to a homogeneous layer	
crystalline sperm oil	Briquettes	Screw probe	From each opened transport container, from different places of each briquette, three samples not less than 0,1 kg	
Invertebrates and processing products	Blocks, boxes, jars	stainless knife	Three split samples of at least 0.2 kg shall be taken from different places of each opened transport container. Two pieces of 0.1 kg each are separated from one of the frozen blocks of the transport container and from the center of the block (about 0.2 kg solid strip). From each transport container 1-2 units of consumer packaging	
Feed flour and cereals, chitin, chitazone	Sack, box	sampler probe	Multiple point samples (from the top, middle and bottom of the pack) of 0,05 kg are taken from different places of the opened transport container	1,0 / 1,0 kg but not less than 1 pack. / 1 pack. for retail

Fish glue	Containers, boxes, packs, barrels, cans	Glass tubes, sampler probe	For each opened transport container 1 pack is taken, and product is packed spilled - plates or scraps up to 0,1 kg. From each transport container of liquid glue select several split samplesfor 0,3 kg, and frozen one – for 0,5 kg each	
pearl paste, pearl preparation	Jars	Stainless spoon	From each opened transport container 3 jars are selected, and at least 10 point samples of 0,5 kg are selected.	
True ambergris	Lumps		From each opened transport container several split samples of small amber and amber crumbs of 0.03 kg each are selected, and large amber - at least 10 pieces of transport container and from them several samples of 0.03 kg are snaped off.	
Liquid feed stuff, krill and krill fodder (except flour)	Jars	Sampling probe	From each opened transport container several split samples of about 0,3 kg	

Note: Consolidated sampl**e**isn't carried out for caviar, caviar paste, ready-cooked foods (including sausages), raw semi-finished products.

3.2.1.3 Products of plant origin

Sampling of fruit and vegetable products

Correct sampling for research along with the correct use of the accepted method for determining a single indicator of product quality is one of the most important tasks.

The composition of the prepared sample should reflect the quality of the entire batch as a whole. it is necessary to take from a homogeneous batch of products such a number of packaging units (cans, boxes, barrels, etc.) that would reflect the quality of the entire batch to compile the initial and average samples. In practice, the number of product units which are selected for the preparation of the initial sample is established by the acceptance rules set indicated in the relevant standards.

Terms and Definitions

Spot sample: The quantity of a product of one denomination taken at a time from one point in a certain batch.

Composite sample: A sample obtained by combining all spot samples taken from various areas in a batch and characterizing the quality of the entire batch.

Shortened (medium) sample: A representative portion of a composite sample obtained by successively dividing or reducing so that the mass or volume meets the requirements for laboratory and control samples.

Laboratory Sample: Part of a reduced sample for laboratory tests (analysis).

Control (arbitrage) sample: Part of the reduced sample stored in the laboratory conducting tests (analyzes) or at the manufacturer of the product and intended for repeated testing (analysis) in case of disagreement in assessing the quality of the product according to the test results (analyzes).

Acceptance number; Ac: The largest number of discrepancies or non-conforming units in the sample in terms of selective control according to the alternative criterion at which batch acceptance is allowed.

Rejection number; Re: The smallest number of discrepancies or non-conforming units in the sample in terms of selective control according to an alternative pattern in which the batch is not accepted.

Sampling of products of different consistency is carried out by different objects. All foodstuff can be combined into 6 groups:

- liquid homogeneous materials;
-heterogeneous liquid materials capable to exfoliate and form emulsions;
- materials of solid smearing consistency, packaged in large containers (barrels, boxes, etc.);
- bulk materials;
- fruits, vegetables, canned goods.

Liquids samples are taken using special probe tubes or a Bakhtin pump (tube with a piston, ball valves and a drain).

Samples of bulk and fine-grained products are taken with a special bag probe from different places - the upper, middle and lower layers of the bag. Samples of bulk products which are in wagons, machine bodies, bins are taken with a wagon probe.

Samples of solid and smearing products packed in boxes or barrels are taken with an oil dipstick. Liquid heterogeneous products are most conveniently selected when unloading containers (tanks, etc.) at the beginning, middle and end of the discharge. Samples of liquid products (syrups, extracts, juices, etc.) packaged in barrels, ballons or bottles are taken from each selected and opened packing unit in the following quantities: from each barrel - 200 cm3, from each bottle - 100 cm3. Moreover, 3% of packing units are subject to opening (but not less than three barrels). The same number of boxes is opened if the products are packed in boxes or cages. Samples of viscous, smearing consistency products are taken from at least 200 g from different layers of the opened packaging unit.

If canned food is packed in boxes, samples of canned food for the compilation of the initial sample are selected on the base of the number of packaging units in a homogeneous batch. So, if there are up to 500 units of packaging (boxes, boxes, cages) in a batch, 5 units are selected for opening. If the lot size exceeds 500 units, then 8 or more packaging units are subject to opening.

Samples are taken from each selected and opened package in accordance with the requirements of the standard acceptance rules and sampling methods. Samples of individual packing units are combined and they make up the original sample.

Sample preparation for physico-chemical research consists in obtaining a homogeneous mass of the product by chopping, grinding, mixing (depending on its type).

The following operations are carried out before grinding the samples:

- remove pits from drupe products; spices, twigs, sepals and impurities are removed in other products;
- products containing animal fats are heated in a water bath, in a thermostat or in an oven until the fat melts;
- frozen foods are pre-defrosted in a closed dish; the liquid phase formed during thawing is added to the ground product.

Depending on the consistency, a product sample is grinded using a meat grinder, mill, homogenizer, mixer or in a mortar until a homogeneous mass is obtained. If the liquid was separated from the product to determine the ratio of the parts, then both phases are combined and mixed after grinding the solid part. The same thing happens in cases where there is no need to determine the ratio of particles of canned food.

Samples of homogeneous liquid and puree products are only mixed.

The prepared product sample is placed in a glass vessel. To determine the vitamins, a sample is taken immediately after preparation of the sample, and for determination of the rest of the physicochemical analyzes the samples are taken during the day. In this case, the sample is stored at a temperature from 0 to 5 °C.

A number of requirements must be taken into account for the preparation of product samples:

- to determine the mass fraction of heavy metals (toxic elements), grinding is carried out in an instrument made of a material that cannot contaminate the product with metals;
- to determine the mass fraction of vitamin C in the product, its excessive aeration, heating and contact with metal surfaces are not allowed;
- to determine the mechanical impurities by flotation method, the sample of the product is not grinded, but only crushed and mixed.

Sampling rules of processed fruit and vegetable

Fruit and vegetable processing products include fruit and vegetable juices, nectars, juice drinks, fruit and vegetable concentrated juices, squashes and concentrated squashes, fruit drinks and concentrated fruit drinks, jelly, compote, jams, marmalade, preserves, fruit and vegetable sauces, ketchups

Acceptance procedure

Fruit and vegetable processing products are accepted by batches. To check the conformity of the marking, trade dress and fullness of the transport packaging from each batch of products, a sampling should be selected randomly, the volume of which is indicated in table 1

Table 1

Batch volume, units of transport packaging, pcs	Normal control			Enhanced control		
	Sample volume, transport packaging units, pcs	Acceptance number Ac	Rejection number, Re	Sample volume, transport packaging units, pcs	Acceptance number Ac	Rejection number, Re
Up to 25	2	0	1	3	0	1
26 - 90	2	0	1	5	0	1
91 - 150	3	0	1	8	0	1
151 -500	5	0	1	13	0	1
501 - 1200	8	0	1	20	0	1
1201 -10000	13	0	1	32	1	2
More than 10000	20	0	1	50	1	2

The verification results are considered to be satisfactory if the number of transport packaging units in the sample is less or equal to the acceptance number Ac, and the batch is rejected if it is bigger or equal to the rejection number Re.

A sample should be randomly selected from each batch of products for checking the products of processed fruits and vegetables in transport packaging for organoleptic and physico-chemical indicators (the volume of sample is indicated in table 2).

Table 2

Batch volume, units of transport packaging, pcs	Sample volume, transport packaging units, pcs	
	Normal control	Enhanced control

Up to 15	1	2
16 - 25	2	3
26 - 90	2	5
91 - 150	3	8
151 - 280	5	13
More than 280	8	20

The verification results are considered to be satisfactory if there is no a single unit of transport packaging in the sample which does not meet the established requirements.

To check the conformity of the marking, trade dress and fullness of the consumer packaging placed in the transport packaging, a random sample should be selected from each batch of fruit and vegetable products, the volume of which is indicated in table 3.

The results of the verification are considered to be satisfactory if the number of consumer packaging units in the sample that does not meet the established requirements is less or equal to the acceptance number Ac, and the batch is rejected if it is bigger or equal to the reject number Re.

table3

Batch volume, units of consumer packaging, pcs	Normal control			Enhanced control		
	Sample volume, consumer packaging units, pcs	Acceptance number Ac	Rejection number, Re	Sample volume, consumer packaging units, pcs	Acceptance number Ac	Rejection number, Re
Up to 25	3	0	1	5	0	1
26 - 90	5	0	1	8	0	1
91 - 150	8	0	1	13	0	1
151 -500	13	0	1	20	0	1
501 - 1200	20	0	1	32	1	2
1201 -10000	32	1	2	50	1	2
More than 10000	50	1	2	80	1	2

Randomly selected sample is collected from each batch of fruit and vegetable products (the volume of which is indicated in table 4) to check the contents of a consumer packaging unit (net weight (volume) of packaged products) and the average content of a batch of packaged products and the mass fraction of component parts of products packaged in consumer packaging.

Table 4

Batch volume, units of consumer packaging, pcs	Normal control			Enhanced control		
	Sample volume, consumer	Acceptance number Ac	Rejection number, Re	Sample volume, consume r	Acceptance number Ac	Rejection number, Re

	packaging units, pcs			packaging units, pcs		
Products in consumer packaging with a bulk of up to 0.35 dm^3 incl.						
Up to 50	2	0	1	3	0	1
51 – 150	2	0	1	5	1	2
151 – 500	3	0	1	8	1	2
501 - 3200	5	1	2	13	2	3
More than 3200	8	1	2	20	3	4
Products in consumer packaging with a bulk of 0,35 - 1,00 dm^3 incl.						
Up to 150	2	0	1	3	0	1
151 - 1200	2	0	1	5	1	2
1201 - 35000	3	0	1	8	1	2
More than 35000 incl	5	1	2	13	2	3
Products in consumer packaging with a bulk of more than 1,00dm^3						
Up to 50	1	0	1	2	0	1
51 - 501	2	0	1	3	0	1
501 - 35000	2	0	1	5	1	2
More than 35000	3	0	1	8	1	2

The verification results (for the mass fraction of the constituent parts of products packaged in consumer packaging) are considered to be satisfactory if the number of consumer packaging units in the sample that do not meet the established requirements is less or equal to the acceptance number Ac, and the batch is rejected if it is bigger or equal to the rejection number Re. A batch of packaged products according to the contents of a unit of consumer packaging (net weight (volume) of packaged products) and the average batch content of packaged products is accepted under the following conditions:

a) the average batch content must be bigger or equal to the net mass (volume) indicated in the labeling;
b) the number of defective packaging units (for which the negative deviation of the contents of the packaging unit exceeds the limit of permissible negative deviations) must be less or equal to the acceptance number Ac;
c) the presence of packing units in which the negative deviation of the contents of the packing unit is not double the value of the limit of permissible negative deviations.

The batch is rejected if at least one of the above mentioned conditions is not met.

To check the physico-chemical parameters of fruit and vegetable products in consumer packaging, a random sample should be selected from each batch of products, the size of which is shown in table 4. it is allowed to use products in consumer packaging, after checking the contents of the consumer packaging unit (net weight (volume) of packaged products) for the physicochemical tests (analyzes), and the average contents of the batch of packaged products and the mass fraction of components, if test conditions (analysis) allow it. If unsatisfactory results are obtained at least one of the physicochemical parameters, tests (analyzes) on a doubled sample should be repeated. The batch shall not be accepted if unsatisfactory results of repeated tests (analyzes) of at least one of the physicochemical parameters are received.

To check the organoleptic characteristics of fruit and vegetable products in consumer packaging, each batch of products must be randomly sampled, the amount of which is shown in table 4. It is allowed to use products in consumer packaging after sampling from it for physical and chemical tests (analyzes) if there were no changes in the organoleptic characteristics of the product (trade dress, consistency, etc.). The results of tests (analysis) on organoleptic indicators are considered satisfactory if the product in consumer packaging does not meet the established requirements is not found.

Sampling equipment

For sampling out of transport packaging:

- a submersible metal vessel;

- sampling tubes for the selection of liquid and viscous products;

- probes of various designs for sampling thick and viscous products;

- scoop for sampling pasty products.

For sampling products from consumer packaging:

- sampling tubes

- scoops

Sampling requirements

Sampling is carried out from undamaged consumer or transport packaging in such a way as to protect the samples from external environmental influences or accidental pollution.

Various sampling methods are used to ensure representativeness of the sample Depending on the physical condition of the product, types of transport and consumer packaging.

Sampling equipment must be clean, dry and free from odors, and the material from which it is made must not affect the quality of the sample.

It is allowed to take samples of liquid products of the same name, presented for one-time acceptance or delivery, in uniform vehicles or in uniform transport packaging, with one sampler, ensuring each time its washing with a portion of the selected product. The portion of the product used for washing is discarded.

All necessary measures should be taken to exclude changes in physico-chemical or organoleptic characteristics During sampling, transportation and storage of samples.

Sampling from transport packaging

Spot samples of liquid processed fruit and vegetable products are taken along the entire height through the hole of the container using suitable equipment.

Table 5

The name of indicators	Minimum weight or volume, kg (dm^3)			
	Consolidated sample	Reduced sample	Laboratory sample	Control sample
Organoleptic	1,00	0,50	0,25	0,25
Physicochemical	2,0	1,0	0,5	0,5
Mineral and foreign impurities	3,0	1,0	0,5	0,5
Mass fraction of particles	8,0	2,0	1,0	1,0

Before sampling liquid products, the content of the container is mixed with one of the available methods to ensure a homogenous product.

At least two spot samples must be from each unit of transport packaging. The mass of the spot sample should be from 0.3 to 3.0 kg, depending on the mass of the consolidated sample, which is shown in table 5. A visual check of the presence of impurities is carried out when mixing the liquid product. In case of detection impurities are taken from the product using the equipment for sampling and sent to the laboratory along with the samples.

Sampling from consumer packaging

Sampling of products from consumer packaging is carried out from a random sample.

Spot samples are taken by selection equal amounts of product from each unit of consumer packaging. Spot samples are mixed to form a consolidated sample. The consolidated sample is thoroughly mixed and part of the product is removed to obtain a reduced sample.

A reduced sample of the product in consumer packaging is divided into two equal parts in order to obtain laboratory and control samples.

3.2.1.4 Dairy products

Sampling of dairy products

Proper sampling of dairy products for research, along with the correct use of the accepted method of determining a simple quality measure is one of the important tasks in assessing the quality of dairy products.
Finished dairy products for sale must meet the requirements of current regulatory documents.
Samples of dairy products are selected in accordance with DSTU ISO 707: 2002 which establishes the rules of acceptance, methods of sampling dairy products and preparing them for analysis.
Regulatory documentation provides for delivery-acceptance of finished products in ***batches***. In this case, a batch is a set of units of products of the same name in a homogeneous container, with the same physicochemical and organoleptic characteristics (of the same grade) produced at one plant, on one technological equipment, during one technological cycle, by one production mode, on one date of manufacture and is drawn up with one accompanying document. In case of batch mixing, the products are sorted into homogeneous batches.
Trade dress and marking of the transport packaging shall be checked for each unit in the batch, and in the consumer packaging each unit of the transport packaging with the products included in the sample are checked before sampling.
Sampling is a complex of units that is selected to control a batch. The sampling volume (the number of transport or consumer packaging units that make up the sampling) for batches of ***drinking milk, cream, liquid cultured milk foods***in the transport container is 5%, ***sour cream***is 10% of the transport container with products. One sampling is selected if there are less than 20 units of drinking milk, cream and liquid cultured milk foods in the batch, or less than 10 units of sour cream.
The sample volume of the indicated products in consumer packaging is given in Table. 1.

Table 1

Number of units of transport pack with products in a batch	The number of units of transport pack with the product in the sampling
Up to 100	2
101 - 200	3
201 - 500	4
501 and more	5

Before opening the package with products it is cleaned of dirtiness, washed with water and wiped. After disclosure the temperature, volume of dairy products for each unit of the sample product is determined, for production in tanks these indicators are determined in each tank or its section.

Samples for microbiological analysisare taken firstly, then for the determination organoleptic parameters, and afyer that for physico-chemical ones.

One unit of consumer packaging is selected from the sample. The volume of one sample of milk, cream, sour cream or liquid cultured milk foods in consumer packaging is equal to the volume of that product included in the sample. From the combined sample of drinking milk a sample of 0.5 dm^3 is isolated after stirring for analysis.

During preparation of samples of liquid cultured milk products or sour cream, they are mixed by 5 times turning the container or a spatula within one minute after unpacking the container. Samples with a thick consistency are pre-heated to a temperature of (32±2) ºC on a water tank with a temperature of (38±2) ºC, and then they are cooled to a temperature of (20±2) ºC. A sample of the combined sample of cultured milk beverages of 0.1 dm^3volumeis selected for analysis, and a sample of sour cream weights about 100 g.

A volume of a sample of the batch of lactic cheese or its products in the transport packis up to 10% of units of transport packaging with products. Point samples are taken by probe, dipping it into the bottom of the container. Three point samples are taken from each unit of transport packaging: one is from the center, the other two are at a distance of 3...5 cm from the side wall of the container, the product is carefully mixed with a spatula, a combined sample of about 500 g is made. 100 g of product of the combined sample is selected.

The sample volume of a batch of **lactic cheese or its products** in consumer packaging is shown in Table. 2.

Table 2

Number of units of transport packaging with products in a batch	Up to 50	51 - 100	101 - 200	201 - 300	301 and more
The number of units of transport packaging with the product in the sample	2	3	4	5	6

2 units of container with products are selected from each unit of transport pack with products included in the selection if a product has a weight of up to 250 g and one unit if a product has a weight of 250 g or more.

Products are exempted from packaging for the compilation of combined sample of lactic cheese in consumer packaging, The product is transferred to dishes and mixed thoroughly. Approximately 100 g is selected for sampling from the combined sample, and about 150 g is selected for filler products.

It is recommended to examine samples immediately after preparation. If it is not possible, the samples shall be stored at 2 ... 8 ºC and not more than 4 hours.

Use tweezers to separate candied fruits, nuts, raisins, and so on from curd products with fillers to prepare them for the analysis.

Sampling of ***ice cream*** in tubes included in the sample is carried out with a probe heated in water up to (38 ± 2) °C, which is immersed in the product at a distance of 2 ... 5 cm from the wall diagonally to the bottom of the sleeve of the opposite wall. A layer of ice cream is removed from the probe with a spatula for the whole length of the probe and transferred to the dishes. The ice cream is left at room temperature until it melts completely. Nuts, candied fruits, raisins and other fillers (if present) are separated from the prepared mass, mixed thoroughly. And a combined sample of about 500 g is made. From the combined sample, a sample of about 100g is taken for analysis. The sample volume from a batch of ice cream in consumer packaging is 10% of the units of transport pack with products, if there are less than 10 units in the batch one is selected. From each unit of transport packaging with the products included in the sample, one unit of consumer packaging with products is selected.

0.1 ... 0.2% of the total packing units are selected for compilation of combined sample of ice cream in consumer packaging included in the sampling. Each unit of packed ice cream is examined separately.

The sample volume of a batch of ice cream cakes is one cake. A cake of less than 500g is used completely as a sample for analysis. From the cake weighing more than 500 g with a symmetrically located decoration, ¼ part of the cake is taken for analysis, cutting it diagonally. If decoration is asymmetrical, the cake is cut diagonally into four parts and selected two of them taking into account the proportional amount of decoration.

The cake or its parts are released from the decoration using tweezers or spatula, thoroughly mixed and a sample of 100 g is selected for analysis. Decoration must be placed in a separate dish and also sent for research. Ice cream is stirred, turning the sample container at least three times during preparation the samples for analysis. The sample temperature must be (20 ± 2) ºC.

The sample volume for each batch of ***butter*** in transport and consumer packaging is 5% of the unit of transport pack with the product. If there are less than 20 units in a batch one unit is selected. For each packaged unit of packed butter, 3% of consumer packaged goods are selected. Spot samples of the butter in the transport packaging are taken by a probe. If butter is packed in boxes, the probe is immersed diagonally from the end wall to the center of the butter monolith. A sample of butter of 10 °C and less is taken with a probe heated in water to a temperature of (38 ± 2) °C.

For compilation the combined sample from the bottom of the of butter tube selected by the probe from each unit of transport packaging with product, point sample of butter weighing about 50g is taken by the knife and it is placed into a dish to compose a combined sample. The upper part of the tube of butter up to 1.5 cm remaining on a probe is replaced and carefully flattened the surface of the butter.

Having removed the packing material and the outer layer of 0.5 ... 0.7 cm from butter a point sample of 50g is taken from each tube of butter.If a briquette weight is 50 g or less, the combined sample is made up of whole briquettes without removing the outer layer.

The combined sample of butter is softened in a water bath with a temperature of (30 ± 2) ºC with continuous stirring. 50 g of butter are selected for analysis from the prepared sample.

The sample is selected in the amount given in table. 3 from the batch of hard cheeses.

Table 3

Number of units of transport package with products in a batch	The number of units of transport package with the product in the sampling
Up to 5	1
6...15	2
16...25	3
26...40	4
41...60	5
61...85	6
86...100	7
More than 100	5 %, but not less than 7

One head of cheese, a bar of cheese or one unit of consumer packaging with foodstaff is selected from each unit of transport pack with the products included in the sampling.

Spot samples of cheese are sampled with a probe from two opposite sides of head of cheese included in the sample. The probe is inserted to a depth of ¾ length. To determine the organoleptic characteristics, a spot sample is selected from one side of the cheese head. The probe is inserted from the end face closer to the center for sselection of point samples of large solid rennet cheeses having the shape of a cylinder or a bar; for selection of point samples of small solid rennet cheeses having a round shape, the dipstick is inserteded from the top of the head to the center. Columns of the cheese about 1.5 cm are separated from the cork layer. Then pieces without acork layer about 4.5 cm in length are placed in a container for combined sample. When sampling of point samples of small solid rennet cheeses which have the shape of a low cylinder, the dipstick is inserted from the cylindrical surface, and for those ones having the form of a bar it is inserted from the diagonal end face.

The sampling of soft cheese (rockfort, camembert, dniprovsky), brine (suluguni, brynza) and combined sampling are carried out in accordance with the requirements of the current regulations for soft cheese.

To make a combined sample of pickled cheeses, entire column of cheese selected by the dipstick is used. The sampling of suluguni cheese and similar cheeses is carried out by cutting with a knife a sector of about 2 cm.

Cheese samples are taken separately to determine physicochemical and organoleptic characteristics. One sample is not allowed for all these analyzes.

Point samples of hard, soft rennet cheeses and close by the consistency to them of pickled cheeses are rubbed through a fine grater, mixed thoroughly, and combined for physicochemical studies; point samples of soft and pasty whizzed cheese are ground in a mortar and made up combined sample. 50 g sample is allocated from the combined sample for the analysis.

From a batch of ***whizzed cheese*** every tenth unit of package is selected and opened, and one cheese is taken from each controlled unit. If whizzed cheese in the package of 30 g, 2 packs of cheese is taken from each control place.

For chemical research 20 g of cheese is cut off from each cheese of the same type and the same mass fraction of fat and placed in one container. The samples selected are crushed thoroughly, mixed and taken about 50 g as a combined sample into a clean glass with a stopper.

Organoleptic evaluation of whizzed cheeses is carried out at a temperature of 14 ... 16 ºC.

The sample volume of a batch***of condensed milk cans and dried dairy products*** in the transport container is 3% of the units of transport container with products, but not less than 2 units of canned condensed milk goods and not less than 3 units of dried dairy products. The sample volume of a batch of preserved milk products and dry products in consumer packaging is also 3%, but not less than two units.

When selling products from each batch of condensed milk, two cans No. 7 are selected for control storage at the milkcanning plant. When packing in a large container, two samples of 500 g are selected from each batch and kept sealed in an airtight container. The control samples are stored for 4-6 months for products of current use and 12-15 months for products of long-term storage and for export.

For each batch of canned dry milk samples are taken for control storage at the plant: 2 places in small packing and two samples of 200 g are taken from large packing, tight packed in plastic bags and sealed. The samples are stored for 8 months in tight packaging and for 3 months in unpressurized containers. After the specified period the samples are removed from storage and sent for industrial processing.

Before sampling, condensed milk in barrels and flasks are thoroughly mixed with a stirrer, and condensed milk in consumer packaging is mixed with a spatula 1-2 minutes after corkage.

If there is a precipitate at the bottom in the cans of condensed milk cans, the can is immersed in water at a temperature of (55 ± 5) ºC and stirred again to obtain a homogeneous mass, the temperature of the product must not exceed (28 ± 2) ºC, then it is cooled to a temperature of (20 ± 2) ºC. After mixing the product in tanks, point samples are taken from different places by a probe or sampler, dipping it into the bottom of the container. For each package, the point samples shall be taken in equal volume and made up of a combined sample of 1 kg. 300 g of product is taken for analysis from the combined sample.

The sampling of dry dairy products in the transport container included in the sample is carried out with a probe from different places of each unit of the trans-sports container with products. A probe is immersed into the product at a distance of 2 ... 5 cm from the wall diagonally to the bottom of the container. The point sample are collected in a container and make up a combined sample of 1-2 kg, and a sample of 200 g is separated from it for the analysis.

Samples of dry dairy products are thoroughly mixed and ground in a mortar.

The selected samples of prepared products the organoleptic, physico-chemical and microbiological parameters are determined for compliance with the current regulatory documents.

The right to draw up documentation and release the finished product for sale has the head of the laboratory or an employee of the laboratory, which is responsible for the production of finished products.

A shift master or technologist of a workshop draws up a passport on a batch of finished products and sends it to the laboratory for quality control. The laboratory worker checks products on organoleptic and physicochemical indicators, checks the state of packaging, marking and packaging for conformity of production with the requirements of regulatory documents and issues a certificate of quality in accordance with the prescribed form. Certificate of quality is the only document that gives the right to release a given batch of products from the enterprise. If there are products without a certificate, the administrative penalties are imposed onto the person who committed the violation.

3.2.1.5 Wines and alcohol beverages

OIV GENERAL PRINCIPALS AND REQUIREMENTS OF WINE AND MUST ANALYSIS

The Compendium of International Methods of Wine Analysis was first published in 1962. All the methods and principles of wine and must analysis, were approved by the ''International organization of vine and wine'' **(**OIV) General assembly and produced each year by the Sub-Commission.

The Compendium plays a major part in harmonizing methods of analysis. Many vine-growing countries have introduced its definitions and methods into their own regulations.

The European Union recognizes all of the methods in the Compendium and makes them binding in all Member States, confirming the close collaboration established between the EU and the OIV.

Thus, through its leading role in the harmonization of methods of analysis, the Compendium contributes to facilitating international trade. With the *InternationalCode of Oenological Practices* and the *International Oenological Codex*, it constitutes a body of considerable scientific, legal and practical benefit.

Layout and wording of OIV method of analysis

Extract of ISO 78-2:1999 standard

1. Title

2. Introduction

Optional

3. Scope

This clause shall state succinctly the method of chemical analysis and specifically the product to which applies.

4. Definitions

5. Principle

This optional clause indicates the essential steps in the method used, the basic principles.

6. Reagents and materials

This clause shall list all the reagents and materials used during the test, together with their essential characteristics, and shall specify, if necessary, their degree of purity.

Shall be given:

Products used in their commercially available form

Solutions of defined concentration

Standard volumetric solution

Standard reference solution

Standard solution

Standard matching solution

7. Apparatus

This clause shall list the names and significant characteristics of all the apparatus and equipment to be used during the analysis or test. Note: each reagent shall be mentioned by a specific reference number

8. Sampling (Preparation of the sample)

Shall be given:

Sampling procedure

Preparation of the test sample

9. Procedure

Each sequence of operations shall be described unambiguously and concisely. This clause shall normally include the following subclauses:

Test portion (this subclause shall give all the information necessary for the preparation of the test portion from the test sample).Determination(s), or test(s) (this subclause shall be described accurately in order to facilitate the description, the understanding and the application of the procedure).

Calibration (if necessary).

10. Calculation (Results)

This clause shall indicate the method for calculating the results. Shall be precised the units, the equation used, the meanings of the algebraic symbols, the number of decimal places to which the results are to be given.

11. Precision (if interlaboratory validation)

The precision data shall be indicated:

The number of laboratories

The mean value of the concentration

The repeatability and the reproducibility

The repeatability and reproducibility standard deviation

A reference to the document containing the published results of the interlaboratory tests.

12. Annex

Annex related to precision clauses

Annex concerning statistical and other data derived from the results of interlaboratory tests.

13. Bibliography

Annex related to precision clauses

This annex shall indicate in particular

- Repeatability statements
- Reproducibility statements

Annex concerning statistical and other data derived from the results of interlaboratory tests.

Statistical and other data derived from the results of interlaboratory tests may be given in an informative annex.

Example of table giving statistical results

Sample identification A B C	A	B	C
Number of participating laboratories			
Number of accepted test results			
Mean values (g/100g sample)			
True or accepted value (g/100g)			
Repeatability standard deviation (Sr)			
Repeatability coefficient of variation			
Repeatability limit (r) (2,8 x Sr)			
Reproducibility standard deviation (SR)			
Reproducibility coefficient of variation			
Reproducibility limit (R) (2,8 x SR)			

Whilst it may not be considered necessary to include all the data shown in the table, it is recommended that at least the following data be included:

- The number of laboratories
- The mean value of the concentration
- The repeatability standard deviation
- The reproducibility standard deviation
- A reference to the document containing the published results of the interlaboratory tests.

Wine and must analysis types

1) **Physical analyses;**
2) **Chemical analyses:**

Organic compounds

- Sugar
- Alcohol
- Acids
- Gas
- Other chemical compounds

Non-organic compounds

- Anions
- Cations
- Other non-organic compounds

3) Microbiological analysis
4) Other analysis

Methods of analysis of wines and musts

General remarks

1. Clear wine or must, must be used for chemical and physical analysis. If the wine or the must is cloudy, it is first filtered through filter paper in a covered funnel or centrifuged in a closed container. This operation must be stated onany required documentation.

2. The reference of the method employed for each determination must be on any required documentation.

3. Units of measure for the various magnitudes (volume, mass, concentration, temperature, pressure, etc.) shall be in accordance with the recommendations of the IUPAC (International Union for Pure and Applied Chemistry).

4. In respect of reagents and titration solutions used, unless otherwise required in the text, the chemicals used are to be of "analytical grade" and the water is to be distilled or of equivalent purity.

5. Enzyme methods, and the determination of a number of parameters, are to be based on absolute measurements of absorbance, which requires spectrophotometers to be calibrated for wavelengths and absorbance. Wavelength may be calibrated by use of Hg lines: 239.94, 248.0, 253.65, 280.4,302.25, 313.16, 334.15, 365.43, 404.66, 435.83, 546.07, 578.0, and 1014.0 nm.

Absorbance may be calibrated by means of commercial reference solutions, obtained from suitable suppliers, or neutral density filters.

6. The essential bibliographical references are given. The references to working documents of the Sub-Commission are marked '*F.V., O.I.V.*' (feuillets verts or 'green pages'), followed by the year of publication and the number of the document.

‘’Malvidin diglucoside’’-description

The presence of anthocyanin diglucoside pigments in red or rosé wine, particularly malvidin diglucoside, is used as an indication of whether the wine has been made using fruit other than Vitis vinifera species, which do not contain appreciable quantities of diglucosides.

‘’Malvidin diglucoside’’- chemical analysis method

1. Principle

Malvidin diglucoside, oxidized by nitric acid, is converted to a substance that, in an ammonium medium, emits a vivid green fluorescence in ultraviolet light. The intensity of the fluorescence of the compound formed is measured by comparison with the fluorescence of a solution titrated with quinine sulfate whose intensity of fluorescence is standardized with the malvidin diglucoside reference. Free sulfur dioxide, which attenuates the fluorescence, must previously be combined with excess acetaldehyde.

2. Qualitative Examination

2.1 *Apparatus*

2.1.1 Ultraviolet lamp permitting measurement at 365 nm.

2.2 *Reagents*

2.2.1 Acetaldehyde solution

Crystallizable paraldehyde ..... 10 g

Ethanol 96% (*v/v*) ..... 100 mL

2.2.2 Hydrochloric acid, 1.0 M.

2.2.3 Sodium nitrate solution, 10 g/L.

2.2.4 Ethanol, 96% (*v/v*), containing 5% concentrated ammonia solution (p0 = 0.92 g/mL).

2.2.5 Control wine containing 15 mg of malvidin diglucoside per liter.

2.2.6 Wine containing no malvidin diglucoside

2.3 *Method*

Into a test tube add:

- 10 mL of wine
- 1.5 mL of acetaldehyde solution wait 20 minutes.
- Into a 20 mL centrifuge tube place:
- 1 mL of wine reacted with acetaldehyde
- 1 drop of hydrochloric acid
- 1 mL sodium nitrate solution

Stir; wait 2 minutes (5 minutes maximum); add:

- 10 mL ammoniacal ethanol

Treat similarly 10 mL of wine containing 15 mg/L malvidin diglucoside (The control wine). Stir. Wait 10 minutes and centrifuge.

Decant the clear liquids from the top into calibrated test tubes. Observe the difference in green fluorescence between the test wine and the control wine under ultraviolet light at 365 nm. For rose wines, it is possible to increase the sensitivity using:

- 5 mL of wine treated with acetaldehyde (2.3)
- 0.2 mL hydrochloric acid, 1 M (2.2.2)
- 1 mL sodium nitrate solution, 10 g/L (2.2.3)
- 5.8 mL ammoniacal ethanol (2.2.4)

Treat the control wine in a similar manner.

2.4 *Interpretation*

Wines that do not fluoresce, or have a distinctly lower fluorescence, than the Control may be considered to have no malvidin diglucoside. Those whose fluorescence is slightly less than, equal to, or greater than the control should have a quantitative determination.

3. **Quantitative Determination**

3.1. *Apparatus*

3.1.1. Equipment for measuring fluorescence:

- excitation wavelength 365 nm;
- wavelength of fluorescent radiation 490 nm.

3.1.2. Optical quartz cell (1 cm path length)

3.2 *Reagents*

3.2.1. See qualitative examination

3.2.2. 2 mg/L quinine sulfate solution

Prepare a solution containing 10 mg very pure quinine sulfate in 100 mL sulfuric acid, 0.1 M. Dilute 20 mL of this solution to 1 liter with sulfuric acid solution, 0.1 M.

3.3. P*rocedure*

Treat the wine by the method described in *Qualitative examination (2)*, except that the aliquot of acetaldehyde treated wine is each case (red wines and roses)1 ML.

Place the 2 mg/L solution of quinine sulfate in the cell, adjust the fluorometer to the full range (transmission T, equal to 100%) by adjusting the slit width or the sensitivity.

Replace this tube with the one containing the test wine: this is the T1 value. If the percentage of transmission, T1 is greater than 35, dilute the wine with wine without malvidin diglucoside whose fluorescence must be less than 6% (this should be ascertained by previous testing.)

Remarks:

1. Salicylic acid (sodium salicylate) added to the wine for stabilization before analysis, causes a spurious fluorescence which can be eliminated by an extraction with ether.

2. Spurious fluorescence is caused by the addition of caramel.

3.4 *Calculation*

A fluorescence intensity of 1, for wine without SO2, for the operating conditions above with the exception of the acetaldehyde treatment, corresponds to 0.426 mg malvidin diglucoside per liter of wine. On the other hand, red and rose wines, containing no malvidin diglucoside, give fluorescence corresponding to a T value of the order of 6%.

3.5 *Expression of the Results*

The amount of malvidin diglucoside is expressed in milligrams per liter of wine to the nearest whole number.

Bibliography

COMPENDIUM OF INTERNATIONAL METHODS OF WINE AND MUST ANALYSIS-OIV, EDITION 2016, VOLUME 1

COMPENDIUM OF INTERNATIONAL METHODS OF WINE AND MUST ANALYSIS-OIV EDITION 2012, VOLUME 2

3.2.2 Human and veterinary medicine

Samples should be selected in accordance with the knowledge of the epizootiology and pathogenesis of the disease under study or previous diagnosis or clinical signs of the disease. This will help to sample the tissues or liquids that most often contain the infectious agent or evidence of infection. Knowledge includes tissue susceptibility or target organ data, duration and location of infection in each tissue type, duration and route of infection, or time frames in which past infection data may be available, such as the presence of antibodies detected by detailed studies. This knowledge can be used to determine the sampling method. In many studies of diseases of herds, itisadvisabletocollectsamplesfromhealthylivestockforcomparativeepizooticorbaselineanalysis (forexample, forrandomcontroland general fordiagnostictesting) andforvalidation. If fixation (immobilization) animals require the use of chemicals, must take into account the impact of the chemical on the results of studies (eg toxicological studies).Some laboratory studies are incompatible with specific blood anticoagulants and tissue preserving agents, such as heparin, formalin, dry ice (exposure of the test sample to elevated levels of CO2) or even freezing.It is important to select the sample aseptically as possible, to avoid contamination with the detergents and antiseptic substances used to clean the sampling site in animals, because these substances can hinder the procedures of laboratory tests.For the study of samples of pathogenic microorganisms, as well as molecular research may be adversely affected by chemicals or detergents,used in the manufacture or preparation of selection tools (for example, chemicals used in the manufacture of certain types of lubricants and detergents used in cleaning glassware). The results of laboratory tests depend on the correct sampling, and therefore the procedure should be planned. When planning sampling, it is necessary to pay attention to the task (purpose) and to decide how accurate the expected response should be (the more accuracy is required, the greater the number of samples to be sampled).

It is necessary to agree on the possibility of conducting research with the specialists of the laboratories where the samples will be sent.

The population of animals to be sampled should be divided into sampling units (from one individual to a group - nest, unit, herd).

Sampling is carried out by the following methods:

- randomization (random selection) - simple (choice of random individuals or systematic (choice of individuals at regular intervals);
- stratometry - the set of animals is first divided into groups (strata) formed on the basis of sex, age, breed, retention, direction of economic activity, and then in the middle of each group carry out simple or systematic randomized sampling;
- clustering - an aggregate of animals is first divided into groups (clusters) formed naturally, through human economic activity or geographical features (herd, farm, individual farm, area), and then within each group simple or systematic randomized sampling is carried out.

The number of samples taken in conditions of the immediate diagnosis of the disease may be limited by the presence of sick (dead) animals and depend on the specific conditions. In monitoring and screening, the sample size / number of samples, to be taken to determine whether or not a disease is present in a given population, depends on the following parameters:

- the volume of the animal population (if more animals are sampled in that population, then the likelihood that positively responding animals are detected, increases with each new sample;
- the likelihood of disease prevalence, if present in the population (the likely level of coverage of a particular disease is estimated by epizootic parameters of the disease or analogues with other herds / populations);
- the level of certainty required for decision-making (95% is considered sufficient for all animals except ducks, geese and turkeys, where this level is 49%). The sample volume in this case is calculated using special formulas or tables.

Samples from each animal are taken with sterile instruments into a separate sterile vessel, identifying each sample. Materials are selected on the verge of healthy and diseased tissue.

For the selection of pathologic / biological material, the animal's corpse is used in the first hours after death or slaughtered a sick animal that has not been treated.

Samples are sent to the laboratory in a non-canned form, with a temperature control (+ 4-8 ° C). If it is impossible to deliver to the laboratory within 24 hours, the samples are frozen in an ice flask or canned.

Depending on the type of infection in clinically ill animals take appropriate, specific for the disease biomaterial, while taking personal safety precautions.

For bacteriological examination pathological material (organs or parts of them) is preserved by 30% aqueous solution of chemically pure glycerol.The solution was sterilized by boiling for 30 minutes. You can use sterile petroleum jelly to preserve the material. The material is poured into the preserving liquid in a ratio of 1: 5.

3.2.2.1 Blood

Whole blood samples are selected for hematology, clinical biochemistry, toxicology, direct testing for bacteria or parasites, for PCR, immunological studies, or for bacterial or virus cultivation.Depending on the needs of the study, whole blood, blood cells and / or plasma samples can be obtained from whole blood collected into appropriate anticoagulants. When selecting an anticoagulant for research, a veterinarian should be aware of the methods of laboratory testing and the possibility of using anticoagulants or preservatives, since some of these may have a negative impact on the research. For the effective use of anticoagulants, it is necessary that the collected blood is mixed thoroughly with the selected anticoagulant during or immediately after its selection.

Microbial contamination and hemolysis are significant problems, especially when receiving blood and serum samples from dead animals.Often, the cause of hemolyzed serum and plasma is due to high temperature or time delays in the separation of serum from erythrocytes, blood sampling using a needle with a diameter too small, or the inability to remove the needle when transferring blood from the syringe.

Whole blood should be collected aseptically by venipuncture of a living animal. Depending on the type of animal and sample, use the jugular, caudal, humeral, main, mammary veins.

Blood for serological studies take in the midst of the disease, and in some cases again after 10-20 days 10 cm3 from two or three sick animals in different tubes.

Horses, cattle, camels, deer, sheep and goat blood taken from the jugular vein in the upper third of the neck in sterile tubes 5-7 cm3. During the selection, the blood should flow freely through the test tube walls. No blood is allowed to enter the floor, soil.

Blood is collected with disposable needles into plastic or glass tubes, vacuum or non-vacuum systems can be used to collect blood. Sampling of blood in disposable syringes of 5-10 cm3 is allowed, as well as blood collection with reusable needles, provided they are pre-sterilized by boiling.

The hair is punctured at the puncture site, the skin disinfected with alcohol or other authorized disinfectant intended for this purpose.

In animals, blood is drawn from the vein of the ear or the tip of the ear, in a bird - from the surface of the crest, the vein or the heart by its puncture (with appropriate skills). Wool, feathers at the site of blood collection are cut (plucked) or shaved, the skin is carefully wiped with cotton swabs soaked in alcohol and then with ether. Instruments (needles, scalpel) must be sterile.

In pigs, blood is taken from the vein of the ear or otherwise (from the tail, ocular sinus, from the anterior cavity (preferably in the dorsal position). The tip of the tail is pre-washed with soapy water and disinfected with

alcohol. After blood sampling, the tip of the tail is treated with a solution of iodine, necessarily ligated, which is removed after 10 - 12 hours.

In rabbits, a small amount of blood can be drawn by incision or piercing of a vein located outside the thin edge of the ear. Animals are wrapped in a towel or placed in a box with a hole for the head, the ear is pre-dipped into warm water or rubbed with alcohol. The place of bloodletting can serve as a chest vein located on the chest side. After treatment (from the elbow joint to the third rib), the vein is pressed with the finger near the elbow. The needle is injected against the flow of blood. When blood is drawn from the heart, the needle is injected into the third intercostal space to the left, at a distance of 3-4 cm from the edge of the sternum.

Venopuncture of the infraorbital vein located on the inner surface of the wing - the simplest method of blood sampling in turkeys, chickens and in most species of poultry, in ducks, in addition to venopuncture of the vena cava wing, blood can easily be taken from the subcutaneous vein of the tibia, near the hopping vein.For most serological studies, it is sufficient to obtain 2 cm3 of blood in a bird.

In chickens and turkeys, blood is also taken by incision or scarification of the comb (earrings). Geese and ducks puncture the crumb of the foot.

In geese, ducks and turkeys, blood is drawn from the inner metatarsal vein located beneath the skin on the medial surface of the metatarsus, closer to the plantar margin.

In foxes, arctic foxes, dogs, blood is drawn from the femoral vein.

From the mink blood is taken into glass capillaries. For this mink is fixed and cut with scissors claw or pulp of one of the fingers of the hind limb. To the protruding droplet, substitute a glass capillary, holding it horizontally.After filling with blood the capillary on one side is closed with plasticine and put in a special tripod with numbered sockets.After sampling, the tripod with capillaries is transferred to a warm place (preferably a thermostat) at t ° 38 ° C for 40-50 min for blood clotting, and then centrifuged at 1500-3000 rpm for 5-10 min. On the same day, they set the reaction.

Blood for research from fish is taken from a gill or tail artery or from the heart. At the site of blood collection, the scales are scalped, the skin wiped with mucus and disinfected with 70% alcohol.The blood is collected into a pasteur pipette, after which it is transferred to a watch glass and quickly selected the required amount. Blood is delivered to the laboratory in hermetically sealed containers in an ice flask.

Test tubes, blood syringes are numbered (the order number and the animal number).

To obtain serum, whole blood is collected without anticoagulants and maintained at ambient temperature (20-30 ° C) for several hours a day. Then the blood clot is separated from the test tube walls with a metal spike (dart), which is burned over the flame. The blood tubes are then placed in the refrigerator at 4-10 ° C. After 18-24 hours, the separated serum (2-3 cm3) is poured into dry sterile tubes (or eppendorf microtubes) and labeled in the same way as blood tubes.The pure serum can be decanted or pipetted after physically removing the clots with subsequent centrifugation to separate the cellular components from the serum. In the absence of a centrifuge, the clot can be separated by tilting the blood tube about 45 ° until it is possible to separate the clot from the tube surface with a sterile rod or pipette, and then remove the clot with forceps.Bacterial contamination and erythrocyte residues in serum samples can lead to erroneously positive reactions when staging an agglutination reaction. Serological studies are negatively affected by hemolysis in the serum sample.

Serum tubes are closed with corks and placed upright for transportation. Blood samples may be sent directly to the laboratory directly in test tubes or disposable syringes

The material is then sent to the laboratory in fresh or canned form.

Serum is preserved by the following methods:

- dry boric acid (4% by volume of serum) to obtain a saturated solution and form a small precipitate of crystals at the bottom of the test tube;

freezing (for testing for viral infections - t ° to -20 ° C).

Non- conserved serum is suitable for research within 6 days of blood collection if stored at 4-8 ° C. Serum preserved with boric acid, suitable for research for 30 days; frozen - for 3-4 days after a single defrost.

Muddy, germinated, hemolyzed serum is not subject to research.

Before sending to the laboratory, make a accompanying letter and a description of the samples (two copies). The first drop of blood is removed with sterile cotton wool (with the exception of blood tests for pyroplasmidosis, when the first drop of blood is taken for a smear).The next free-falling drop is taken on a pre-prepared glass with a quick and light touch of a drop to its surface.Then the glass quickly turn up drop between the fingers of his left hand in a horizontal position. To the left edge, the droplets touch at an angle of 45 ° with the sanded edge of another slide (or cover).When the drop is evenly distributed on the edge of this glass, it is quickly carried out on the surface of the slide from right to left, without bringing to the edge of 0.5 - 1 cm. The width of the

smears should be narrower than the slide. For each new smear take a fresh drop of blood. Ready blood smears are air dried, not dried over flames or in the sun is not recommended. In the cold season, smears are made in a warm room or on heated glasses.

The method of fixation of smears depends on the purpose of the study.

Properly made blood smears should be thin, uniform and of sufficient length. Dried brush and the prints are signed with a simple pencil or marker (fiberglass), indicating the number or nickname of the animal and the date of the smear.

Blood samples should be collected and distributed as quietly as possible to avoid red blood cell damage causing hemolysis.Blood and serum are transported and stored in cool or frozen form (for serum). For some studies, the samples can be dried on a piece of unprocessed or specially treated filter paper designed to transport and store a stabilized sample.

The blood is stabilized with ethylenediaminetetraacetic acid (EDTA).

3.2.2.2 Faeces

The faeces can be freshly squeezed or taken directly from the rectum / cloaca, or can be collected using cotton, dacron or gauze tips, depending on the volume of sample required by the specific test method. Samples collected on smears should remain moist and should be placed in a transport vehicle (sterile solution, culture medium containing antimicrobials or stabilizers) recommended for relevant research. Fecal samples should be stored refrigerated at t ° + 4 ° C or on ice and examined as soon as possible after selection to minimize the adverse effects of the test results from the death of the target micro-organism, bacterial germination, or parasite eggs. Pack faeces samples in a double-pack with a tight lid or in tight containers, which are then placed in airtight plastic bags that will prevent cross contamination of the samples and packaging materials.Plastic bags or tubes with a rubber stopper for transporting faeces taken from the rectum are unsuitable as the samples very often contain gaseous bacteria that can tear the plastic bags, displace the plugs and lead to leakage.

Faeces for research are sent in sterile glasses, test tubes or jars, tightly covered with parchment paper.From dead animals feces can be sent in a segment of intestine tied at both ends.

The material is delivered to the laboratory not later than 24 hours from the time of its selection and with obligatory observance of the temperature regime. The material is not preserved.

3.2.2.3 Urine

Urine sampling is carried out in the morning before feeding the animal or 4-5 h after the last urination. An average urine sample is taken. Urine sampling is performed by piercing the bladder, using a catheter or naturally. The choice of method may affect the result of the analysis. Urinary sampling in the last two methods is accompanied by bacterial contamination. Selected urine can naturally reflect changes that occur in the kidneys or lower urinary tract.For some diseases, it is necessary to select the daily amount of urine, and for routine research is selected in a sterile vessel of 30-200 cm3. Urine is delivered to the laboratory within 2-4 h after its receiving or it has to be frozen. Formaldehyde (1 drop of 40% formaldehyde per 30 cm3 of urine) or antimicrobials (thymol, toluene, boric acid, etc.) are used for the preservation of urine.

3.2.2.4 Dead animals' tissue

The selection of tissue from an animal's corpse should only be carried out by a qualified veterinarian and pathologist.Pathological and anatomical incision is performed not only for the purpose of sampling, but also to provide information on pathological changes in the body.Such observations are an important complement to epizootic and clinical observations in a comprehensive veterinary study of cases or outbreaks of disease.

Samples of relevant pathological material are selected to determine or confirm the cause of the disease, the death of animals (birds, animals, bees, fish) in suspected infectious, invasive disease or poisoning or slaughter of a diseased animal that has not been treated.

The autopsy should be dissected in the first hours after death, because in the case of corpse decomposition, the secondary intestinal microflora contaminates all organs and tissues.

Depending on the suspected disease, the condition of the carcass, and objects available for post-mortem sampling, samples are taken from one or more organs and sent to the laboratory for fresh (non-canned) or canned for further laboratory testing.

For research, the parenchymatous organs and tubular bone are sent to the laboratory.The carcasses of small animals or pathological material are packed in a waterproof container and added to the accompanying.When selecting material for post-mortem diagnosis, pathological material is selected as soon as possible: no later than 12

h after the animal has died in winter and 6 h in the warm season, which is preserved or sent fresh.Autolysed patmaterial for isolation of pathogens is not suitable.Blood samples are taken with a sterile syringe from the left ventricle of the heart.The puncture is performed after treatment of the selected area with a sterile, moistened with 3% hydrogen peroxide solution with a napkin and subsequent removal of the sterile sterile moistened with sterile saline tissue.

Disinfection of the selected area for puncture can be done by cauterizing the tissues with a spatula. For the sample from the left ventricle of the heart with a monovet or sterile syringe select 10 cm3 of blood, which after selection is transferred to a sterile bottle with double medium and closed with a cotton-gauze cork. For the determination of bacteremia, the selected blood samples (10 cm3) are transferred into containers for aerobic and anaerobic cultivation.

Samples (pieces) of organs and / or tissues are selected at least 3-5 cm3 (necessarily samples of the spleen, regional and remote lymph nodes, inflamed foci), each placed in a separate sterile vessel (disposable sterile containers with plugs that are screwed or screwed Petri dishes with a diameter of 55 mm. You can use containers with transport setting that are permitted for use for this purpose in the prescribed manner. Containers with material are marked with an organ and / or fabric.

The pus from inflamed cavities, cerebrospinal fluid and other fluids are aspirated with a sterile syringe of at least 7 - 10 cm3 in volume and placed in sterile disposable tubes or left in syringes sealed with sterile rubber stoppers or in transport containers. Samples are marked.

The material from the corpses with purulent-inflammatory pathology is delivered to the laboratory within 1 h after selection. Liquid patmaterial (blood, mucus, urine, bile, etc.) can be collected into disposable syringes or pasteur pipettes, which after sealing the material, avoiding heating, are sealed at both ends and then wrapped in cotton wool and placed in test tubes.

In addition, different selections can be sent in the form of smears or imprint smear, which are fixed in the air, wrapped individually in parchment and labeled.

Tubular bone is necessarily taken from stale corpses. Bone marrow is sent from pathological material of undetermined freshness for bacteriological research. The ear or other material is taken from stale corpses to exclude anthrax.

Fresh carcasses of small animals, parts of carcasses of large animals and individual organs are sent on purpose.

Fresh samples are given particular attention to their processing and storage, avoiding autolysis and contamination by bacterial and fungal pathogens. Freshly selected samples are stored at a constant cool temperature (2-8 ° C) from the time of sampling and until the study. If cold storage cannot be provided, fresh samples for some studies may be placed in special solutions, such as ethylene glycol, that inhibit the growth of secondary microorganisms. Formalin is most commonly used to save posthumous samples.

After sampling, all residual tissues or parts of the carcass and fluid must be collected and disinfected by appropriate methods or destroyed, and the carcass opening section carefully disinfected.

For bacteriological examination, pieces of skin, mucous membranes, parenchymatous organs, tubular bone, cerebro-spinial axis, lymph nodes, samples of fluid from the thoracic and abdominal cavities, intestinal segment, isolated ligatures, fetal membranes, fetuses, are sent to the laboratory. Each organ sample is placed in a separate container and labeled. In each case, it is necessary to select material in which it is possible to identify characteristic changes for a particular disease. Hair and skin areas - with skin diseases.

For *virological studies*, samples are sent from animals in three stages of the disease:

- from sick animals with pronounced clinical signs indicating the body temperature, pulse rate and respiration (blood, bone, lymph nodes and affected organs);
- from those killed in the agonized state - blood, bone, lymph nodes and affected organs;

from animals in the stage of recovery (blood).

For *virological examination*, samples should be taken as early as possible after clinical symptoms have been identified. The likelihood of virus detection is greatest in the first 3 days and decreases sharply over the next 5 days. Samples are taken in accordance with aseptic conditions, each sample is selected in a separate sterile screwdriver container. You can use sterile conical centrifuge tubes (15-50 cm3), which are screwed, as well as small (4 cm3), which bring 1-2 cm3 viral transport medium. Samples are delivered to the laboratory in a transport environment or in ice containers.

Forvirologicalexamination, afreshorpreserved 30% glycerolsolutionpreparedonphosphatebuffersolution (pH 7.2-7.4) issenttothelaboratoryfromdeadbodiesorslaughteredanimals.

For virological examination, the material is packed in a plastic bag and placed in an ice flask or canned with a 30-50% solution of chemically pure glycerol in sterile saline solution.The saline was pre-autoclaved at 120 ° C for 30 min. Blood or its serum, rinses from the nasopharynx and other body fluids, walls, afts content, papules (nodules), vesicles (serous vesicles), pustules (purulent vesicles), pieces of the brain, liver, lungs, spleens or pieces of other organs and selected tissues in which the virus of the suspected disease is contained in the highest amount according to the tropism of the virus.

For *bacteriological and virological examinations*, areas of the intestine with the most characteristic pathological changes are selected. Then the intestines are laundered from fecal matter and placed in glasses separately from other organs. If necessary, preserve 40% glycerol solution in the ratio of 1:10.

For *histological examination* patmaterial selected only from fresh corpses. To the laboratory, send pieces of the affected tissues of 3-4 cm2, with a thickness of not more than 1 cm, bordering on unchanged tissue sections.The area of the surface of the organ or tissue of the corpse from which the sample is to be taken is cleaned of contamination, contaminated with alcohol or baked with a heated metal plate (spatula). The material is selected with sterile instruments and placed in sterile dishes (penicillin vials, test tubes, etc.).When selecting pathological material for histological examinations, the suspicion of disease and the results of the pathological and anatomical incision of the animal's body should be taken into account.For *histological examination*, the material (organs and tissues that have marked pathological changes) is taken from fresh corpses or slaughtered animals.From different sections of organs (tissues) with pathological changes (on the border of pathologically altered tissue and placed near the normal), cut small pieces 2 × 3 cm thick.When cutting a piece, take into account the microscopic structure of the organ and tissue. So, the kidney pieces should consist of cortical and brain layers. In the case of the death of small animals, the parenchymal organs are selected whole. When cutting out samples from an organ of the same structure, its capsule is also captured.Immediately after selection, the material is transferred to the fixing fluid, the volume of which should be 10-20 times greater than the volume of material taken. For fixation often use 10% aqueous neutral formalin solution. In the absence of formalin, 96% ethyl alcohol can be used. When using alcohol, the thickness of the tissue pieces should not exceed 0.5 cm. The fixing fluid is changed 24 hours a day. The pathological material is fixed in a sealed glass container.

For *histochemical studies*, the pathological material is fixed in 96% ethyl alcohol, Carnu's fluid or Buen's fluid. The name of the fixing solution must be indicated on the label.In order to prevent freezing of the material during shipment during the winter, the material fixed in formalin is transferred into a 30-50% solution of glycerol prepared in 10% formalin or in 70% alcohol, or in a saturated salt solution.Frozen pathological material is not suitable for histological examination.

Pathological / biological material submitted for molecular genetic testing is not allowed to be preserved by any substance.For *molecular biological research*, macrobioptate (macroautoptate) (pieces of tissues: spleen, lymph nodes, etc.) weighing 50-100 g) is sent, placed in containers with 0.85% sodium chloride solution or transport medium. The storage and transportation of biological material for PCR testing should be carried out with a cold regime: blood samples at 2–8 ° C for 1 day; plasma and serum samples at 2-8 ° C for 5 days, at -70 ° C without restriction.

3.2.2.5 Epithelial tissue

Carried out for diseases that cause damage to the epithelial cover, accompanied by the formation of ulcers and abscesses (FMD, vesicular stomatitis, smallpox, tuberculosis, etc.).From living animals, blood and epithelium samples are taken from lesions, and from the dead or killed animals send pieces of skin on the border of healthy and affected areas.

Epithelial tissue is selected by biopsy or scraping of the skin, mouth swabs, nose, pharynx and gastrointestinal tract.Cut hair or wool can be used for clinical diagnosis or laboratory testing to detect surface parasites such as ticks and lice, fungal, bacterial and viral infections, allergic reactions and neoplasia.Samples are aseptically sampled and stored according to scheduled studies. Epithelial tissues, which are associated with vesicular lesions and must be collected in viral transport environments, may be useful in the laboratory diagnosis of specific viral infections (FMD, plague, etc.).

Skin samples are taken from the periphery of lesions that have not been treated.Crusts with residues of wool, as well as some wool, are pulled out by tweezers from contaminated areas (where possible less contaminated).Hair is plucked and skin scrapes made with a scalpel on the border of the affected and healthy tissues. To the laboratory send scrapings of the epidermis from the lesion and crusts from the periphery of the lesion, as well as the allocation of the contents in its presence. In the case of multiple foci, the selection should be

made separately from the foci located in different parts of the body. The material is placed in wadding tubes or paper bags.

If necessary to made the skin studies, select the most affected parts of the size not less than 3 × 3 cm are selected. The material is sent in a sterile, hermetically sealed container.

For bacteriological examination, wool is taken from different places with at least 5 samples weighing about 2 g each (it is better to take a bundle of contaminated wool). If the wool is packed in packs, take at least 10 samples from different places of each pack, as well as dust collected inside the sheath. Samples from one pack are combined and packed together. Each sample is placed in a dry sterile glass jar and closed with a sterile lid, cork or parchment. You can use plastic bags that tie with twine.

Select 5 pieces of size 3 × 3 cm from the peripheral, not rotten and without mold areas of the skin. If the skins are stored in bales, piles (large batch), at least 3 samples from each location are collected from each bale and combined into one sample.When the skin of hemorrhages or infiltrates is found on the inside, samples are taken from these areas.

For bacteriological and virological examinations, areas of the intestine with the most characteristic pathological changes are selected.Then the intestines are laundered from fecal matter and placed in glasses separately from other organs. If necessary, preserve 40% glycerol solution in the ratio of 1:10.

3.2.2.6 Samples from the eye

Samples from the surface of the eye are collected by washing or purifying the eyes for cell selection, not mucous secretions or tears.Samples from the conjunctiva are collected by holding the fingers of the eyelid and lightly touching the surface of the eye with a cotton, dacron or gauze swab, pre-moistened with sterile saline or equivalent medium. Such smears should be stored in humid physiological or transport media specifically recommended for research. A biopsy from the third eyelid of sheep, which is rich in lymphoid tissue, is used to detect prions.

The sample can be selected with a sterile cotton swab, bacteriological loop or sterile eye stick. The discharge is taken from the inner surface of the lower eyelid toward the inner corner of the eye slit. Care should be taken to ensure that the eyelashes do not touch the tampon when blending. In the absence of visible pus, swabs moistened with sterile isotonic solution are used. The secret from the lacrimal sac is selected with a sterile cotton swab after a careful massage. Material from the cornea is selected with a platinum loop after local analgesia. In the presence of crusts, they are pre-removed with tweezers, and samples are taken with a sterile moist (0.5 cm3 saline) cotton swab from ulcers.

3.2.2.7 Reproductive organ samples

Fluids and smears from the birth canal, vagina, uterus and urethra are used to study diseases of the reproductive organs. Samples should be stored moist after being collected by placing them in a transport medium (sterile saline or culture medium). Spermsamples are obtained by artificial vagina or penis extrusion and artificial stimulation.The contamination of the sample with the antiseptic or detergent solutions used to prepare the animal / sampling site must be avoided.

3.2.2.8 Nasal fluid, saliva and vesicular fluid

For the sampling of nasal secretions, the wings of the nose and the front of the nasal passages are washed with water, after which the discharge are collected with sterile swabs from deep portions of the nose. Tampons are placed in sterile tubes containing 0.5 cm3 of sterile saline solution. Samples from a nose are selected by means of a tampon placed on a flexible wire handle. The tampon is injected into the nostril for a few seconds parallel to the palate. Then in a circular motion slowly remove the probe, clamping it to the lower inner surface of the nose. Samples from both nostrils are taken with the same swab. The tampons should be in contact with the secret for up to 1 min, then they are placed in a test tube with the transport medium and sent to the laboratory without delay at a transport temperature of 4 ± 0,5 ° C. The purulent secretions and (or) crusts are pre-removed with a cotton swab dipped in sterile saline, from the inside out. The sampling of the material is carried out in circular motions of a sterile applicator with a cotton tip and placed in a sterile tube with a transport medium or in a sterile tube.

Secrets are taken directly from the bottle or tube or by using a swab. The selected liquids provide a highly concentrated source of pathogen for diagnostic testing and are collected from undiscovered vesicles by a sterile needle and syringe and immediately transferred to a securely sealed vial or tube. Specific instruments (sample bowls) for sampling cell material and mucus from the pharynx of the animal. Cotton harnesses that animals chew are used to sampling saliva from domestic pigs.

3.2.2.9 Sputum

Sputum is best taken before feeding the animals in the morning. Before sampling, it is necessary to thoroughly wash the oral cavity of the diseased animal with clean water (cattle are not allowed to mix sputum with gum). Sputum is obtained by sampling the contents of the oral cavity after coughing. If it is not possible to select in this way, then the upper respiratory tract will be obtained by tracheobronchial probes (or elastic tubes) and sucked out contents.After selection, the sputum is placed in a clean, dry wide-necked vessel or a Petri dish.Selected material should be investigated as soon as possible. Store sputum at 3-6 ° C for 6-8 hours. Cattle sputum is collected by the method of coughing, wrapping a piece of cloth around his nose and mouth and squeezing his nasal openings. After coughing, a small amount of sputum is found on the canvas.Forcing an animal to cough while keeping its tongue out of its mouth, tilting its head down, large amounts of sputum can be collected on a plate.After coughing, the mucus remains on the pharyngeal wall, and in some cases between the tongue and the molars.From there it is obtained with a tampon or sterile gauze cloth, which is sent in a sterile vessel to the laboratory.The mucus can be collected from the trachea by inserting a sterile swab on the wire through the tracheotube.

3.2.2.10 Milk

For the diagnosis of certain infectious diseases, samples of milk, which is taken individually from each animal, are sent to the laboratory in a clean and labeled container.If necessary, pools are made of milk samples according to appropriate procedures.

Before taking milk samples from cows, the udder is washed with warm water; the dugs are treated with 70 ° alcohol. From each portion of the udder take the last portions of milk of 10-15 cm3 in separate sterile tubes with rubber stoppers.

In sheep and goats milk samples are taken by puncture of the udder cistern.For this purpose the animal is fixed in the lateral position, the udder at the base of the dug is rubbed with 70 ° alcohol and greased with iodine tincture.A sterile syringe with a needle is punctured at the base of the dus and after the needle gets into the tank (as judged by the free movement of the needle end), the milk is injected into the syringe and transferred to a sterile tube with a rubber stopper.

Milk is thoroughly mixed before sampling. The sample is taken with a 9 ml diameter metal tube that is vertically immersed in the bottom of the tank.The upper opening is closed with a finger, the tube is removed from the tank, and the milk is poured into a tightly closed vessel. The average sample of milk for the study is 250-500 cm3.

wo-day milk samples are preserved with 40% formalin solution at the rate of 1-2 drops per 100 cm3 of milk.

Milk samples are stored and delivered to the laboratory during 3-4 hours from the time of sampling in special containers ensuring a temperature not exceeding 4-10 ° C or in an ice-cream flask.

It is forbidden to send milk from cows for the first 2 weeks after calving.

3.2.2.11 Samples from abscesses, lymph nodes and discharge from wounds

The wool at the location of lesion is cut, the skin covering is treated with 70% alcohol and greased with iodine.

Then a sterile syringe with a large needle needle puncture and transfer punctate into a sterile tube with a rubber stopper. The purulent discharge and crust should be removed with a cotton swab dipped in sterile saline, from the center to the periphery. The sampling of the material is carried out in circular motions of a sterile applicator with a cotton tip and placed in a sterile tube with a transport medium or in a sterile tube taken in the laboratory. The entire surface of the abscess disinfect, make skin puncture, picking matter into the syringe. In the same way, the selection will contain pustules and vesicles. The pus from the opened abscesses, apostasis, boils and discharge from wounds take a sterile swab from deep.

The material from ulcers and wounds is obtained by scraping on the border of the affected and healthy tissues.

When the puncture is taken from the lymph node, the animal is well fixed, wool is excised at the puncture site, the skin is wiped with a cotton swab dipped in alcohol or iodine solution. With left hand pull back a lymph node and hold between thumb and forefingers.

Then a sterile needle is inserted into the depth of the node, a syringe is put on it and the lymph is sucked off.The syringe is disconnected, the needle is extracted, and the contents of the syringe piston squeezes on a glass slide. Make a thin smear and dried. The puncture site is disinfected with iodine solution. Manure, secretions from

different cavities, natural openings for microscopic examination (for detection of microorganisms, parasites) are sent in the form of smears.

Subject glasses previously boiled for 10-15 minutes in a 1.2% aqueous solution of soda, then washed well with clean water and wipe dry.Dry glasses are placed in a solution of alcohol-ether, taken equally, and stored for use.

Samples from purulent wounds are collected by syringe with or without needle by aspiration, which is closed with a sterile rubber stopper or injected with a needle into a sterile rubber stopper.

If the manure is very thick, it is scraped and collected in a sterile disposable container or placed in a dry sterile disposable tube or container with a medium using a sterile swab (cotton, viscose, alginate).

When sampling during surgery, tissue pieces (3-5 cm3) are placed in a sterile container or glass container, 3-5 cm3 of non-bacteriostatic saline solution is added to prevent the material from drying out.

3.2.2.12 Bones

Pieceless tubular bones with intact ends are cleansed from muscles and tendons, sent in a sealed plastic bag.

From small animals, you can select a small piece of rib or tubular bone, previously freeing them from muscle and cartilage. From large animals it is possible to scrape a bone marrow tissue from any tubular bone, scapula, vertebrae, or chest bone with a knife or scalpel. To do this, it's necessary to bare the articular surface, cut the cartilage and with help of the end of a knife or scalpel, rotating them, scrape up and put in a bottle required number (5-10 g) bone and brain tissue. Bones can also be preserved with kitchen salt (pouring bone material with salt and tight wrapping (wrapping).

3.2.2.13 Samples of brain

Pathological material (brain) is selected from animals with clinical signs of damage to the central nervous system.The brain for histological, electron microscopic and immuno-chemical studies is selected immediately after slaughter or death of the animal until the onset of tissue lysis or reproduction of other associated microflora.

For this purpose it is necessary to detach the head from the neck along the atlanto-occipital junction and well fix. Remove the skin and muscles from the skull, making incisions between the ocular cavities and from them to the nape of the neck. Use a saw, ax, scissors and tweezers to remove the skull. Separate the main nerves, remove the brain and place it on a cell or board.Take samples of the cerebral cortex and the bases of the spinal cord. For research on rabies, it is necessary to select samples of ammon horns. For this purpose it is necessary to open longitudinal sections of each central hemisphere at a distance of 2 cm (for dogs) from the midline of the brain, to remove the upper parts to the gap (space), in which are ammon horns, which are semicylindrical bodies of white color. It should take a few pieces of Amon horns and base of the spinal cord. The total weight of each sample should be 5-10 g. Separated from the corpse, the head is packed in plastic wrap, placed in a metal box with ice and transported to the laboratory at 2-5 ° C.

3.2.2.14 Sampling for suspected diseases with signs of nervous system damage

In diseases accompanied by signs of nervous system damage (Teschen, Aujeszky's disease, otitis media, rickettsiosis, sporadic bovine encephalomyelitis, rabies, etc.), brain, bone, lymph nodes, blood and, if present, other organs are taken for sampling. Blood from diseased animals should also be sent to detect antibodies in serological reactions. For convincing results, it is desirable to send blood samples from a large number of diseased animals (up to 10 samples). For suspected rabies, it is necessary to send the whole head whenever possible without resorting to brain sampling. If a whole head is sent to the laboratory, it should be wrapped in paper, additionally packed in a plastic bag, placed in a non-leaking bucket or tin, and urgently delivered to the laboratory. Brain sampling can only be done to ensure the safety of the staff working with this material, and then disinfect the workplace. Brain samples may only be taken by a specialist who has no injuries on the hands, must be in rubber gloves and who is vaccinated against rabies.Personnel not involved in this operation should be away from this place of work.

3.2.2.15 Samples from honey bees

For bacteriological, microscopic, serological, biological, chemical and other studies, samples of live bees and their corpses are taken; honeycombs with brood, honey, parchment; dried bee larvae, feces or stool scrapers; hemolymph smears or muscle imprints on the slide; waxy crumb from the bottom of the hives; parasite insects and bee pests. Depending on the type and method of research, from 50 to 500 live / dead bees from different families are selected, the samples of 10 × 15 cm of honeycombs with dead larvae and pupae, garbage from the bottom of

beehives (not less than 200 g from apiary), spring - bee brood on a honeycomb 3 × 15 cm in size, 200 g of pumped unsealed honey, 50 g of parchment in a honeycomb. Pathological material is packaged and sent in accordance with the following rules: live bees are placed in glasses, which are bound by two layers of gauze or cloth; samples of brood cells and honeycomb frames are sent in plywood or wooden box, not wrapping paper, but separating them from each other and from the walls of the box with wooden strips; sick living bees - on fixed cellular frames with feed in quantity sufficient for the time of transportation, in plywood or wooden box; dead bees and debris from the bottom of the hives - in paper bags.Honey - in tightly closed glasses.

3.2.2.16 Samples from fish

Sick or suspected infectious and invasive disease fish are sent to the laboratory alive. 15-20 individuals with well-defined signs of the disease are selected for the study.

The fish are transported in clean baths or other tanks intended for the carriage of live fish filled with ¾ volume of water from the same reservoir from which the fish was selected or from an artesian well. Fish sent to the laboratory in paper; gauze or other packaging materials are not suitable for research.

In the summer, with long transportation, the water with the fish is gradually cooled to a temperature of 12-15 ° C, adding small pieces of ice. To avoid temperature shock, it is not possible to remove fish into colder water than in a reservoir (7 ° C or more difference). If it is not possible to send live fish, then from large fish take pieces of the affected organs and tissues. The samples are placed in a sterile glass vessel, poured into a sterile 40% aqueous glycerol solution, sealed and delivered to the laboratory. Blood, exudate, intestinal contents sent in sealed sterile Pasteur pipettes. In summer, pathological material is suitable for bacteriological examination within 2 hours of its collection. In winter, you can freeze samples.

For virological examination, live fish are packaged in a double plastic bag filled with 1/3 volume water and placed on ice. The package is packed in a box and delivered to the laboratory. Dead fish is sent only if it was killed after being caught before being sent to the laboratory. The selected fish is packaged in a plastic bag, which is placed in an ice flask. For virological examination, internal organ samples (organs from 5 fish are combined into one) are aseptically collected and placed in a sterile vial, which is tightly closed and placed in a thermos or ice pack.

If immediate dispatch is not possible, the package is stored in the refrigerator at temperatures not higher than + 4 ° C during the day. Pathological material from a sick or suspected viral infection of fish is allowed to preserve in 50% phosphate-buffered glycerol solution (pH 7.2-7.4). Material for histological examination is taken from diseased asleep fish. Small fish (juveniles, annuals) after opening the abdominal cavity is fixed whole, and from large take organs or pieces of organs in size 2 × 3 cm, 0.5-1 cm thick.

Pieces from the affected organs are cut out, capturing normal and damaged tissue.

Regardless of the degree of damage, samples of different organs (skin with muscles, gills, liver, spleen, heart, intestines, swimming bladder, brain) are taken.

The intestine is cut open or made several cuts before fixation to allow the fixing fluid to penetrate into its cavity. The brain is removed whole after opening the cranial box. The samples are placed in a wide-necked glass jar and fixed.

To the blood for biochemical studies add lemon or oxalic sodium (1 cm3 - 2 cm3), 1- 2% solution of heparin (1 cm3 - 0,01-0,04 cm3) and delivered to the laboratory in hermetically sealed glasses (test tubes) with labels. In case of suspected fish poisoning, water samples are taken from the reservoirs directly at the site of the death of fish, sewage from industrial enterprises and agricultural objects located near the catchment area of the reservoir.

3.2.3 Biopsy

Biopsy is a diagnostic procedure involving extraction of sample cells or tissues for examination to determine the presence and extend of a disease. Biopsies are commonly performed for differentiation between inflammatory, cancerous or other conditions.

Types of biopsy procedures:

1. excisional biopsy – entire tumour is removed;
2. incisional biopsy – few smaller samples are collected;
3. fine needle biopsy – cytology – sample or fluid collected mainly with needle.

1. Excisional biopsy

This is a common approach for mass removal in veterinary medicine, but it remains controversial. It may provide a diagnosis and, therefore, a therapeutic plan, but the mass is removed without knowing the tumour type. It is much more invasive.

These days a less invasive biopsy procedure is recommended to guide the veterinary surgeon in choosing the appropriate method of surgical resection, which may be more suitable to maintain an adequate tissue margin.

Excisional biopsy is best reserved for cases in which adequate surgical margins are easily achievable with biopsy alone (eg, small cutaneous mass on trunk of a dog).

a. Sampling

Sampling takes place under general anaesthesia. Surgical margins should be determined before the surgery; in general – 1 cm in benign masses, 3 cm in aggressive tumours. Once it is collected and properly prepared, histopathologist evaluates it.

b. Preparation and fixation

The time between sampling and fixation should be minimizing. The biopsy sample should be placed into a fixative as soon as possible after the procedure. The sample should not be necrotic.

When the margin evaluation is important, cranial and caudal margins should be marked (i.e. by sutures or ink).

Smaller samples (3-5 mm) could be fixed in their entirety. Larger samples should be cut at least in half before fixation or smaller tissue section could be prepared. If the sample is surrounded by thick capsule, also it should be cut (Fig. 1). Fixation could be performed with 10% buffered formalin, alcohol or by freezing in low temperature (-80 °C). At least 10 volumes of formalin for 1 volume of tissue should be used (Fig. 2).

Fig. 1. Excisional biopsy. Tumour on the right side should cut at least in half or sliced into smaller pieces.

Fixative penetration is usually directly related to tissue thickness. If specimens are very large, it takes a great deal of time for the fixative to penetrate the inner portion of the sample. As a result, autolysis can occur in the centre of thick tissues.

c. Transport

UN 3373 - Biological substance, Category B. The histopathological samples must be transported in compliance with 49 CFR, Part 173.199 or IATA Packing Instruction 65.

Any packaging for biological substances must include three components: - a primary receptacle: the tube, vial or other container typically made of glass or rigid plastic (including the stopper, cap or other closure elements) that is in direct contact with the specimen (Fig. 2 and 3);
- a secondary packaging (including cushioning and other materials) that fully encapsulates the primary receptacle (Fig. 3 and 4);

- an outer packaging for shipping or transit.

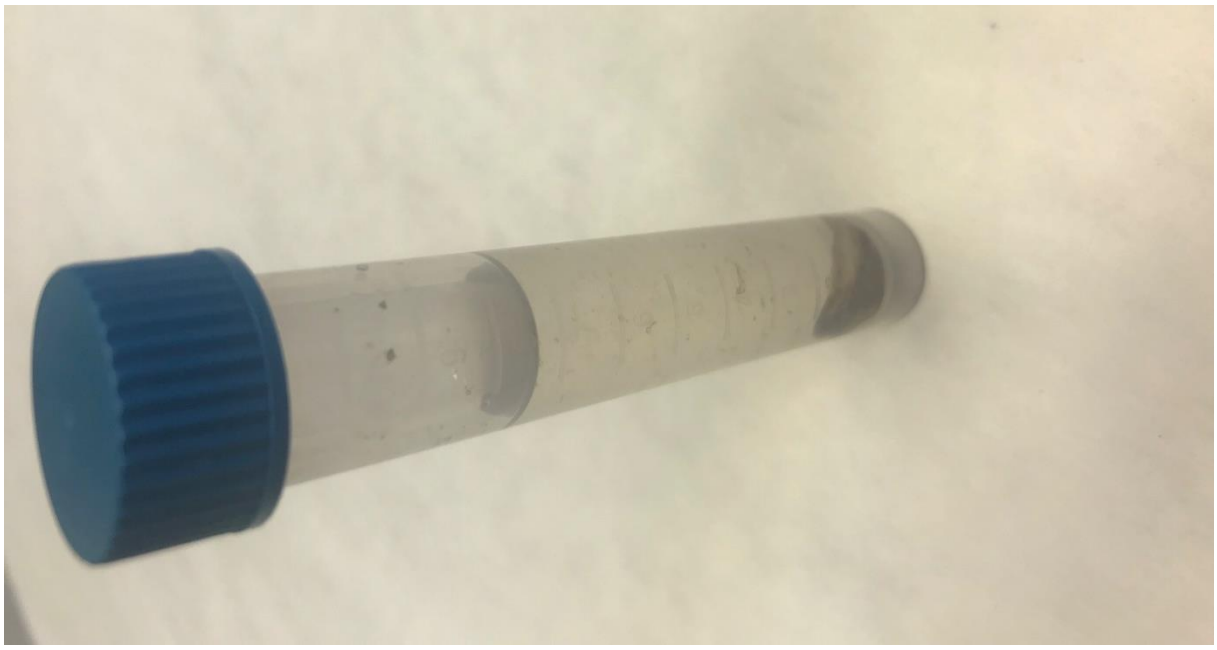


Fig. 2. Primary packaging, enough amount of formalin was used.

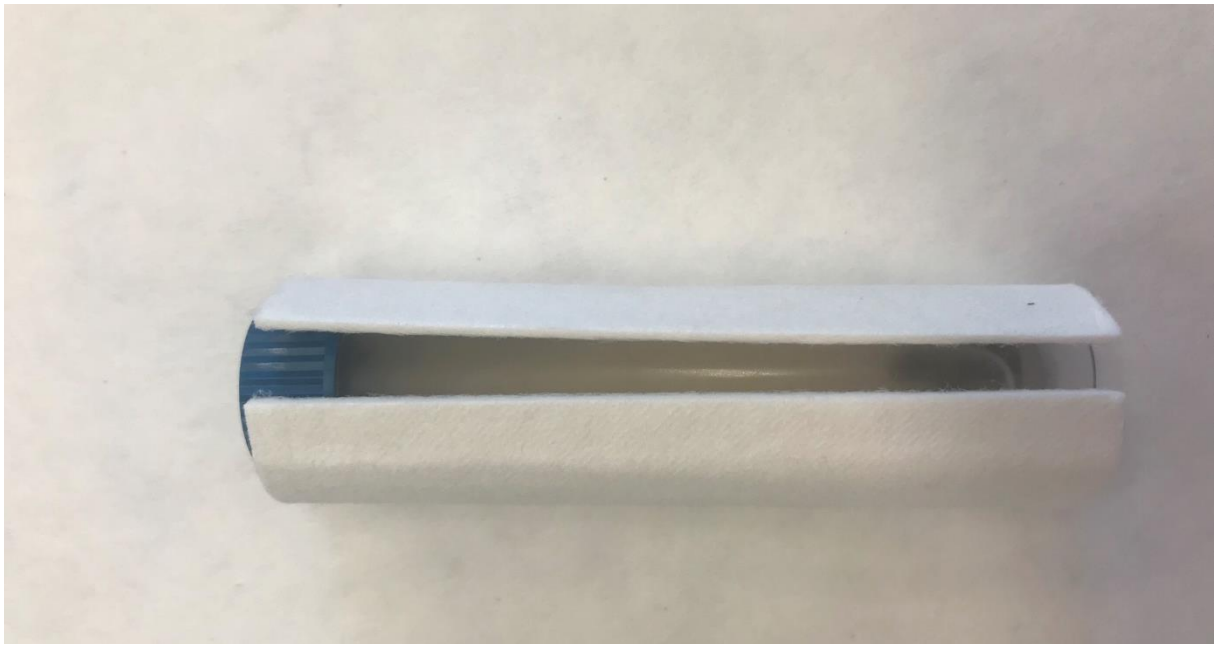


Fig. 3. Package cushioning.

Fig. 4. Secondary packing with visible cushioning.

2. Incisional biopsy

Incisional biopsy is performed when less invasive techniques fail to yield a diagnosis. Wedge and punch biopsies are examples of incisional biopsy techniques.

It is especially useful for diagnosis of soft, friable, inflamed, and/or necrotic tumours. The border between normal healthy tissue and abnormal tissue also could be examined, which allows the pathologist to determine extent of tumour invasion into normal tissue.

a. Sampling

Sampling takes place under general anaesthesia. Incisional biopsy varies in size (e.g., 2–10 mm). Material which volume is less than 3 mm may be too small for histopathological evaluation and should be avoided. Typically 4 to 8 mm biopsy is collected. Once it is collected and properly prepared, histopathologist evaluates it.

b. Preparation and fixation

Fixation agents are the same as during excisional biopsy. Small samples could be placed directly into histology cassette.

Very small samples (< 3 mm) can be easily lost during shipping or in processing, because sample shrinkage during the fixation and processing. This kind of samples could be placed on paper before fixation (surgical glove paper is appropriate). Extremely small samples could be dyed with eosin or commercially available ink (i.e. India ink).

c. Transport

The transport is the same, as in the case of the excisional biopsy.

3. Cytology

The cytology is a rapid, cost-effective, minimally-invasive procedure that is useful for differentiating non-neoplastic from neoplastic diseases and, in many situations, obtaining a definitive diagnosis. It has a relatively low risk of complications.

Cytology is usually reserved for cutaneous and subcutaneous masses, fluids (effusions, joint fluids, cerebrospinal fluid, bronchoalveolar lavages, urine and prostatic washes), but internal organs also can be examined by sampling with ultrasonography or computed tomography guidance.

Cytological biopsy technique needs minimal amount of tissue sampled, but lack of organized tissue architecture prevents tumour grading. Some of tumour types, especially mesenchymal tumours (i.e. sarcomas) usually do not exfoliate cells well, leading to low-cellular samples and false-negative results. Interpret negative aspirates or aspirates with questionable results with caution, and if needed perform more aggressive biopsy techniques.

a. Sampling

Aspiration cytology

Fine-needle aspiration (FNA) is the most common biopsy technique used for skin masses, lymph nodes and internal organs. Different techniques may be used to collect the sample. After collecting the sample, the smear should be done.

- **Needle only method.** It is commonly used for delicate, soft masses and lymph nodes. During this technique sample is collected without syringe, only with needle (size 20-23 G; 0,9-0,6 mm). The needle is moved back and forth and directing in many directions within the tumour. The needle-only method leads to the accumulation of the sample in the hub of the needle (Fig. 5).

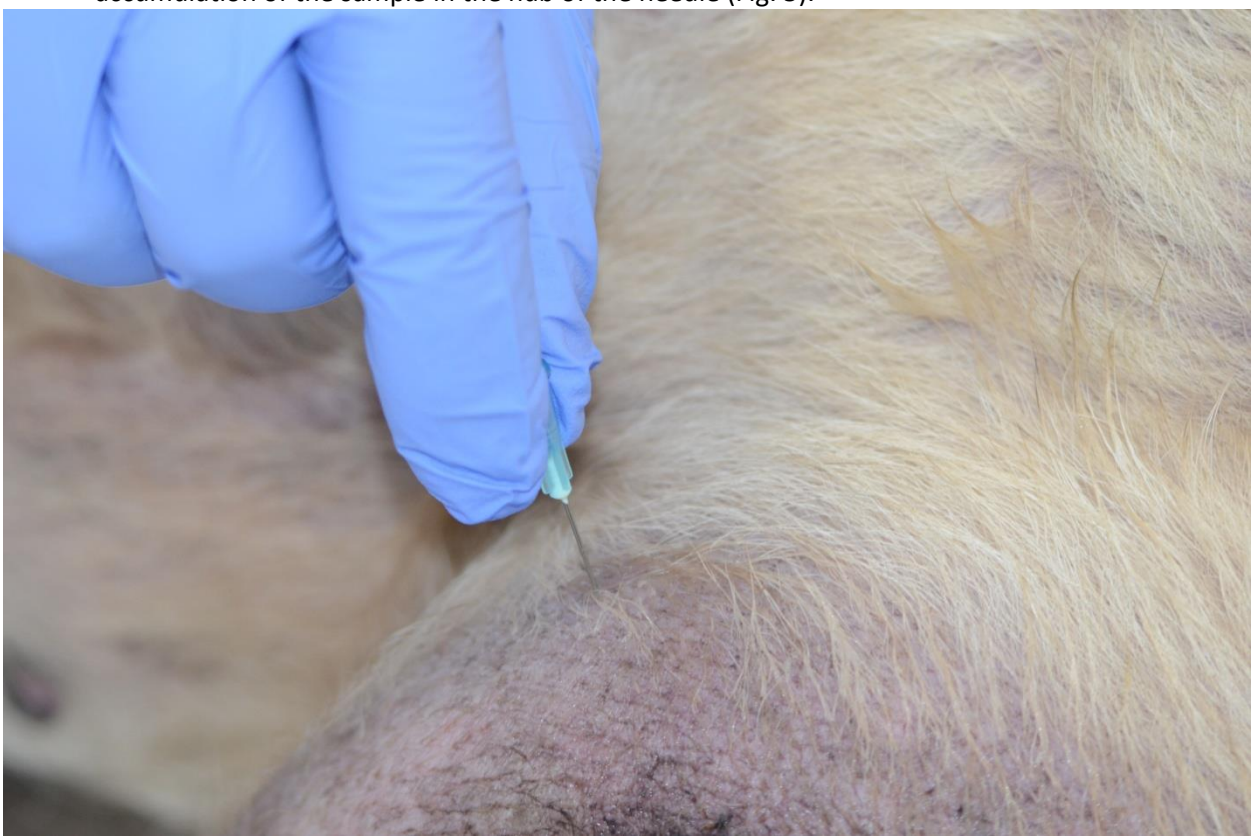


Fig. 5. Needle only method.

- **Continuous suction method.** It is recommended for more firm or poorly exfoliative masses. During this technique sample is collected with syringe (2,5-5 ml) and needle (size 20-23 G; 0,9-0,6 mm). The needle is moved back and forth and directing in many directions while the suction is applied by withdrawing the plunger. Suction is released before removing the needle from the tumour (Fig. 6).

Fig. 6. Continuous suction method.

Impression smear cytology

This method is used for superficial ulcerated lesions or intraoperative cytology during surgery. This sample may be not representative for entire tumour.

Before procedure lesion surface should be cleaned. Glass slide should be gently appose to mass and quickly removed. Several imprints on one slide could be done. If the collected material is thick, additionally smear should be done (Fig. 7).

It should be mentioned, that this method is perfect for superficial part of the lesions or intraoperative cytology. The sample is not representative of the entire lesion.

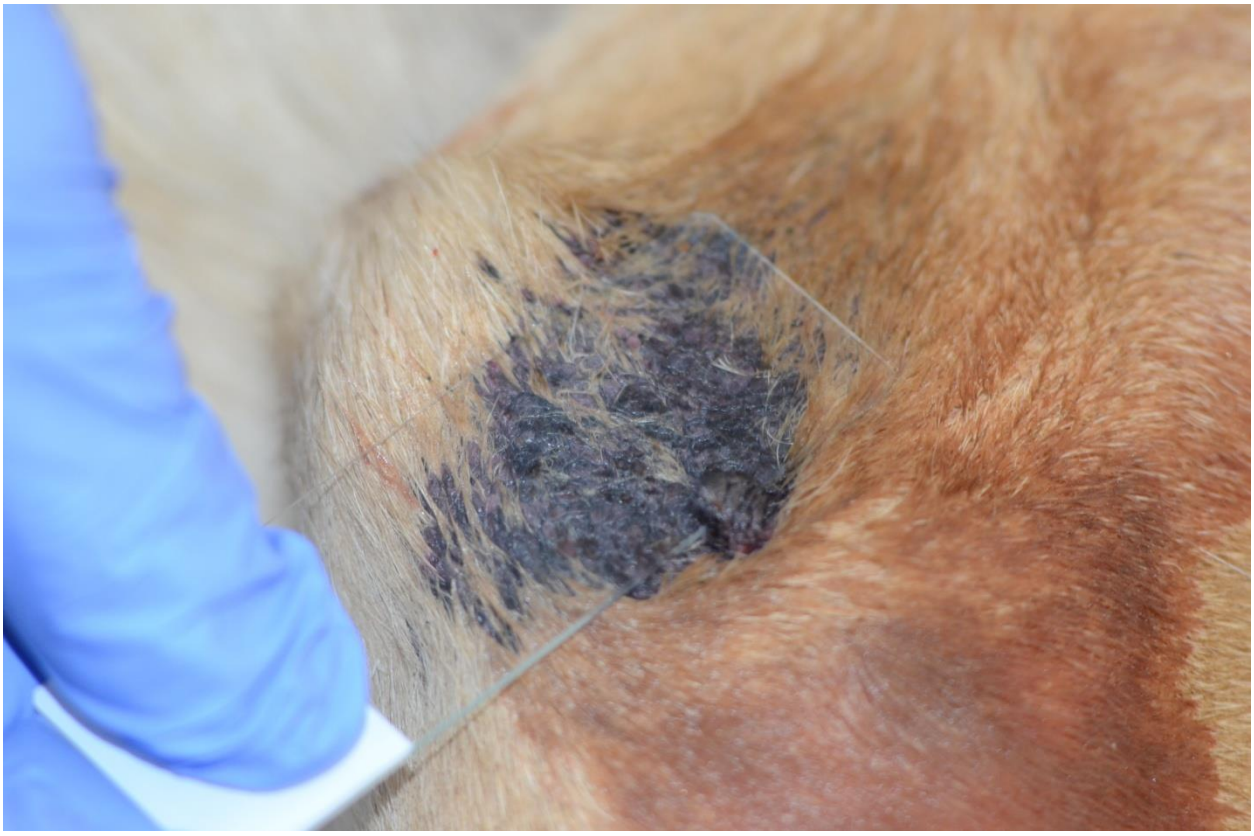


Fig. 7. Impression smear cytology.

Scrape cytology

Scrape cytology can be divided into two main types. Superficial scraping gives us information about epidermis, deep scraping – about dermis.

Exfoliative cytology

The microscopic study of cells shed or obtained from the body, e.g. fluid, urine, CSF, ear or vaginal cytology.

b. Preparation and fixation

The smear should be thin, thick smear is often not representative. There are two main smear preparation techniques:

- blood smear technique. Small drop of deposit should be placed near to the end of the glass slide. Drop should be touched by the second slide. the second slide should be pushed across the surface of first slide (Fig. 8).

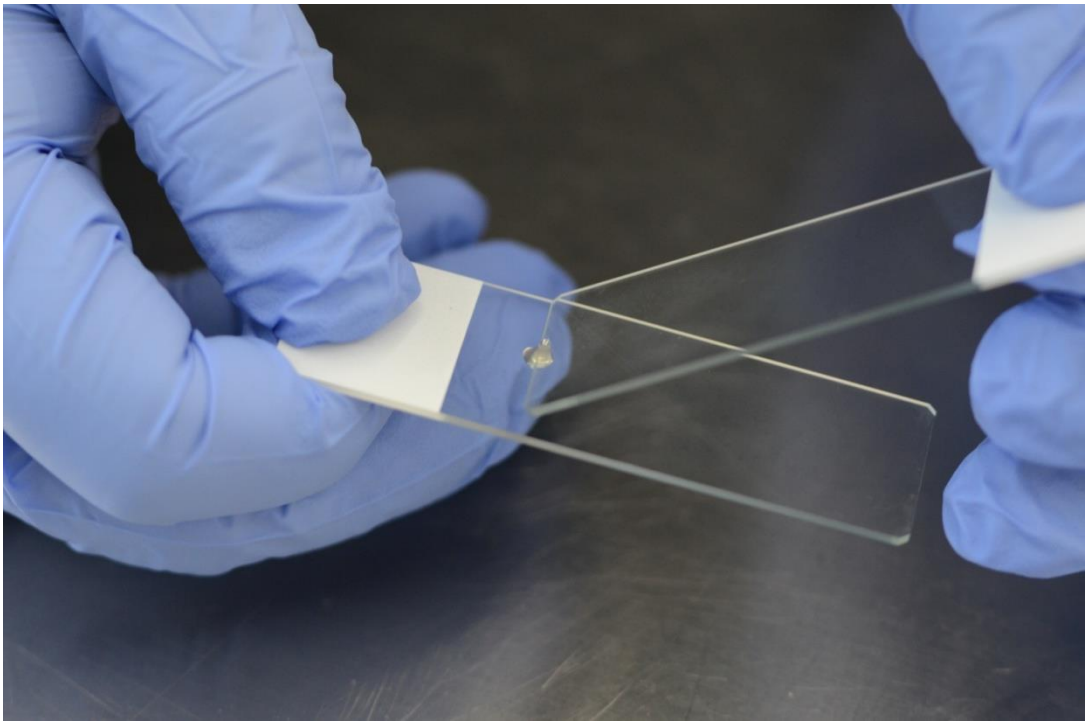


Fig. 8. Blood smear technique.

- squash preparation. The collected material should be placed on the first slide. The second slide should be squashed against the first slide and then – pulled (Fig. 9).

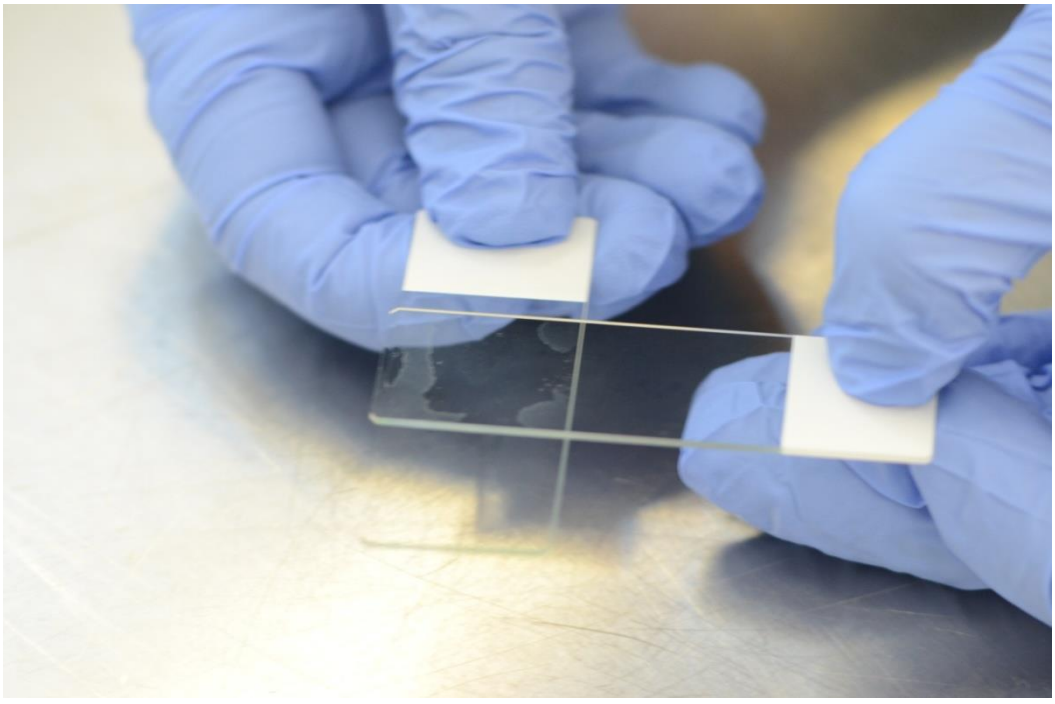


Fig. 9. Squash preparation.

The sample should air-dried and fixed. Fixation could be with alcohol or commercial fixative spray.

There are plenty of staining methods. One of the most popular is Diff-Quick® commercial Romanowsky stain variant, commonly used in cytological staining to rapidly stain and differentiate a variety of smears.

c. Transport

If the transport is needed, the appropriate container (transport kit) should be used (Fig. 10).

Fig. 10. Cytological biopsy kit.

3.3 Agronomy

3.3.1 Soil

Soil preparation for analysis

To determine the chemical composition of the soil and its physical properties, take in the field samples from places that are homogeneous in relief, vegetation and agrotechnical condition.

Soil samples are taken by borer of Nekrasov, Kaczynski or others, or directly from the pits. On a plot having a flat terrain, samples are taken diagonally, on an incongruous surface, too, diagonally, but from each ground feature.Samples are taken from the appropriate depths and from several points, mixed and each mixed sample is taken on an average sample of 1 kg, transferred into a bag with a label, on which indicate the graphite pencil field number, pits, depth of the sample, the name of the household, and the signature of the person who signed .

When study the large tracts of land, samples are taken from genetic horizons - separately from all detected soil types in the territory.

The selection of soil samples from the cut (on the example of sod-podzolic soil). On the front wall of the cut using a measuring tape or woodenmeter divide the profile into genetic horizons, in the field diary or log indicate their indices (NO, E, I, P) and depth of occurrence. Then they clean the wall (from up to down) and with a wide knife mark the places where the samples will be taken. Samples are taken from the bottom up, starting from the lower horizon and ending with the upper (arable layer). The samples are taken as monoliths from the middle of the genetic horizon 10 in length, 8 - 10 in width and 6 - 8 cm in thickness. Two samples are taken in the arable layer - from a depth of 0 - 10 and 10 - 20 cm, and one in the subsurface - from its middle. In the illuvial (I) horizon, depending on its magnitude, two samples are taken: in the lower, middle and upper parts. Each sample is placed in a numbered pouch, where a label is placed on which to record the address, field or experiment name, section number, horizon, depth of sampling, date and name of the executant. In the laboratory, the soil is crushed, dried to air-dry state, selected plant residues and sifted through a sieve with openings up to 1 mm.

Preparation of soil samples for laboratory analysis

The selected soil samples are placed in paper bags or cloth bags and labeled. The label indicates the location and depth of the sample, the sample number or test area, and at the end put date and signature. All records are made with a simple (non-chemical) pencil. Each sample should have two labels - one inside, the other outside. It is recommended that samples with undisturbed structure be sampled in cylinders or aluminum cups in the order of cylinder numbers,

specifying at the same time in the field log the cylinder number, location, depth and date of sampling. The samples are then placed in special boxes and transported to the laboratory without severe shocks or strokes.

In the laboratory, soil samples are air-dried in a dry, well-ventilated and protected from the access of vapors of acids, ammonia and other gases to the room, to an air-dry state. To dry the soil, spread a layer of 2-3 cm on a sheet of thick paper, gently crushing large pieces. The dried samples are placed in cardboard boxes and stored until research.

Most soil analyzes are performed in air-dry specimens. An average sample is taken from the dried soil sample. To do this, it is thoroughly mixed, scattered on paper in the form of a square or rectangle and divided diagonally into four equal parts. Two opposite parts are poured into a cardboard box, and the other two are mixed. The operation is repeated until the weight of the average sample is 300-400 grams, and the ground that is not included in the average sample do not rub.

To determine humus and total nitrogen the average sample is prepared as follows. The soil is strewn with an even layer of paper in the form of a rectangle, which is further divided by vertical and horizontal lines into small squares of 3x3 or 4x4 cm in size, and from each square take a small spatula of soil, which is poured into one sample so that its mass is about 5-10 g. The samples selected in this way carefully select the roots (with tweezers and loupe). Then the soil is pound in an agate mortar and sift through a sieve with holes with a diameter of 0.25 mm. The resulting sample is stored in a paper bag. Similarly, an average sample is taken to determine the particle size distribution of the soil, its mass is 30–40 g. It is necessary to pound this part of the soil in small portions in a porcelain mortar with a rubber tip tip, to sift through a sieve with openings with a diameter of 1 mm and to store in a paper bag. Prepare the rest of the soil sample (250-350 g) in the same way and store it in a glass with a ground stopper. From this part of the sample, samples are taken for other laboratory tests.

Sampling for determination of soil properties under laboratory conditions

Sampling to determine the specific gravity of the soil

To determine the specific gravity, use a pycnometer or measuring flask with a capacity of 100 cm3.The principle of the method is to determine the volume of a piece of soil by the volume of displaced water. Selected dry soil. The amount of dry soil in the sample for laboratory analysis should be 10 g. The amount of absolutely dry soil in 10 g of air-dry can be determined using hygroscopic moisture, calculated as a percentage of air-dry soil.

Sampling for determination of bulk(volume) mass

Volumetric mass of soil– is the mass in grams / 1 cm3 of soil with undisturbed structure, ie the mass of 1 cm3 of soil together with its pores. The bulk mass depends on the mineralogical, granulometric and structural composition of the soil and the amount of organic matter in it. The bulk mass is always less than the specific mass and ranges from 1-1,8. There are two methods of soil sampling:

Method 1: Cylindrical method: Insert a 10 cm diameter metal cylinder with a volume of 1 dm3 into a ground with undisturbed structure. Dig the soil around the cylinder, carefully trim it from below and remove the cylinder. Carefully trim the soil protruding from the cylinder, even with its edges. When performing the above operations, it must be ensured that the soil structure is not disturbed and the soil does not spill out of the cylinder. The correctness of the obtained results depends on this. If the soil partially spilled out of the cylinder, then a new sample must be taken. The cylinder must be closed with a lid so that the soil does not spill out of it. After that, the cylinder with soil must be weighed. Weigh the empty cylinder with the lid. And from the soil under study, take a sample to determine the humidity. Knowing the weight of the empty cylinder and its weight with the soil, the weight of the volume of soil taken is calculated.

Method 2. *Paraffin treatment method:* From the soil of the undisturbed structure cut in the form of a ball or other shape of size 5x5 cm and weighed on technical or electronic scales. Melted paraffin is prepared at the temperature about t-2-3° C above the melting point. A piece of soil is then immersed in paraffin on one side or the other to create a paraffin film on the soil surface. After formation of 5-10 layers of paraffin, the soil with paraffin film is cooled and weighed. The difference between the weight of the paraffin (n) soil and the pre-paraffin weight (m) is the paraffin weight (n-m). To find the volume occupied by paraffin (WP), the weight of the paraffin must be divided by the specific gravity (P), equal to 0.89.

Sampling to determine the moisture content of the soil

Determination of field and hygroscopic moisture

Field moisture characterizes the amount of water in the soil during sampling. Defining it, we learn about the total soil moisture in the soil and its dynamics during the growing season of plants.

Hygroscopic moisture for plants inaccessible and is a dead water supply in the soil. The amount of hygroscopic moisture in each soil depends on the humus content, particle size distribution and the presence of hygroscopic salts. The more humus and mineral colloids in the soil, the greater the amount of hygroscopic moisture.

When determining the field moisture, take in boxes 10-20 g of soil and 3-5 g of air-dry soil (when determining hygroscopic), cover them with lids and determine the moisture according to the method of analysis.

Sampling to determine soil moisture capacity

When determining the total and capillary moisture capacity of the soil, you must take soil samples with undisturbed structure. But in the laboratory sometimes determine the moisture content of the soil in samples that have a broken structure, that is, pounded and sifted through a sieve with holes of 1 mm.

Sampling to determine the capillary moisture content of the soil. Put a circle of filter paper in the grid, moisten it with distilled water, put the grid on the cylinder and weigh on the counter balance to the nearest 0,01 g. Further, remove the grid, insert the cylinder into the drill chuck and take a soil sample at a given depth by Nekrasov or Kaczynski drill. After taking the sample, remove the cylinder from the drill cartridge, close the lids and transfer to the laboratory.

Sampling to determine the total moisture content of the soil. Soil sampling is carried out in the same way as for sampling to determine the capillary moisture capacity of the soil.

Sampling of soil for biological indication after the action of herbicides

Samples are sampled by random sampling from conventional soil and agrochemical surveys. The edges of fields, elevations, locations of possible sprayer stops, and other places where herbicides may accumulate should be selected separately for analysis.

Soil samples for biological indication are selected by the « envelope » method.

In normal soil tillage, samples are taken in layers: 0-10, 10-20, 20-30 cm. Using zero technology, the sample is taken in a layer of 10-15 cm.

The common sample (for layer-by-layer selection, each layer separately) must be at least 5 dm3 (= 5 l). A control sample of soil (5 dm3) was collected on untreated areas.

Sampling and preparation of the soil for granulometric analysis

A sample of soil from the study area weighing from 0.5 to 2.5 kg is dried to air-dry state and scattered on a piece of paper in the form of a square. The soil sample is then divided diagonally into four parts using a glass rod. One part is completely removed, weighed and passed through sieves with openings 10, 7, 5, 3, 2, 1mm. Sifting of all parts of the selected soil sample with an approximate weight of 100 g is carried out gradually, in small portions, separately through each sieve. The residue of the aggregate particles of each fraction on a sieve is transferred to a pre-weighed porcelain cup (or a tattered sheet of paper) and weighed on a balance. According to the obtained data, the percentage content of each fraction relative to the mass of the soil sample taken is calculated.

Soil preparation for granulometric analysis (by NA Kachinsky)

The basic preparation of the soil for analysis is the preliminary destruction of soil aggregates. For this purpose it is pound in a porcelain mortar with a rubber tip tip and sifted through a sieve with a hole diameter of 1 mm. The skeletal part remaining after sieving on the sieve is washed from the clay particles, dried, weighed and calculated as a percentage of the total weight of dry soil. Then proceed to chemical preparation in accordance with the method of NA. Kaczynski. It depends on the presence of calcium carbonate and magnesium in the soil.

Preparation of carbonate-free soil

A soil lot of 10 or 20 g is poured into a porcelain cup (the lighter the granulometric composition of the soil, the larger the soil lot should be taken) and portions are poured there from a measuring flask of 200 ml of 0.05 n hydrochloric acid, stirring with a glass rod with a rubber tip, allowing, settle to the bottom; thereafter, the suspension is filtered through a medium-density moistened with distilled water, a medium-density filter calibrated to a volume of 300 ml. The last portion of the acid, along with the soil, was transferred to a filter and washed with distilled water up to 300 ml, avoiding turbidity. If there is a turbidity in the flask indicating the passage of colloids through the filter, the washing is stopped. The wash water together with the filtrate should be equal to 300 ml. The filtrate is thoroughly mixed and used to determine losses from soil treatment with hydrochloric acid and the amount of absorbed bases. The magnitude of the losses from treatment with hydrochloric acid is of independent importance. It characterizes the presence of readily soluble salts and carbonates in the soil.

With a pipette, 50 ml of the filtrate is collected and transferred to a pre-weighed porcelain cup, placed in a water bath, evaporated, dried in a drying oven for 2 hours, cooled in a desiccator and weighed on an analytical balance. The calculations are performed by the formula.

Preparation of carbonate soil

A soil lot of 10 g is treated in a 0.2 n HCl porcelain cup, topping up with approximately 50 ml of acid each time. The soil treated with the first portion of acid is stirred with a glass rod 5-6 times for one hour. After that, the liquid on a stick is poured onto a medium density filter into a 500 ml volumetric flask and made sure that all the soil remains in

the cup. A new portion of acid is added to it. The dissolution of carbonates is repeated until the formation of CO2 bubbles ceases. After the last portion of the acid is poured, the soil cup is left overnight for the final dissolution of the carbonates, and then washed with 0.05 n HCl until no calcium reaction occurs.

Calcium test. Collect from the funnel about 3 ml of filtrate and neutralize it with a 10% solution of ammonia, adding the latter to a distinct odor, acidified with a few drops of 10% acetic acid, add to a test tube 2 ml of a saturated solution of ammonium oxide and heated to boiling . If there is calcium in the filtrate, the CaCrO7 precipitate precipitates.

In the presence of calcium, the soil is still treated with hydrochloric acid. If calcium is absent, allow the hydrochloric acid to drain completely from the filter. The filtrate determines the amount of dissolved substances in the acid. To do this, measure its volume, or bring to 500 or 1000 ml, mix well. Pipette of 25 ml and evaporate in a pre-weighed cup.

The filter soil is washed with water in a 500 ml conical flask, gradually adding 0.1 n NaOH to a slightly alkaline reaction. The suspension is boiled as in the preparation of carbonate-free soil.

Preparation of soil for granulometric analysis by rubbing with sodium pyrophosphate solution

From an air-dry sample of soil sifted through a sieve with 1 mm openings, take a sample of 10 g, weighed to the nearest 0,01 g and place in a porcelain cup with a diameter of 10-12 cm. Pour a small volume of 4% sodium pyrophosphate into a small glass. For unsalted and non-gypsum soils of light granulometric composition take ml for heavy-loam, clay and carbonate soils - 10 ml, and for saline and gypsum soils - 20 ml.

A piece of soil is moistened with a drop of sodium pyrophosphate solution to a paste-like state and carefully, without pressure, rubbed with a rubber tip tip for 10 minutes. After grinding, pour into the cup the residue of a solution of pyrophosphate, add distilled water and stirring with the same pestle with a rubber tip. After grinding, pour into the cup the residue of a solution of pyrophosphate, add distilled water and, stirring with the same pestle, bring the mixture to a state of suspension. The saturation reaction of the absorbent soil sodium complex is by equation.

3.3.2 Seeds

3.3.2.1 Sampling of seeds and plant material for analysis

A batch of seeds is a certain number of homogeneous seeds of one culture, variety, reproduction, varietal purity, physical qualities, year of harvest and one origin, numbered and certified by the relevant documents. The batch size depends on the size of the seeds (from 250 centners (grain) to 2 centners (tobacco)). To analyze the physical and sowing qualities of the seeds of any seed lot, it is necessary to take an average sample from it - this is a relatively small sample of seeds, which should characterize all the features of a large seed lot.

Accuracy in taking the average sample can be achieved only when it is taken not from one place, but consists of a large number of small samples - recesses taken from different places of the seed batch. This explains the complexity of the method of taking the average sample and the selection of samples.

The accuracy of the selection of the average sample is affected by the following: taking a sample from too many seeds, insufficient size of the average sample and violation of the rules of its selection (a small number of places from which the recesses are taken).

Medium samples for determining the quality of seeds are taken from prepared batches of seeds, ie cleaned, sorted, dried (in case of high humidity), weighed, numbered and having labels of the established form.

The assembly of the initial sample begins with the taking of recesses.

Before taking the grooves, the seed lot is carefully inspected, paying attention to the color of the seeds, their luster, odor, clogging, moisture and homogeneity, check all the documents for this seed.

If a batch of seeds weighs more than the control unit established for this crop, it is first divided into parts (control units), and then set the number of recesses from each of them to compile the initial sample.

Simultaneously with the preliminary inspection of seeds check the conditions of their storage (storage procedure, care for them, the state of storage). All features identified as a result of the inspection are recorded in the act of sampling. After that, proceed to the selection of excavations.

The main task when taking extractions is to correctly identify the places of their selection. It is necessary to follow the following order: to take samples from three layers - the top, average and bottom.

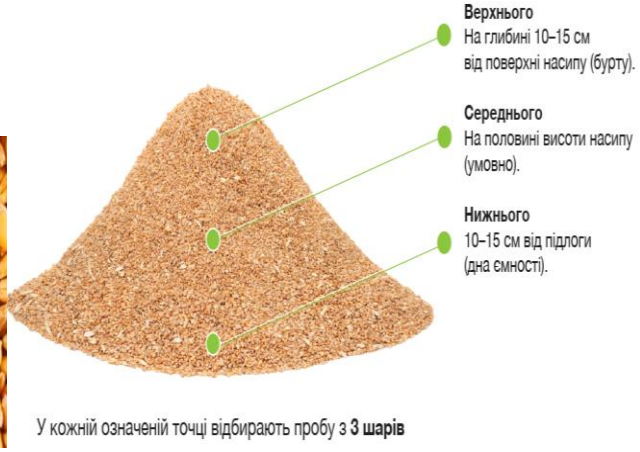
After screening each sample separately for seed homogeneity, they are pooled to obtain the original sample. In the case of a sharp difference between the samples, depending on the number of seeds is divided into two or more control units and from each of them make up the original sample.

Two medium samples are taken from the obtained initial sample by means of cross-section: one for determination of humidity and damage by barn pests (it is contained in a glass vessel which is tightly closed and filled with wax, wax or paraffin), the second - for determination of physical and sowing qualities of seeds.

Pointsampling from the embankment

Spot samples are taken from each compartment or collar in the form of an envelope in five places for batch sizes up to 20 t or 20 m² of surface. If the surface area of the seeds is larger in area and weight than the previously established one, it is conditionally divided into
sections, approximately 20 m, each, and selected from each in five places.

More than 20 m²



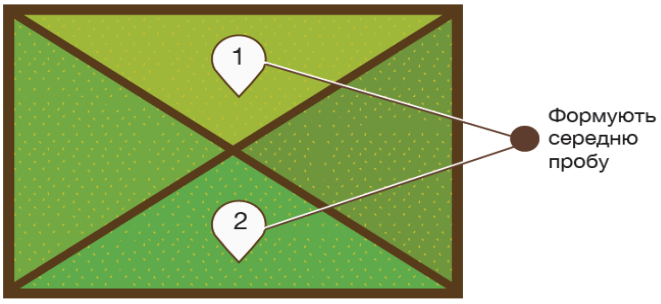
Selection of point samples from bags

The weight of the average sample should be up to 2 kg

Кількість мішків/ контейнерів, шт	**Number of samples, pcs**
Up to 5	From each container, but not less than 5
From 6 to 30	From 5 containers or one from each of the third, but not less than 5
From 30 to 400	From 10 containers or one from every fifth, but not less than 10
More than 400	From 80 containers or one from every seventh, but not less than 80

Forming an average sample

The seeds are poured on a separate board and evenly unfolded into a square. They are divided into four parts with the help of wooden plates (rulers). The two opposite parts combine and indicate the information on the branded package, the other two are discarded (stored on the farm). Selection of the average sample is also possible when using a grain divider (DP-5, DP-10 or other modifications).



Sampling of plant material

When sampling plant material, the field should be carefully inspected to determine the deviations from normally developed plants. Plants should be selected from one field in ten different places (the number of spot samples may vary depending on the size of the field and the available deviations) - only from 20 to 100 pieces depending on the culture and the phase of development:

Plant	Stage of development	Part of a plant	Number of plants for analysis
Maize	In the germination phase (less than 30 cm)	everything above ground	20-30
	Before throwing the panicle	Upper, fully developed leaf	15-25
	From the stage of ejection of the panicle to the stage when the pistil columns become brown	The whole leaf near the cob	15-25
	Selection afterthe corn silk turn brown is not recommended		
Soybeans and other legumes	Germination phase (less than 30 cm)	Everything above ground	20-30
	The initial stage of flowering	2 or 3 fully developed leaves on top of the plant	20-30
	Selection after the beginning of filling of pods - is not recommended		
Wheat, rye, oats, barley	Germination phase (less than 30 cm)	Everything above ground	50-100
	stem elongation	The uppermost mature leaves or flag leaf	20-30
	Selection after the stem elongation - is not recommended		
Sunflower	Germination phase	Everything above ground	15 -20
	Vegetative stages to the stage of full flowering	The ynew and fully ripe leaves	25-30
Potato Analysis for nitrate nitrogen	Germination phase	Everything above ground	20-25
	Vegetative stages to the stage of full flowering	The new and fully ripe leaves	25-30
	The appearance of tubers	Stalk	25-30

To ensure optimal conditions for plants during transportation, it is necessary to leave at least 100 g of soil in the root layer. If the plants are large in size, the affected and unaffected organs (roots, stems, leaves, flowers and fruits) should be separated. The selected material must be packed in bags / boxes that will protect the plants during transportation.

Selection of plant material to establish typicality

Establishing the typicality of plant material of sunflower and corn is carried out during the phases (germination phase - physiological maturity).

Sampling is recommended in rubber gloves, which should be wiped with ethyl alcohol before each sampling. Tools are also treated with alcohol in the case of their use in the selection (scissors, knife).

On the diagonal of the field from different plants to select 60 leaves or other parts of plants. Each part of the plant should be placed separately in a clean plastic bag. The plant material of each test sample (60 parts of plants) should be placed in a branded bag and completely fill the label.

Physico-mechanical properties of seeds and sampling for their determination

Sampling to determine the weight of 1000 seeds in crops

The weight of 1000 seeds characterize their weight, ie is related to the size and density of their internal structure, and therefore determines the supply of nutrients accumulated in the seeds. Heavy seeds are usually more complete, it provides greater germination and better plant growth. The weight of 1000 seeds is required to determine the weight of sowing.

Determination of the mass of 1000 seeds by conditioned humidity is carried out in parallel with the analysis of their purity. From the fraction of pure seeds, two samples of 500 seeds are weighed without sampling and weighed to the nearest 0,01 g, and if the difference between the weighing results is not more than 3%, the mass of 1000 seeds is calculated as the arithmetic mean of the two samples.If the difference between the weighing results is more than 3%, the third determination is made, and the weight of 1000 seeds is determined by the two samples that have the smallest difference. Sometimes determine the absolute mass of seeds, which means the mass of 1000 seeds in a completely dry state. This figure is calculated by the formula.

Sampling to determine the nature of the grain

The nature of the grain is the mass of a certain volume of grain, often 1 liter, expressed in grams. It depends on the shape and size of the seeds: elongated grain is often smaller in nature than short. With increasing humidity, its nature decreases. Well-filled seeds are characterized by high nature; slender, poorly filled seeds have a low nature. Determination of nature is carried out on special scales - purka. The mass of grain in a liter of purk expresses its nature (Table 1).

Table 1

Nature of grain (g per 1 liter) of some cereals.

Culture	High	Medium	Low
Wheat	>785	725-785	< 725
Rye	>730	685-730	< 685
Barley	>605	545-605	< 545
Oat	>480	420-480	< 420

Sampling to determine seed purity

To determine the purity of the seeds are isolated from the average sample by means of excavations or by dividing two portions by weight: corn, peas, beans and other large-seed crops - 200 g of wheat, rice, barley, oats, buckwheat - 50 g, millet - 20 g, flax - 10 g, clover, alfalfa – 5. Each portion, sifted through a sieve with appropriate holes for the selection of small and large seeds, is placed on a folding board or paper sheet and carefully disassembled with a spatula, separating the two main fractions: a) the seeds of the main culture; b) care.

The seeds of the main culture include: well-developed seeds, regardless of their color, insufficiently fulfilled, only thin; without germ or with partial damage; with endosperm or cotyledons reflected by 1/3 or less; naked or with a cracked shell; pecked, in which the end pierced the shell, but has not yet emerged from the seed.

The waste includes: small sized, underdeveloped, sprouted, addle and pest-damaged seeds of the main crop, if more than 1/3 of the seeds are lost; weed seeds, seeds of other cultivated plants, dead debris.

Sampling to determine seed germinative capacity and germination energy

Seed germinative capacity means the number of seeds in the sample that germinated normally, expressed as a percentage of the total number of seeds in the sample. *Seed germination energy* characterizes the simultaneousness of the appearance of normal seedlings for the time set for each crop. The greater the energy of seed germination, the faster and more simultaneous sprouts appear after sowing.When sampling for germination, use the seeds of the main culture, obtained by determining their purity, for which count four consecutive samples of 100 seeds each. In large-seeded crops, the number of seeds in the sample is reduced to 50. The seeds are germinated in Petri dishes placed in thermostats at a certain temperature.

To determine the quality of a shipment of seed, it must be sampled in such a way so that the samples taken are representative of the entire quantity of seed. The seed quality testing is performed on part of the representative sample. Therefore a technically sound sampling methodology is very important so that the seed testing results are valid. Seed sampling and testing are part of the seed procurement process but it may also be used by emergency staff and local officials to verify the quality of seed before delivery to farmers or to verify seed quality if the seed has been stored for several months.

The objectives of sampling are to obtain a sample that is suitable in size for testing and to obtain a sample that is representative of the lot being tested. All sampling should be done quickly with limited exposure to room air in order to minimize changes in seed moisture if this is to be tested. Seed practitioners are reminded that any

movement and settling of seed in containers can easily bring empty lighter seeds to the surface, so mixing just before any samples are taken is important to ensure an accurate representation of the entire seedlot.

Primary samples are small portions of seed taken at random from the seedlot. All primary samples taken from one seedlot are then combined and mixed to form the composite sample. Composite samples are reduced to a smaller subsample called the submitted sample. Submitted samples are portions of seed that are submitted to the laboratory for testing. Working samples are subsamples of the submitted sample and is the portion of seed on which a test is made.

The following sampling intensities shall be used:

containers	samples
1-4 containers	3 primary samples from each container
5-8 containers	2 primary samples from each container
9-15 containers	1 primary sample from each container
16-30 containers	15 primary samples from the lot taken from randomly selected containers with no more than one sample per container
31-59 containers	20 primary samples from the lot taken from randomly selected containers with no more than one sample per container

Primary samples

The method for obtaining primary samples must meet ISTA (2015) standards. Alberta recommends the use of a trier/sampling stick or sampling by hand to achieve cheap but effective sampling. Primary samples taken by hand should be taken from the middle of the container if one sample is taken, the top & bottom if 2 samples are taken, the top/middle/bottom if 3 samples are taken, etc. to ensure a representative composite sample.

Composite sample

The primary samples are combined and thoroughly mixed to make a uniform composite sample. A random method must be used to reduce the composite sample to the required submitted sample size.

The submitted sample is to be transported in an intact, hermetically sealed container with as little air as possible. Plastic Ziploc type bags are not recommended and are only acceptable for very short storage periods of ＜1 hour.

Glass vials, rubber sealed glass jars or heat sealed foil bags are recommended as containers that are most often hermetically sealed at room temperatures, with the heat sealed foil bags being the most reliable and cost effective option.

Every effort must be made to start germination tests within 2 weeks of receiving the submitted sample. Interim storage should be in refrigerated conditions. Moisture tests must be started within 24 hours of receipt of the submitted sample; however, samples must be brought to room temperature before containers are opened.

The best time to sample is at or just after seed cleaning. This minimises the number of times the seed is likely to be augered or handled after the test is done. It also provides an ideal way to get a good representative sample. However, if you think a seed lot is likely to have reduced germination, testing should be done before seed cleaning. This minimises expenses and provides time to obtain replacement seed. When you do the test before or after seed cleaning, the germination tray or ground temperature is likely to be higher than at sowing. This does not matter as the aim is to identify the number of normal seedlings and this is not affected by temperature.

Sampling

The key to a good germination test is getting a representative sample. A test should be done for each 20 tonne seed lot. Sampling should be random and include numerous subsamples to give the best results. Small amounts (1 cup) of seed should be taken regularly while seed is being moved (perhaps out of the seed cleaner, storage or truck) or from many different bags. Do not sample from a silo as it is dangerous and diffi cult to obtain a representative sample. When the sub-samples have been bulked, mix thoroughly and take a seed sample of 1 kilogram.

Appendix

composite sample – A sample that is made by mixing together the primary samples drawn from containers of the seed lot for testing purposes.

primary sample – A small portion of seed taken from one point in a seed lot during the sampling process.
sampling – The method by which a representative sample is taken from a seed lot to be sent to a laboratory for analysis.
submitted sample – Is a sample submitted to the testing laboratory. It must be of at least the size specified by ISTA regulations and may comprise either the whole or a sub-sample of the composite sample
sub-sample – Is the portion of a sample obtained by reducing the sample using one of the sampling methods prescribed in ISTA regulations.

REFERENCES

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3.4.2.2 Seeds quality parameters sampling and determination

Standard procedures forseed quality evaluation and sorting which aremainly based on the assessment of variousphysical, morphological and physiologicalproperties of seeds are used in many Agricultural laboratories in the world. Now aday, a strongneed was felt for the development of moreaccurate, quick and non-destructive methods ofseed quality evaluation. Machine vision orcomputerized image analysis system is found tobe very convenient method for seed relatedstudies as it is free from human errors, morerapid and provides close analysis of seeds andgerminating seedlings. The declining cost andincreasing capability of computer hardware ofimage processing and its integration withcontrolled environmental condition systems areother advantages associated with this technique.

An additional advantage of this method is thatthe images, vigor indices and other informationpertaining to the specific seed lot/type are storedand a database can be developed for futurereference. Assessing of eachindividual seed within a large seed sample helpsin the development of non-destructive and moreefficient methods for sorting seed subsampleswith different germination capabilities. Dataobtained through this technique can further beprocessed statistically and displayed graphically,and a database may be developed to integrateimage analysis data with taxonomic andbiomorphologicalfeatures of plant species. This approach highly useful for present and futureprospectus of seed quality research.

Definition of Seed:

A true seed is defined as a fertilized ovule by pollen and some growth within the mother plant. The embryo is developed from the zygote and the seed coat from the integuments of the mature ovule that possesses embryonic plant, stored material, and a protective outer covering (coat or coats).

The formation of the seed is part of the process of reproduction characteristic of all phanerogams (seed plants) the spermatophytes, including the gymnosperm and angiosperm plants.

The term "seed" also has a general meaning that antedates the above – anything that can be sown, e.g. "seed" potatoes, "seeds" of corn or sunflower "seeds". In the case of sunflower and corn "seeds", what is sown is the seed enclosed in a shell or husk, whereas the potato is a tuber.

Many structures commonly referred to as "seeds" are actually dry fruits. Plants producing berries are called baccate. Sunflower seeds are sometimes sold commercially while still enclosed within the hard wall of the fruit, which must be split open to reach the seed. The structure of seeds may be studied in such common types of pea, gram, bean sunflower or almond.

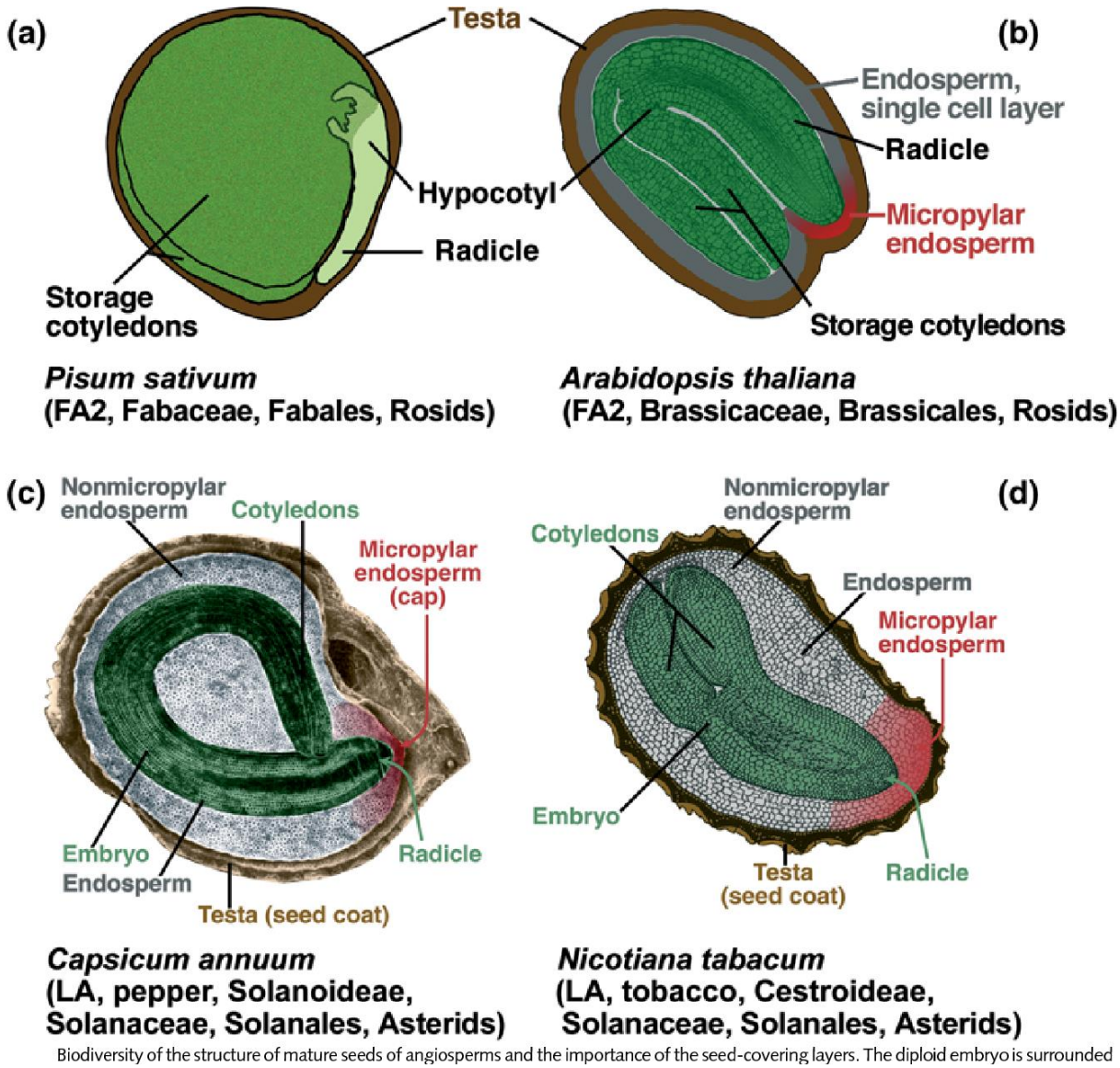
Different groups of plants have other modifications, the so-called stone fruits (such as the peach) have a hardened fruit layer (the endocarp) fused to and surrounding the actual seed. Nuts are the one-seeded, hard-shelled fruit of some plants with an indehiscent seed, such as an acorn or hazelnut.

They are all built on the same plan although there may be differences' in the shape or size of the seed the relative proportion of various parts.

There are hundreds of variations in the seed size, shape, colour and surface. The seeds range in size from tiny dust particles, as found in some orchids, to large double-coconuts. The seed surface may be smooth, wrinkled, striate, ribbed, furrowed, reticulate, tuberculate, alveolate, hairy, and pulpy or having patterns like finger prints.

In the seed, life activities are temporarily suspended in order to enable the plant to successfully pass through unfavourable and injurious climatic conditions. On the approach of favourable conditions, the seed

resumes active life and grows into full plant. In the form of seeds, a plant can be carried to long distances without special precautions.

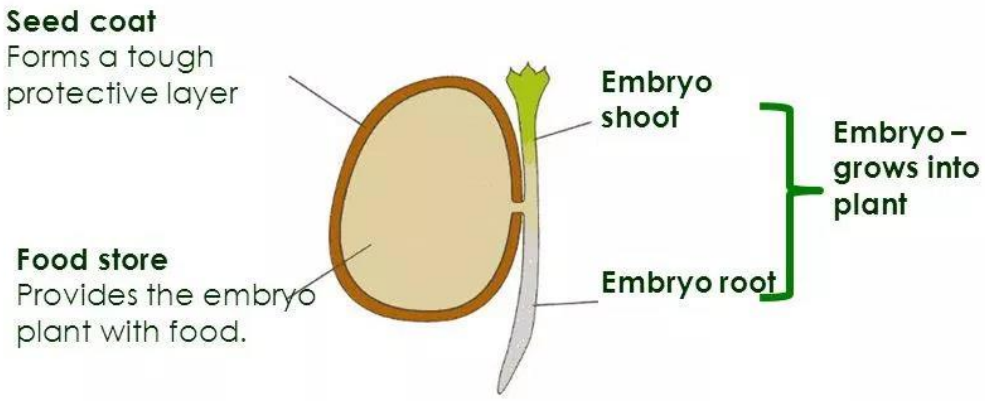


Biodiversity of the structure of mature seeds of angiosperms and the importance of the seed-covering layers. The diploid embryo is surrounded

Biodiversity of the structure of mature seeds of angiosperms and the importance of the seed-covering layers. The diploid embryo is surrounded by two covering layers: the triploid (in most species) endosperm (nutritive tissue; mostly living cells) and the diploid testa (seed coat; maternal tissue; mostly dead cells). In several species the endosperm is completely obliterated during seed development and the nutrients are translocated to storage cotyledons. Mature seeds of (a) pea (Pisum sativum) (without endosperm) and (b) Arabidopsis thaliana (single cell layer of endosperm) are characterized by embryos with storage cotyledons. The micropylar endosperm (several cell layers) is known to be a germination constraint of Solanaceae seeds (c, d). FA2 and LA are seed types. Part (c) is modified from Watkins & Cantliffe (1983) and reprinted with permission from the American Society of Plant Biologists. Parts (a), (b) and (d) are modified from 'The Seed Biology Place' (http://www.seedbiology.de).

Seed Structure

- Parts are:



Seed quality:

Quality of seed is very crucial for healthy andvigorous seedlings, good plant stand in field andultimately a good crop harvest and yield. Thefundamentals of seed quality are genetic purity,physical purity, germination, vigour and diseasefreestatus. In commercial seed chain, this qualityis maintained by following standard seed testingprocedures by means of which we measure theviability and all the physical and physiologicalfactors that regulate the performance of seeds.

Seed testing is basically done for the evaluationof a seed lot and it tells us about its seedlingemergence potential. But these testingprocedures have their limitations, like most ofthese are time consuming, labor intensive andsometimes the results are not reproducible inactual field conditions. An implication of newtechniques for testing of seeds should befocused and also attention is being laid atinternational level for the development ofappropriate methods like image analysis of seedand other plant organs, bio-chemical andmolecular markers.

Selection Of Seed Samples For Analysis

Seed Sampling

To determine the quality of a shipment of seed, it must be sampled in such a way so that thesamples taken are representative of the entire quantity of seed. The seed quality testing isperformed on part of the representative sample. Therefore a technically sound samplingmethodology is very important so that the seed testing results are valid. Seed sampling and testingare part of the seed procurement process but it may also be used by emergency staff and localofficials to verify the quality of seed before delivery to farmers or to verify seed quality if theseed has been stored for several months.

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Every effort must be made to start germination tests within 2 weeks of receiving the submitted sample. Interim storage should be in refrigerated conditions. Moisture tests must be started within 24 hours of receipt of the submitted sample; however, samples must be brought to room temperature before containers are opened.

Seed shape analysis

Morphological variation in seed characters includes differences in seed size and shape. Seed shape is an important trait in plantidentification and classification. In addition it has agronomic importance because it reflects genetic, , and ecologicalcomponents and affects yield, quality, and market price.Theuse of digital technologies, together with development of quantificationandmodelingmethods, allows a better description of seed shape. Image processing systems are used in the automatic determinationof seed size and shape, becoming a basic tool in the study of diversity. Seed shape is determined by a variety of indexes (circularity, roundness, and J index).The comparison of the seed images to a geometrical figure (circle, cardioid, ellipse, ellipsoid, etc.) providesa precise quantification of shape.The methods of shape quantification based on these models are useful for an accurate descriptionallowing to compare between genotypes or along developmental phases as well as to establish the level of variation in different setsof seeds.

The shape of the seed isinterpreted by different methods involving several traits anddiverse indices. Technically, the data for shape analysis may be obtained
in two ways: manual and computational. The simplest way is to measure seed length and width with calipers.

Figure : Digital processing of seed images. (a) Images corresponding to 25 seeds. (b) Binary images (black and white) ofthe seeds obtained by of the figure. (c) Silhouettes of seed images. (d) Ellipses adjusted to each seed image (fitted ellipses aregiven by the programImage J). (e) A seed with its image after segmentation and the silhouettes of the image (top) and the adjusted ellipse. (f)

An example of the seed with its bounding rectangle (top) and the seed with the fitted ellipse, showing in both cases themajor andminor axes.

Seed shape can be determined by the length/width ratio. Though not giving an accurate description of the seed shape, it is the simplest index to estimate and frequently used bymany authors. Eccentricity Index (EI):

EI $=\frac{L}{W}$

The aspect ratio of the particle's fitted ellipse is given by

AR $=\frac{MajorAxis}{MinorAxis}$

Flatness Index (FI) is based upon the relationship betweenthe particle imensions along the three principal axes. It wasdeveloped and used to characterize seed shape. The index is given by

FI $=\frac{L+W}{2H}$

where L, W, and H are the length, width, and height of theseeds, respectively. It ranged from a value of 1 for spheres tovalues greater than 2 for spindly seeds. Shape is related to seed length, width, and height, but thisis still incomplete and other shape descriptors may be moreprecise. The following shape descriptors are useful.

Circularity index [18–20] or form factor is as follows

$$:I = \frac{4\pi \text{Ч} \text{area}}{\text{perimeter}2}$$

This index (I) is a measure of the similarity of a plane figureto a circle. It ranges from 0 to 1 giving the value of 1 forcircles and it is a useful magnitude as a first approximationto seed shape. More and detail information could be find on ***Updated Methods for Seed Shape Analysis:Emilio Cervantes, José Javier Martín, and Ezzeddine Saadaoui. Hindawi Publishing Corporation ScientificaVolume 2016, Article ID 5691825, 10 pages*** ***http://dx.doi.org/10.1155/2016/5691825***

Seed shape is one of the features discussed for seed descriptionand the analysis of intra- and interspecific variability.Theavailability of software for digital image analysis helps withthe development of several indices enabling the modeling ofseed shape, according to virtual curves (cardioid, ellipse, circle,ovoid, etc.).This allows quantification of seed shape thatcan be used in comparative taxonomy, genetics, physiology, and biochemistry. Seed shape is influenced by genetic andenvironmental factors. It is related to the taxonomic statusand may be, as well, related to the physiology of germinationand yield of seed products (starch, fixed oils, protein, etc.).The morphological description of plant structures isa requisite for understanding the relationships betweenstructure and function in evolution and may contribute todefining developmental situations associated with genomic composition and activity. Changes in shape may be eitherthe result of developmental programs in a "regular" environmentor the response to changes (stress) in environmentalconditions. Modeling seed shape by geometric figuresis an easy approximation that may help to understand andquantify morphological variation in seeds, changes in thecourse of imbibition, and alterations in mutants as well asdifferences between related genotypes. Analysis of seed shapehas unexpected applications in botany and agrobiology.

Seed testing

Seed testing provides essential information for determining the quality of a shipment of seedconcerning such parameters as germination, physical purity and moisture content. In this way oneknows that it meets the technical specification of the order and that quality seed is being providedto the vulnerable farmers. Seed testing should be carried out in a national seed laboratory or ISTAaccredited laboratory

Quality declared seed (QDS)

Seed for emergency operation should comply with quality standards to ensure quality seed isprovided to the vulnerable farmers. The FAO developed Quality Declared Seed scheme providesseed quality standards that are used as a minimum standards for seed purchased in seed reliefactivities.

Seed deterioration

Temperature and relative humidity of the storage environment are two critical factors to payattention for an environment favourable for seed storage. The moisture content of the seed andthe particular crop are also important factors in seed storage. The lower the temperature andrelative humidity, the longer the seeds can be safely stored. Therefore in emergency operationseeds should not be stored for extended periods in tropical conditions to avoid problems withseed deterioration due to high temperature and relative humidity.

Seed storage

Effective seed storage requires: the seed to be dried to the prescribed moisture content, a cleanwell ventilated storage area, if needed treatment of the seed to prevent insect attack, and periodicinspection of the stored seed. Seed should not be stored for extended periods when there is hightemperature and relative humidity.

Physical-mechanical properties of seeds and methods of their determinationseed quality attributes

In seed relief operations, the **physical, physiological, phytosanitary** and **genetic qualities** of theseed require attention so that vulnerable farmers are provided with quality seed of the appropriatecrops and varieties of those crops.

The provision of quality seed of appropriate crops and varieties to farmers in a timelymanner to increase their seed security and food security is one of the primary strategies of FAOin emergency operations. An understanding of the technical and operational aspects of seedquality by project implementers is essential to carrying out this strategy. Quality seed is criticalto agricultural production: poor seed limits the potential yield and reduces the productivity of thefarmer's labour. There are four basic parameters to seed quality attributes:

• Physical qualities of the seed in the specific seed lot1(An identifiable quantity of seed of one variety, of known origin and history, andcontrolled under one reference number in a seed quality assurance scheme.)

• Physiological qualities which refers to aspects of performance of the seed.

• Genetic quality which relates to specific genetic characteristics of seed variety.

• Seed Health which refers to the presence of diseases and pests within a seed lot.

When seed has good physical, physiological, health and genetic qualities, farmers havegreater prospects of producing a good crop. High quality seed is a major factor in obtaining agood crop stand and rapid plant development even under adverse conditions although otherfactors such as rainfall, agronomic practices, soil fertility, and pest control are also crucial.

Seed Quality Attributes :

Physical qualities of the seed in a seed lot are characterized by the following:

• **Minimum of damaged seed:** Damaged (broken, cracked or shrivelled) seed may notgerminate and is more likely to be attacked by insects or micro organisms. It is possible toeliminate most of the damaged seed during seed processing (conditioning).

• **Minimal weed seed or inert matter:** Good quality seed should be free of weed seeds(particularly noxious types), chaff, stones, dirt and seed of other crops. Almost all theseimpurities can be discarded during processing/conditioning.

• **Minimum of diseased seed:** Discoloured or stained seed are symptoms of seed that maycarry micro organisms that already have attacked or will attack the seed when it starts togrow. The plant may live and spread the disease to other plants.

• **Near uniform seed size:** Mature medium and large-size seed will generally have highergermination and vigour than small and immature seed. In the conditioning (processing) ofseed lot, undersized and light seed is normally eliminated.Physical quality parameters such as seed uniformity, extent of inert material content, anddiscoloured seed can be detected by visually examining seed samples. **Closely examininghandfuls of seed is the first step to better understanding the quality of seed** that are beingprovided to farmers and it gives the first but not the only opportunity to decide seed cleaningneeds.

Seed Quality Attributes- Physiological

High germination and vigour:

The germination percentage is an indicator of the abilityof the seed to emerge from the soil to produce a plant in the field under normalconditions. Seed vigour is the capacity of seed to emerge from the soil and survive underpotentially stressful field conditions and to grow rapidly under favourable conditions. Theloss of a seed's ability to germinate is the last step (not the first step) in a long process ofdeterioration (gradual loss of viability). Decrease in seed vigour and other physiologicalchanges happen before loss of germination. Therefore seed with acceptable germinationcan be low in vigour.

The importance of physiological quality can not be over emphasized. Seed can only fulfil itsbiological role if it is viable. Therefore, physically uniform seed of an adapted variety will beuseless if it is low in germination and vigour or if it fails to germinate when planted.

Thedifference between grain and seed is that the former may or may not germinate whilethe latter must germinate. This is why the germination, particularly high percentage of it, issuch an important technical specification for seed.

Seed Quality Attributes – Genetic

Seed of the same variety:

Within crops (species) such as maize, rice or groundnuts thereare thousands of distinct kinds of these crops. **These distinct kinds of the particular crop are referred to as varieties or cultivars**. Plants produced by seeds of a varietypresent the same characteristics and that these characteristics are reproducible from ageneration to another. The definition of a cultivar is an assemblage of cultivated plantswhich is clearly distinguished by any characteristics (morphological, physiological,cytological, chemical or others) and which, when reproduced (sexually or asexually) retains its distinguishing characters.

There are **modern varieties** that are the result of plant breeding and varietaldevelopment programmes, multi-location trials, national variety release systems andformal seed production systems. Another kind of crop varieties are**traditional varieties (landraces)** that are produced and conserved by farmers which canbe local

population of plants selected by farmers or sometimes are modern varieties thatwere released many years ago. Seed of different varieties of the same crop are oftendifficult or impossible to distinguish once it is harvested. Mixing of different varieties ofthe same crop or species can occurs when the grain/seed is sold and it enters into theformal and informal marketing system. A mixture of varieties can be a problem because:

Mixed varieties may mature at different times which lead to problems in harvesting, postharvest handling, and results in lower yields. Additionally, each seed of an undesiredvariety in a mixture will produce seed when it is planted and those seeds will producemore seed so that each year the proportion of the undesired variety becomes greater. Fieldinspection followed by roguing (removal of undesirable plants) during the growing periodof the seed crop is one of the steps taken to insure varietally pure seed in certified seed.

However it must be pointed out that traditional varieties or landraces particularly of crosspollinated varieties used by subsistent farmers are often populations of plants that are notvery uniform. This heterogeneous character can be an advantage in some circumstancesof low rainfall, low fertility and pest and disease pressure. In other situations such as seedfor bean in Burundi, farmers prefer to plant a mixture of several different kinds of beans.

Determination of the mass of 1000 seeds in different crops

Only pure seeds are used, as per the definition given in the Purity Analysis section above; however, seed from the official purity analysis may not be used to determine TSW due to the possibility of moisture changes in seeds during longer exposure to ambient conditions. For this reason, TSW tests should be completed quickly to minimize weight errors.

Eight (8) pure seed replicates of 100 seeds must be drawn randomly from the submitted sample. Each replicate weight is recorded in grams to three decimal places and the mean weight determined from these 8 replicates. The mean weight of 100 seeds is then used to calculate the weight of 1000 seeds. Variance, standard deviation and coefficient of variance must be calculated using the following formulas:

$$\text{Variance} = \frac{n(\Sigma x^2) - (\Sigma x)^2}{n(n-1)}$$

where : x = weight of each replicate in grams n = number of replicates Σ = sum of

$$\text{Standard deviation } s = \sqrt{\text{Variance}}$$

$$\text{Coefficient of variation } CV = \frac{sx100}{x}$$

where: x = average (mean) weight of 100 seeds

If the coefficient of variation does not exceed 4.0 then the thousand seed weight is accepted and is reported to 3 decimal places. For grass seed the coefficient of variation must not exceed 6.0. An Excel tool can be provided for the above calculations upon request.

If the limit is exceeded, eight more replicates must be drawn and weighed. The standard deviation must then be calculated using all 16 replicates and any replicate that diverges from the mean by more than twice the standard deviation must be discarded. The remaining replicate weights are then used to determine the weight of 1000 seeds.

Determination of humidity

The objective of a moisture test is to determine the overall moisture of a seedlot to establish optimal conditions for storage and to maximize the lifespan of the seed. The two moisture testing methods below are not interchangeable, i.e. an eRH reading should not be converted to an MC for test result submissions and vice versa. Moisture testing must commence within 24 hours of receipt at the testing facility.

Moisture Content

The moisture content of seed in Alberta is defined as the quantity of water in a sample expressed as a percentage of the weight of the original sample, also called a wet weight basis. A balance (weighing scale) capable of gram weights to at least 3 decimal places must be used. Moisture content is determined using the low constant temperature oven method: 103°C ± 2° for 17 hours ± 1 hour. The oven must be capable of drying samples in accordance with section 9.1.4.2 of ISTA (2015) and only metal sample containers should be used.

Sampling

Tests are carried out in duplicate on two independently drawn working samples weighing 4 to 5 grams each. For very small seedlots of less than 200 grams, reduced sample sizes and/or sample numbers are found in Table 2.

Table 2: Acceptable reduced sample sizes for moisture content testing on very small seedlots.

Total seed in seedlot	Sample size
50 to 200 grams of seed	Use two 1-gram samples
30 to 50 grams of seed	Use two 0.5-gram samples
10 to 30 grams of seed	Use one 0.5-gram sample
<10 grams of seed	Moisture content testing is not mandatory. Water activity measurement recommended. Special emphasis should be given to proper handling procedures, drying times, and stirring/mixing.

Seed cannot be exposed to the ambient air for longer than two minutes from the time it is removed from the submitted sample container until it has been placed in the drying containers and weighed. Each container and its lid must have matching labels.

Method

Containers and lids should be wiped with alcohol and dried before each use. The sample is evenly distributed over the surface of the container. The empty container and lid is weighed and this weight recorded. The sample is placed into the container and the seed, container and lid weight recorded. The samples, containers and lids are dried at 103°C ± 2° for 17 hours ± 1 hour. At the end of the drying period, the lid must be placed on the container before being moved to a desiccator (with appropriate desiccant) to a maximum of 30 minutes. After cooling, the container with its lid intact plus the seed inside are reweighed. All weights are recorded in grams to three decimal places.

Calculation

The following formula is used to calculate moisture content:

Moisture content%=$\frac{lossofweight}{Initialweight}x100$

Moisture content%=$\frac{M2-M3}{M2-;1}x100$

where M1 = weight of the container and its cover
M2 = weight of the container, cover, and contents before drying
M3 = weight of container, cover and contents after drying
The average of the two replicates is the percentage moisture content of the submitted sample, rounded to 0.1%.
The average MC submitted for registration and storage must be a value of 4.0-8.0% MC.

Tolerances

The difference between the two replicates is rounded to 0.1%. If the difference between the two replicates is greater than 0.3% then the test is out of tolerance and must be repeated.

If the duplicate results of the second test are within tolerance limits as above, report the second result. If the results of the second test are outside tolerance limits, then the first and second test results are compared for tolerance (≤ 0.3%). If they are within tolerance then an average of the first and second tests is reported. If the two tests are out of tolerance then the equipment should be checked, instructions reviewed and the whole test repeated from the beginning.

Equilibrium Relative Humidity

Seeds are 'hygroscopic', meaning that they absorb (and lose) water relatively quickly in their environment. This transfer of water back and forth will eventually reach an equilibrium between the seed and the environment. The relative humidity of the air in an enclosed environment containing seeds at equilibrium is called the equilibrium relative humidity, written as '% eRH'. In much the same way that evaporation is used to measure seed moisture content, a type of hygrometer called a water activity meter is used to measure equilibrium relative humidity. Measuring seed moisture in this way is usually a fast, accurate and non-destructive method. The hygrometer should be set to a 'water activity' type of measurement. The measurements given by the meter are identical to equilibrium

relative humidity (eRH). However, water activity is expressed on a decimal basis, i.e. on a scale from 0 to 1 and the units are 'aw ',whereas equilibrium relative humidity is expressed as a percentage, i.e. 0 to 100% eRH. In simple terms, this means that the water activity reading should be multiplied by 100 or in effect, the decimal is moved 2 places to the right.

e.g. a reading directly off the meter might be 0.226 aw → this is reported as 22.6% eRH

All eRH measurements must only be taken when the seed and equipment is 20-30°C; however, the temperature does not need to be reported with the eRH.

Sampling

Sampling requirements are similar to those for **Moisture Content** testing:

• Tests must be carried out in duplicate on two independently drawn working samples. Seeds must fill at least 20% of the volume of the container used with the meter.

• The submitted sample size can be the smaller value but only if the eRH test will be non-destructive.

• As with sampling for **Moisture Contents**, large tree/shrub seed where the thousand seed weight often exceeds 200g (e.g. whitebark pine & hazel nuts) must be cut in half before testing. Due to the normally high value of these seeds, only 1 sample consisting of 5 seeds need be tested and again, this should be noted with the submitted result.

• For very small seedlots where two independent samples are not possible following the 20% volume rule above, it is acceptable to use the entire seedlot in only one working sample but this should be noted with the result. If the seedlot size is so small that a single sample volume of the testing container is less than 20%, the volume should be approximated and also noted with the result.

e.g. 17.5% eRH, 1 sample, 10% filled

Method

• Seeds must be approximately the same temperature as the equipment and air in the room. RH measurements are only acceptable when taken between 20-30°C.

• Wipe containers with alcohol and dry before each use. The seeds are evenly distributed over the surface of the sample container and fill at least 20% volume of the container.

• Measurements may be taken on instruments set on full (actual measurement) or 'quick mode' (estimated/extrapolated measurement). Quick mode is to be set at no less than 5 minutes and all submitted measurements must wait for the final reading.

• If choosing to use a full equilibrium measurement, the time to completion (10-30 minutes) is mostly dependent on the stable temperature of the seeds inside the chamber, so using seeds at room temperature and positioning the meter away from drafts/heaters/fans will help.

Calculation

The hygrometer will give a reading of water activity (0 to 1.0 aw) when used on the correct setting. This is converted to equilibrium relative humidity (eRH) by multiplying the water activity measurement by 100.

e.g. 0.278aw X 100 = 27.8% eRH

The reported eRH is the average reading of the two samples to one decimal place, e.g. 23.2% eRH.

Tolerances

The difference between the two replicates should be rounded to 0.1%. If the difference between the two replicates is greater than 3.0% then the test is out of tolerance and must be repeated.

Determination of seed purity

The objective of purity analysis is to determine the percentage composition by weight of pure seeds versus seeds of other species and debris (inert particles) that make up the sample.

A working sample must be drawn from the submitted sample, weighed and recorded in grams to three decimal places. For species not listed here either, a working sample of a weight estimated to contain at least 2,500 seeds should be used.

The working sample is then separated by hand, sieve or blower into two components: pure seed of the test species, and seed of other species or debris. For clear definitions of pure seed, other seed and inert matter.The two components must then be weighed and recorded in grams to three decimal places.

The sum of the weights of the two component fractions must be compared to the original weight of the purity working sample for any gain or loss. If a discrepancy of more than 5% of the original sample weight is found, the test must be discarded and a re-test is required.

The pure seed percentage should be reported and is calculated by the following formula:

$$\text{Pure seed\%}=\frac{\text{weight of pure seed fraction}}{total\ working\ sample\ weight}x100$$

Pure seed percentage is rounded and reported to one decimal place.

Determination of the similarity and energy of germination of seeds

The objective of the germination test is to determine the germination potential of a seedlot. In any location the percent germination is the estimated viability of a seedlot, tested under specified conditions and within a specified period of time. Each test must consist of four hundred seeds which are drawn from the working sample and then randomly divided into four replicates of 100 seeds.

Similarities and germination of seeds is an important indicator of their sowing qualities. Seeds with good germination and high germination energy during normal farming practices are always friendly and give full seedlings. Germination of seeds is of great industrial importance: it determines their suitability for sowing, the norms of their seeding.

Under the germination of seeds meant an amount of normally germinated seeds in the sample taken for analysis, expressed as a percentage. Germination germination of seeds is determined at their optimal conditions established standard for each culture.

Standard for high-quality and sowing qualities of seeds to the high demands of similarity standards. Seeds that do not meet the similarity requirements of the standard may not be used for planting. When sowing seeds with low germinative capacity decreases yield; such seeds is better to use for food and technical purposes.

Simultaneously with the similarity determined germination energy. Under the vigor that characterizes the friendliness of seed germination, is meant an amount normally germinated seeds for the period of time determined for each culture, expressed in percentage.

Calculation of the weight norm of seeding

The correct plant density is an important factor in maximising yield of pulse crops. To obtain the targeted density it is necessary not only to have quality sowing seed but also be able to accurately calculate seeding rates. It is surprising the difference a slight variation in seed size or germination makes to the seeding rate required to achieve a target plant density. Seed size, quality and germination varies between varieties, from year to year, from paddock to paddock and should be checked for each seed line to be used.

Quality of sowing seed

The large size of pulse seed makes it vulnerable to mechanical damage by the header at harvest and during subsequent handling. This damage is not always visually apparent. Damage can be reduced by slowing header drum speed, opening the concave, taking care when augering and reducing the number of augerings. Ideally a rotary header and a belt grain mover or elevator should be used.

Seed to be used for sowing should be treated with special care. Ideally seed to be used for next years crop should be produced as a specifi c seed crop and not just randomly kept from an area of the whole paddock at harvest. If this is not possible, seed should be kept from the best part of the crop where weeds and diseases are absent and the crop has matured evenly. Grain to be used for seed should be harvested fi rst, to avoid any weed and disease contamination from other pulse crops or parts of the paddock. Store the seed, with minimal handling, separate to the bulk seed. A seed that has been damaged will produce an abnormal seedling –the shoot, the root, or both may be damaged. If the root is damaged the seedling will germinate, emerge and then generally die. This is because the taproot is weak and cannot grow normally. If the shoot is damaged the seedling will germinate and may emerge.

In damaged field pea and faba bean seedlings, where the cotyledons remain below ground level, the shoot takes longer to emerge, looks deformed and may be yellow or pale green.

Abnormal seedlings which do emerge lack vigour making them vulnerable to the rigours of fi eld establishment. Factors such as temperature, disease, insects, seeding depth and soil crusting are more likely to affect the establishment of weak seedlings. Those that do emerge are unlikely to survive for long, producing little dry matter and making little or no contribution to fi nal yield.

Unsatisfactory establishment of commercial crops can often be linked to poor quality sowing seed.

The quality of pulse seed should always be checked before it is sown. A visual check of the seed lot should be done for any seed coat cracking or other damage from insects and disease and a germination test carried out to identify the number of viable normal or undamaged seeds.

Testing for the presence of seed borne diseases can be conducted by specialist laboratories for a number of diseases such as cucumber mosaic virus in narrowleaf lupins, bacterial blight in fi eld peas and ascochyta blight in chickpeas. Albus lupins should be checked by 'UV screening' for possible bitter (high alkaloid) seed contamination.

Germination testing

All pulse crop seed to be used for sowing should be germination tested. Ideally only pulse seed with greater than 80% germination should be used. Germination testing can be done in a laboratory or at home.

When to do the test

The best time to sample is at or just after seed cleaning. This minimises the number of times the seed is likely to be augered or handled after the test is done. It also provides an ideal way to get a good representative sample. However, if you think a seed lot is likely to have reduced germination, testing should be done before seed cleaning. This minimises expenses and provides time to obtain replacement seed. When you do the test before or after seed cleaning, the germination tray or ground temperature is likely to be higher than at sowing. This does not matter as the aim is to identify the number of normal seedlings and this is not affected by temperature.

Sampling

The key to a good germination test is getting a representative sample. A test should be done for each 20 tonne seed lot. Sampling should be random and include numerous subsamples to give the best results. Small amounts (1 cup) of seed should be taken regularly while seed is being moved (perhaps out of the seed cleaner, storage or truck) or from many different bags. Do not sample from a silo as it is dangerous and diffi cult to obtain a representative sample. When the sub-samples have been bulked, mix thoroughly and take a seed sample of 1 kilogram.

Home germination tests

Setting up the test

A convenient method is to use a flat tray about 30 cm square and 5 cm deep. Put a single sheet of paper in the bottom to cover the drainage holes and fi ll with clean sand, pottingmix or freely draining soil. If you do not have a tray the test can be done in any sort of self-draining container or in a cool part of the garden. Laboratory germination tests are normally conducted at 20°C, so if the test is to be done indoors aim to conduct it at this temperature. Count out 100 seeds (including damaged ones) and sow 10 rows of 10 seeds —the rows make it easier to count seedlings. Seeds should be sown at normal seeding depth of 2-3 cm. Place the seeds on top of the sand or soil and push them in with a piece of dowel or a pencil and cover with a little more sand. Larger seeds, such as faba beans, can successfully be tested in the same trays, and should be sown as deep as possible. Gently water! Keep moist (not wet). Over-watering will result in fungal growth on the seeds, causing possible seed rot, affecting normal germination. If you do not have a tray, sow 100 seeds If you do not have a tray, sow 100 seeds in rows in the garden at normal depth, carefully counting the number sown. Keep moist.

Counting Seedlings

should be counted after 7 to 10 days when the majority of seedlings are up. Do not wait until the late ones emerge–these are the damaged, weak ones. Only normal seedlings should be counted. Do not count badly diseased, discoloured or distorted seedlings or, in the case of lupins, those missing a cotyledon. Remember, you want to know the total number of normal, vigorous, healthy seedlings. If you count 83 normal seedlings then your germination percentage is 83%

Calculating seeding rates Seeding rate can be calculated using target density, germination percentage, 100 seed weight and establishment percentage (see equation following).

$$\text{SEED RATE} = \frac{\text{Target plant density} \frac{pl}{m2} \times \text{100 seed weight (grams)} \times 10}{\text{Germination percentage} \times \text{Establishment percentage}}$$

Step 1 – Target plant density

What is the optimum plant density? This will vary depending on which pulse is being planted, the region, the rainfall, and the sowing time (on time or later than preferred?). The example used here is :

• field peas

• with 425 mm of annual rainfall

• The target plant density for this example is 40 plants/m2 .

Step 2 – determining 100 seed weight This is done by counting a set number of seeds (at least 200) and weighing them. Seeds per kilogram or seed size can also be obtained on request with a laboratory germination test. The more seeds counted the more accurate the answer. Always count each individual seed lot, never assume they are the same if from different paddocks, varieties, or years. In this example, 100 seeds weighed 21 grams. If you have seeds per kilogram from a laboratory test this can be easily converted to 100 seed weight, as follows:

100 seed weight = 1000 x 100 seeds per kg

Step 3 – adjust for germination and establishment percentage Assume that only 80% of normal germinated and emerged seeds will establish under fi eld conditions—temperature, moisture, soil type, sowing depth, insects and disease will all affect survival. Establishment percentage can be adjusted according to your confi dence in your sowing operation and fi eld conditions. A realistic estimate of establishment is 80%.

In the example the field pea seed germination is 93%, target density is 40 plants per m^2 , and 100 seed weight is 21 grams. When calculating seeding rate use the decimal equivalent of establishment percentage (0.80 for 80%).

Your seeding rate	Example	Your seed
Step 1 target density (plants/m2)	= 40 pl/m2	TD
Step 2	= 42	÷ 2
wt of 200 seeds 100 seed wt (grams)	= 42 ÷ 2 = 21 grams	100sw
Step 3 germination (%)	= 93%	G
SR seeding rate (kg/ha)	= 113 kg/ha	SR

Determination of the seed suitability of the seed.

Seed viability is the ability of the embryo to germinate, and is affected by a number of different conditions. A variety of factors can affect seed viability such as the ability of the plant to produce viable seeds, predator and pathogen damage, and environmental conditions like flooding or heat. The age of the seed also affects its health and germination ability. Seeds are living embryos and, over time, cells die and cannot be replaced. The amount of time a seed remains viable can be influenced by both genetics and environment. Some seeds can remain viable under optimal conditions for many years, and others for only a season cycle.

The viability of the seed accession is a measure of the percentage of seeds (how many seeds) are alive after storage. and could develop into plants which will reproduce themselves, given the appropriate conditions.

Viability is usually measured in order to assess the suitability of the seeds for particular purpose, most commonly to produce a crop but also for industrial purposes, especially the malting of barley. The results of germination test are used to determine the suitability of seed lot for sowing.

Seed viability can be tested in many easy ways. A seed germination test is probably the most simple: seeds are given the needed resources (air, water, warmth, and light) to germinate and grow into a seedling. Simply place seeds in the soil or in a pot of soil and see how many grow. However, one disadvantage of using soil, pots, and outdoor resources is enviro fluctuation that can cast doubt on the true viability of the seeds.

Two main reasons for the failure of seeds to germinate in suitable conditions are because they are either dead or dormant. Dead seeds can be identified because they usually soften and rot during the test as a result of

attack by bacteria and fungi. Seeds which remain hard or absorb water, but remain firm and in good condition during the germination test are probably dormant. Seed dormancy is common in some crops straight after harvest (post harvest dormancy) and in many wild species related to crop plants.

It is important to know that the seeds that are stored in a genebank will grow to produce plants. Therefore they must have a high viability at the start and during storage. The viability of seeds at the start of storage will also determine, within the environmental conditions, the storage life of the accession.

Viability will need to be determined at the start of storage and at regular intervals during storage to predict the correct time for regeneration of the accession. The viability test takes from a few days to weeks or even months to give an accurate result. If possible the results should be available before the seeds are packaged and placed in the genebank so that poor quality seeds can be identified and regenerated before storage. Where the viability cannot be determined before storage, the seeds should be placed into long-term storage to ensure their safety whilst awaiting the results of the test.

The most accurate test of viability is the germination test and this will be described here. The germination test is made under controlled conditions to find out how many seeds will germinate and produce normal seedlings which could develop into normal reproductively mature plants. The IBPGR Advisory Committee on Seed Storage recommends that for the initial germination test of species where a reasonable germination technique is available, a minimum of two replicates using 200 seeds (100 seeds per replicate) is acceptable, providing that germination is above 90%. If not, a further 200 seeds should be tested as before and the overall result for seed viability taken as the mean of the two tests. Other biochemical tests are available to test viability. These have the advantage of being quicker, but are not as accurate and require considerable skill and practice in their implementation and interpretation. These are not recommended by the IBPGR Advisory Committee on Seed Storage for general use as tests for seed viability.

Procedures

The various methods for determining seed viability all serve to provide seeds with a substrate that makes water available at the pr amount for the seeds to imbibe (take up into themselves) and to germinate.

All seeds have specific light and temperature requirements, but a general rule of thumb is that most seeds will germinate when the temperature is between 20 and 30ºC, sufficient water is present in the substrate, and some amount of light is given to the seeds .

Seeds for the test should be randomly selected from your entire lot of seeds. International standards for seed testing suggest that 200 seeds be used in a germination test. If this quantity of seeds is difficult to attain, 100 or even 50 seeds may be used. Divide your total number of available seeds by two, so that you will have two replications for your germination test. If you have an abundance of seeds (lettuce, cabbages, tomatoes, etc.), then four replications of 100 seeds will provide very robust results.

Testing the viability of your seeds by conducting a seed germination test is an important way to deduce the quality of your seeds, to determine the efficacy of your seed storage methods, and to help you plant the proper amount of seeds. By conducting these simple seed viability tests, you can increase your seed saving efficacy and help to empower farmers in the saving and planting of important genetic diversity. Consider the mean percentage viability of the accession. If it is above 90%, accept the test as valid and use this value as the true viability. If the result is 90% or below, repeat the test using a further 200 seeds following the same procedures. Calculate the mean percentage viability from the results of the two tests and use this as the overall test result.

Seed vigor

Seed vigour is defined as "the Sum total of those properties of the seed which determine the level of activity and performance of the seed or seed lot during germination and seedling emergence". In any seed lot, losses of seed vigour are related to a reduction in the ability of seeds to carry out all the physiological functions that allow them to perform. This process, called physiological ageing (or deterioration), starts before harvest and continues during harvest, processing and storage. It progressively reduces performance capabilities, due e.g. to changes in cell membrane integrity, enzyme activity and protein synthesis. These biochemical changes can occur very quickly (a few days) or more slowly (years), depending on genetic, production and environmental factors which are not yet fully understood. The end point of this deterioration is ultimately death of the seed (i. e. complete loss of germination). However, seeds lose vigour before they lose the ability to germinate. That is why seed lots that have similar high germination values can differ in their physiological age (the extent of deterioration) and so differ in seed vigour and therefore the ability to perform. These seed vigour differences exist in seed lots of agricultural, horticultural and silvicultural species (ISTA, 2009).

Effect of storage on viability and vigor The highest seed yield in agriculture achieved in normal condition of nutrition and environmental conditions.The storage condition affected on seed quality and in final on seed yield after cultivation. The storage potential of seed lots is related to their stage of deterioration (vigour status) on entering storage. If the storage environment exerts any form of stress (e.g. changes in temperature or relative humidity in uncontrolled storage), high vigour seed lots will be better able to withstand such environmental stresses and will decline in quality at a slower rate than lower vigour seed lots. Even under controlled storage conditions (i.e. low temperature and low seed moisture content), performance after storage is dependent on the vigour status of the seed lot (IATA, 2009). Use of hermetically sealed containers, desiccants and low temperatures improves storability as several physiological and biochemical processes and products are being regulated during dry storage. Accelerated ageing of seeds induced by several days of exposure to high temperature and humidity is recognized as an accurate indicator of seed viability and storability . Some of the deleterious effects of ageing are associated with damages occurring at membrane, nucleic acids and protein levels . Standardization of appropriate seed conditioning, packaging and storage conditions could ensure satisfactory planting quality of onion seeds at the time of sowing. The rate of seed deterioration is influenced by confounding environmental and biological factors. High temperatures during storage enhance seed deterioration as does high seed moisture content . Relative effects of seed moisture content and temperature on longevity differ with species, and the structural and biochemical composition of seeds. A complete pattern of loss in viability could be understood on the basis of seed moisture and storage temperature . Drastic fluctuations as well prevailing high humidity and temperatures under subtropical Indian conditions aggravate the loss of germination in stored onion seeds.

Seed viability after many years of storage Genetic erosion of material maintained in genebanks is considered a relevant problem at the international level (FAO, 1997). For this reason, the monitoring of the main factors causing genetic erosion in ex situ collections is strongly recommended to minimize the loss of genetic diversity. These factors include low quality of the original material, overdrying of seed before storage, increase of temperature or moisture content of seed during preservation, lack of regeneration, losses of germplasm in multiplication, physiological changes in seed during storage and no detected loss of germination caused by lack of viability monitoring (FAO, 1997). In general, the combination of 3±7% moisture content and storage temperature below 8C would permit long-term seed preservation (FAO/IPGRI, 1994). But, even for those seed stored under controlled conditions, viability may decrease as a result of a deterioration process.

Testsing seed vigor

Standard germination test is an indicator of seed quality, which can be used to predict the field emergence, if soil conditions are nearly ideal . However, conditions in which the seed is found during examination are often in conflict with the conditions in the field. Field germination depends on seed viability. Seed viability or seed vigour are the set of characteristics that determine the activity and behaviour of the seed lots of commercially acceptable seed germination in different environmental conditions. In addition to the above mentioned, longevity of the seed is determined by the seed vigour without adverse consequence (ISTA, 2009). To obtain more precise information about the quality of the seed lot different vigour tests are used .Testing of seed viability using different seed vigour tests is very significant, since vigour tests give results, which are often better correlated with the results of field germination under unfavourable environmental conditions, than the results obtained by application of standard laboratory germination test. Vigour tests could be grouped into three groups:

*Physical tests determine seed characteristics such as size and mass. These tests are inexpensive, quick, can be applied to large number of samples, and are positively correlated with seed vigour. The main feature of seed development is accumulation of nutritive materials, which is also in direct correlation with vigour, i.e. with size and mass of seed.

**Physiological tests using germination and growth parameters. There are two types of these tests. First type, when germination is done under favourable conditions (standard laboratory germination, and test of growth intensity). Second type, when seed is exposed to unfavourable environmental conditions (cold test, accelerated aging test, and Hiltner test).

***Biochemical tests are considered as indirect methods for estimation of seed value. These are Tetrazolijum test, conductometric measurements, enzyme activity and respiration.

Two criteria have been employed by the ISTA seed vigour committee to evaluate, the performance of seed vigour test methods for different crops: Reproducibility ofvigour method and the relationship between vigour test results and seedling emergence in field soil. There is no universally accepted vigour test for all kinds of seeds. The determination of following vigour tests will be useful in gaining additional information on seed quality

1. Growth Tests

Principles: Growth tests arc based on the principle that vigorous seeds grow at a faster rate than poor vigour seeds even under favourable environments. Vigorous seeds rapidly germinate, metabolize and establish in the field. 'Iherefore, any method used to determine the rapidity of growth of the seedling will give an indication of seed vigour level.

Apparatus and equipment: All the equipment,and materials needed to conduct a germination test are required. Additionally, a toploadingbalance and an air oven are also required.

Procedure

(a) First count: The test is done along with the regular germination test. 1he number of normal seedlings, germinated on the first count day, as specified in the germination test for each species, are counted. The number of normal seedlings gives an idea of the level of seed vigour in the sampIc. Higher the number of normal seedlings greater is the seed vigour.

(b) Seedling growth rate and dry weight: The seedlings arc grown either in laboratory, green house or field. 1n laboratory, in between rolled towel paper method should be followed. Ten seeds are planted in the centre of the moist towel papers in such a way that the micropyles are oriented towards bottom to avoid root twisting. The rolled towel papers are kept. in the germinator maintained at a temperature recommended for crop in reference. After a specified period of time (5-10 days) towel papers are removed and five seedlings arc selected, their length is measured and mean seedling length is calculated. Seed lots producing the taller seedlings are considered more vigorous than the seed lots producing shorter seedlings. For dry weight determination, the seedlings are removed anddried in an air oven at 100°C temperature for 24 hours. The seedling dry weight provides additional information for assessing seed vigour.

(c) Speedof germination : One hundred seeds each in four replications are planted in recommended substratum for germination. The substratum is kept in a germinator maintained at recommended temperature for the crop in reference Crable S.l). Number ofseedlings emerging daily are counted fromday of planting the seeds in the medium till the time germination is complete. Thereafter a germination index (G.I.) is computed by using the followingformula:

Germination Index G.I.=$\frac{n}{d}$

where, n =number ofseedlings emerging on day 'd'

d = day after planting

The seed lot having greater germination index is considered to bl~ more vigorous.

(d) Seed vigollr index (S, v.I,) : This is calculated by determining the germination percentage and seedling length of the same seed lot. Fifty seeds each in four replications arc germinated in towel papers as prescribed for the crop specie..')in germination test. While evaluating the number of normal seedlings at the time of final count. the seedling length of 5 randomly selected seedlings are a1<;omeasured. Seed vigour index is calculated by multiplying germination (%) and seedling length (0101).The seed lot showing the higher seed vigour index is considered to be more vigorous.

Hiltner Test (Brick gravel test) Principle:

The test was developed by Hiltner in Germany in 1917. The seeds of cereal crops affected by Fusarium disease were able to germinate in regular test but were not able to emerge from brick gravels of 2-3 mm size. Compared to this, healthy seeds were able to emerge from the brick gravel. The principle is that the weak seedlings are not able to generate enough force to overcome the pressure of brick gravels, so this method can be used to differentiate vigour levels in cereal seeds. This method reproducible and associated with field emergence in case of wheat.

Apparatus and equipment: Germination box, aluminium tray, sand, sand marker brick gravclof 2-3 mm size, germinator, seed sample.

Procedure: The sand is seived, moistured and filled in the germination box leaving about 3 em empty at the top. One hundred seeds are 'placed in each box in the impressions made by a sand marker. After this 2-2.5 em of porous brick gravel is spread over the seeds. The box is kept in the germinator at appropriate temperature. Afterthe period required for germination, the box isremoved and the seedlings which have emerged through the

brick gravel layer are counted. The percentage of emerged seedlings are used to compare seed vigour of different lots. The test should be repeated 3-4 times to get authentic value.

Cold Test Principle: The ccold test has been developed in USA to evaluate the seed vigour of maize (com). In USA when the corn is planted in late spring, the soil is humid and cold. The weak seeds do not germinate and establish. Therefore, to simulate the actual field conditions witnessed at the time of corn planting, cold test has been developed. The test aims to differentiate between weak and vigorous seed lots by subjecting them to low temperature prior to germination at optimum temperature. The test has been criticized for using field soil which greatly varies from place to place.

Apparatus and cquipment : Aluminium tray, field soil, sand markel', germinator, seed sample. Procedure: After grinding and properly sieving the soil is field in tray upto 2 cm depth. Fifty seeds are placed over the sand and covered with another 2 cm thick layer of soil. The soil is compacted and enough water is added to make the soil about 70% of its water holding capacity. The temperature of the water should be l0°C. After watering the trays arc covered with polythcne bags and placed in the refrigerator maintaincd at 10°C("temperature for one week. After one week the trays are removeed and placed in the gcrminator at 25°C temperature. The seedlings emerged after 4 days arc counted. –Thegermination percentage is computed by counting the number of normal seedlings as in germination test. Higher the germination percentage greater is the vigour.

Accelerated Ageing Test

Principles : The accelerated ageing test has been developed at the Seed Technology laboratory, Mississippi State University, USA for determining the storage potential of seed lots. The ageing process is accelerated by subjecting the seeds to high temperature and relative humidity in a chamber before standard germination. Theseed lots that show high germination in accelerated ageing test arc expected to maintain high viability duringambient storage as well. Thus. ageing test gives an indication of the performance of the seed lot during ambient storage. Tests conducted at Pantnagar with Bragg soybean seeds have shown positive relationship between 3 days accelerated ageing test (42- 45°C temperature, 95- 100% RH.) and viability after 6 months after ambient storage. The test also suffers from fungal growth on seeds at high temperature and humidity .

Apparatus and equipment: Acceleratged aging chamber, equipment for germination test, seed samples, tight jar, muslin cloth, wire mesh etc

Procedure: One hundred seeds each in four replications are tied in a fine muslin cloth. The tied seeds are placed in jar on a wire mesh. The lower part ofthe jar is filled with water. There should not be a direct contact between water and the seed. The jar is covered with the lid and sealed with parafin wax to make it air tight. Thejar is thenplacedin the acceleratedagingchambermaintainedat 45 ±2°C temperature for3-5 days. The jar is removed after this period and the seeds are cooled in a dessicator. The seeds are then tested in a normal germination test specific to different crops.The percent germination gives levelof seed vigour. Higher the germination percentage greater is thevigour of the seed.

Future Role of Seed Vigour

Testing Seed vigour is an important component of seed quality and satisfactory levels are necessary in addition to traditional quality criteria of moisture, purity, germination and seed health to obtain optimum plant stand and high production of crops. As agricultural and horticultural techniques become progressively more sophisticated, the need for high vigour seeds will increase and testing standards, similar to those reconginzed for germination will be required. The technology . of seed vigour testing has not been perfected so far, so much so that there is not a single universally accepted seed vigour test method.Research is needed to further refine the current seed vigour test methods and to develop new methods which are more related to field/storage conditions.

Appendix

analytical purity - The percentage of the seed that is of the same crop species but not necessarilythe same crop variety. The balance can include inert matter, weed seed, damaged seed. Whileregular seed testing procedures may not, in all cases, distinguish between different varieties ofthe same species, the seeds of different crop (species) can be identified in the seed laboratory byclose examination of the seed.

certified seed - Seed of a prescribed standard of quality produced under a controlledmultiplication scheme either from basic seed or from a previous generation of certified seed. It isintended either for the production of a further generation of certified seed or for sowing toproduce food, forage, etc.

commercial seed – Seed which is intended for crop production, but has not been produced undera recognized certification scheme.

composite sample – A sample that is made by mixing together the primary samples drawn fromcontainers of the seed lot for testing purposes.
germination – Initiation of active growth of all essential embryonic parts required for asuccessful seedling establishment. In a seed test it is regarded as the emergence and developmentfrom the seed of those essential structures which indicate the ability of the embryo to developinto a normal plant under favourable field conditions.
germination capacity – The percentage of pure seed which germinate in a standard test to givenormal seedlings as defined in the Rules for Seed Testing.
ISTA – The International Seed Testing Association that with it member laboratories establishesthe international standards and procedures for seed testing.
moisture content – The weight of available water in a seed sample expressed as a percentage ofthe total weight of the seed at the time of determination.
phyto sanitary certificate – A certificate issued by a legally constituted authority of federal orstate government stating that a seed lot has been inspected and found to be free of quarantinedisease infestation. These certificates are frequently used in international seed trade agreementsto prevent the spread of seed borne diseases among countries.
primary sample – A small portion of seed taken from one point in a seed lot during thesampling process.
pure seed – Refers to the species stated on the label or found to predominate in the test and shallinclude all botanical varieties and cultivars of that species including both whole seed, immatureseed, diseased seed, and seed larger than one-half their original size or as defined by ISTA rulesfor seed testing.
Registered seed – A class of seed in a certified seed scheme which is produced from foundationseed and planted to produce certified seed.
relative humidity – The ratio, expressed as a percentage of the quantity of water vapour actuallypresent in the air to the greatest amount it could contain at that temperature.
sampling – The method by which a representative sample is taken from a seed lot to be sent to alaboratory for analysis.
seed – The ripened ovule consisting of an embryonic plant together with a store of food or otherstructure including the ovule used by farmers as planting material.
seed lot – A quantity of seed of one cultivar, of known origin and history, and controlled underone reference number.
seed equilibrium moisture content- the percentage of moisture in a seed at a particulartemperature and relative humidity.
seed vigour – Is the sum of the properties that determine the activity and performance of the seedlots of acceptable germination in a wide range of environmental conditions. A vigorous seed lotis one that is potentially able to perform well even under environmental conditions which are not
optimal for the species.

submitted sample – Is a sample submitted to the testing laboratory. It must be of at least the sizespecified by ISTA regulations and may comprise either the whole or a sub-sample of thecomposite sample
sub-sample – Is the portion of a sample obtained by reducing the sample using one of thesampling methods prescribed in ISTA regulations.

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Chapter 4. Analysis and its performance

General Scope

The goal of this chapter is to briefly introduce some general concepts and practical tools that are important for performance of lab analysis in any applied researches. This chapter covers material that lays out some of the foundation of conducting research with good laboratory practice. It contains principal definitions, requirements and guidelines for laboratory set-up, method validation requirements, general description of methods, test

4.1 The concept of analysis (applied researches)

analysis and reports. It identifies and describes the basic required elements of managing an analytical testing and scientific study, its planning, performance, reporting, monitoring and ethic at laboratory.

4.1.1. Introduction

It's well known, that all knowledge and theory in science have originated from practical observation and experimentation: this is equally true for disciplines as diverse as microscopy and molecular genetics. Investigations in applied science, agro-food science and technology, whether by the agro and food industry, government food safety agencies, or universities, often require determination of raw material, food and feed composition and characteristics. Trends and demands of consumers, national and international regulations, and realities of the food industry challenge agro-food scientists and analytical testing laboratories as they work to monitor food composition and to ensure the quality and safety of the food supply.

The nature of the sample and the specific reason for the analysis commonly dictate the choice of analytical methods. Speed, precision, accuracy, robustness, specificity, and sensitivity are often key factors in this choice. Validation of the method for the specific testing matrix being analyzed is necessary to ensure usefulness of the method. Methods of analysis developed and endorsed by several nonprofit scientific organizations allow for standardized comparisons of results between different laboratories and for evaluation of less standard procedures. Such official methods are critical in the analysis of raw material, food and feed, to ensure that they meet the legal requirements established by governmental agencies. Regulations and international standards most relevant to the analysis of raw material, food and feed, are mentioned here but covered in more detail in other chapters.

4.1.2. General laboratory and procedural requirements

Basic requirements

Any laboratory work that involves working with living organisms, animals or microorganisms must be carefully considered. Health and safety law requires laboratories and academic institutions to provide a working environment that is safe and without risk to health. Where appropriate, training and information on safe working practices must be provided. Lab staff must take reasonable care to ensure the health and safety of themselves and of others, and must not misuse any safety equipment. Safe working means following a code of safe practice, supported by legislation, alongside a moral obligation to avoid harm to yourself and others. Ethical and human aspects that must all be considered before any work is carried out include: possible damage to fauna and flora; safety of others within group; implications of your work to others, e.g. landowners, or other users. Without an ethical framework, individuals can only make subjective value judgments, based solely on personal opinions and viewpoints, rather than objective analysis, as discussed in more detail in other chapters.

Laboratory organization

The laboratory shall have available the facilities, equipment, qualified personnel, systems and support services necessary to manage and perform its laboratory activities. For all applied research and agro-food testing laboratories compliance with applicable requirements with respect to safety regulations and manufacturers' safety recommendations shall be followed and should be in accordance with the guidelines outlined in GLP and ISO/IEC 17025 standards. Basis on of these standards, addressing both risks and opportunities establishes a basis for increasing the effectiveness of the laboratory management system, achieving improved results and preventing negative effects. The laboratory is responsible for deciding which risks and opportunities need to be addressed. It is very important, that in any laboratory conflicts of interest do not exist, or are resolved so as not to adversely influence subsequent activities of the laboratory. The laboratory shall document the competence requirements for each function influencing the results of laboratory activities, including requirements for education, qualification, training, technical knowledge, skills and experience.

The facilities and environmental conditions shall be suitable for the laboratory activities and shall not adversely affect the validity of results. Separate laboratory space shall be provided, as needed, for the performance of the routine and specialized procedures. Physical separation through the use of different rooms is the most effective

and preferable way of ensuring separate work areas, but other methods may be used as a protection against contamination, provided their effectiveness is comparable. For example, accidental DNA contamination can originate from dust and spreading aerosols. As a consequence, the organization of the work area in the laboratory is logically based on systematic containment of the methodological steps involved in the production of the results, and a "forward flow" principle for sample handling. Staff shall wear different laboratory coats in different work areas, also wear disposable gloves. Gloves and laboratory coats should be changed at appropriate frequencies. Reagents and solutions should be stored at room temperature, unless otherwise specified. Influences that can adversely affect the validity of results can include, but are not limited to, microbial contamination, dust, electromagnetic disturbances, radiation, humidity, electrical supply, temperature, sound and vibration and all of these parameters must be monitoring, controlling and recording.

In generally, laboratory have equipment, including: measuring instruments, which shall be calibrated, software, measurement standards, reference materials, reference data, reagents, consumables or auxiliary apparatus, that is required for the correct performance of laboratory activities and that can influence the results. Each piece of equipment must have standard operating procedures (SOPs) for operation, calibration, and routine maintenance. Apparatus and equipment shall be maintained according to manufacturers' instructions.

Personnel shall use appropriate methods and procedures for all laboratory activities and, where appropriate, for evaluation of the measurement uncertainty as well as statistical techniques for analysis of data. Measurement results have to be traceable and demonstrate with the International System of Units (SI). All methods, procedures and supporting documentation, such as instructions, standards, manuals and reference data relevant to the laboratory activities, shall be kept up to date and shall be made readily available to personnel. The laboratory shall validate non-standard, laboratory-developed, in-house methods. The performance characteristics of validated methods, can include: measurement range, accuracy, measurement uncertainty of the results, limit of detection, limit of quantification, selectivity of the method, linearity, repeatability or reproducibility, robustness against external influences or cross-sensitivity against interference from the matrix of the sample or test object, and bias. This information covered in more detail in other chapters.

Protocols and standard operating procedures

Laboratories shall have standard operating procedures (SOP), which is a written procedure that describe how to perform certain routine laboratory tests or activities normally not specified in detail in study plans or test guidelines. Study protocols define "what" and "when" it will be done during the testing; SOPs define "how" to carry out protocol specified activities. SOPs should be detailed enough to provide meaningful direction to study personnel for the conduct of routine laboratory activities. If exceptions from SOPs will apply for the study, then those exceptions should be described in the protocol. The protocol should list the SOPs used in a particular study. If a study activity or research is not yet "standard" or is intended to be a one-time event, it is acceptable to incorporate a detailed description of the "how-to" for that activity in the study protocol or in a laboratory notebook. If such activities become routine, however, an SOP should be prepared. The first SOP that should be written is the SOP for writing SOPs. This SOP should contain the guidance for the content of each SOP, the numbering system for SOPs, and the system for review, revision, and acceptance of SOPs. Copies of SOPs must be easily and readily accessible by laboratory personnel. Standard Operating Procedures are living documents and require care and maintenance however. Keeping SOP manuals up to date continues to be a major effort for the labs.

Good laboratory technique has always included proper labeling of reagents and solutions. Which include the four pieces of information: "Identity", "titer or concentration", "storage requirements", "an expiration date". For such materials there is no known expiration date, and it is acceptable to indicate "NONE" or "N/A" (not applicable) on the label for expiration date. In this case, it is sufficient to assign expiration dates based on literature references and/or laboratory experience.

The general procedure of applied and agro-food researches/testing includes the following steps:

– Sampling;
– Obtain a representative sample;
– Homogenize the laboratory sample;
– Reduce the laboratory sample to the test sample;
– Prepare and grind the sample;
– Extract the analyte;
– Test, interpret and report the results.

Performing the assay is unique for each component or characteristic to be analyzed and may be unique to a specific type of raw material, food and feed. Selection of a method depends largely on the objective of the measurement.

For example, methods used for rapid online processing measurements may be less accurate than official methods. Methods referred to as reference, definitive, official, or primary are most applicable in a well-equipped and staffed analytical lab. In analyzing samples all results depend on obtaining a representative sample and converting the sample to a form that can be analyzed. Sampling is the initial point for sample identification. Analytical laboratories must keep track of incoming samples and be able to store the analytical data from the analyses.
To make decisions and act based on the results obtained from performing the assay that determined the composition or characteristics of a raw material, food and feed, one must make the appropriate

Test report

General, the test report shall contain at least the following information:

1) all information needed to identify the laboratory sample;
2) any particular information relating to the laboratory sample (e.g. insufficient size, degraded state);
3) reference to the International Standard(s);
4) statement about date and type of sampling procedure(s) used (e.g. reference to the sampling plan and sampling method used by the laboratory or other bodies where these are relevant to the validity or application of the results);
5) date of receipt;
6) storage conditions, if applicable;
7) analysis start/end date, if applicable;
8) person responsible for the analysis;
9) size of the laboratory sample and test sample;
10) results according to the requirements of the specific method and the units used to report the results and the calibrators and the calculation method used;
11) any particular observations made during testing;
12) any deviations, additions to, or exclusions from, the test specification;
13) Information shall be given with regard to the units.
14) the measurement uncertainty and its level of confidence shall, on request, be made available to the user of the results.

The laboratory shall be responsible for all the information provided in the report, except when information is provided by the customer. Data provided by a customer shall be clearly identified. In addition, a disclaimer shall be put on the report when the information is supplied by the customer and can affect the validity of results. Where the laboratory has not been responsible for the sampling stage (e.g. the sample has been provided by the customer), it shall state in the report that the results apply to the sample as received.
Any laboratory that conducts nonclinical laboratory studies must provide dedicated space for the storage of raw data, documentation, protocols, specimens, and interim and final reports from completed studies. Storage conditions (e.g., temperature, humidity) in the archives should be reasonably related to the nature of the stored documents, specimens, and samples.

Besides the testing of samples, laboratory perform specific activities, including but not limited to, the following: development, modification, verification, validation of methods and participate in proficiency testing.

4.2 Method validation

Introduction

Analytical validation, quality assurance and quality control are terms currently widely used by testing laboratories.

According to ISO, analytical validation as first level of quality assurance in laboratory is “confirmation through the provision of objective evidence that the requirements for a specific intended use or application have been met.”

Quality Assurance (CA) is “quality management focused on providing confidence that quality requirements will be met.”

Quality control (CC) is “part of quality management, focused on meeting quality requirements.”

Laboratories must use analytical methods appropriate to the tests they carry out.

Even if standardized methods are used in the laboratory, the laboratory must make sure that the degree of validation of a particular analytical method is appropriate to the required purpose and that the laboratory is capable of meeting any performance data set.

Testing laboratories develop a quality control program to lend credibility and relevance to the results of measurements obtained in the laboratory.

Definitions

VALIDATION – confirmation by examining and providing objective evidence that the specific requirements for a particular purpose or a particular application are met and there is a balance between costs, risks and technical possibilities.

QUANTITY (characteristic) – attribute of a phenomenon, of an object or of a substance, which is likely to be determined qualitatively and quantitatively.

VALUE OF A QUANTITY – value compatible with the definition of a specific quantity.

MEASUREMENT – a set of operations aimed at determining a value or a quantity.

RESULT OF A QUANTITY (analysis result) Xi – value of a quantity, obtained by measurement.

ARITHMETIC MEAN Xi – sum of results of a quantity (sum of analysis results), related to number of values.

DEVIATION xi – difference between the individual values of a quantity and the average of the same individual values.

STANDARD DEVIATION D – numerical value in units of values observed that measure the tendency of data dispersion tendency.

METHOD PRECISION – difference allowed between the test results obtained under the same testing conditions. Precision measurement is expressed by repeatability and reproducibility.

REPEATABILITY – difference allowed between the test results obtained on the same subject matter analyzed, on the same device, by the same test operator.

REPRODUCTIBILITY – difference allowed between the test results obtained in two or more laboratories.

ACCURACY – difference allowed between the test result and an accepted reference value (a reference material).

PRECISION (systematic error) - difference between the average of the results obtained from the test and the accepted reference value (a reference material).

REFERENCE MATERIAL – material or substance with one or more properties sufficiently well-defined to be used for calibrating a device, for the value of an analytical method or for assigning material values.

ANALYSIS INTERVAL – limits of the range of values that, with a certain probability, include the value of the measurand.

Reference Documents

● **SM SR EN ISO/CEI 17025:2006** General Requirements for the Competence of Testing and Calibration Laboratories;

● ISO 5725-3: 2014 (1-6) Accuracy (Trueness and Precision) of Measurement Methods and Results;

● **Government Decision no. 265 of 06.04.2009** harmonizing EC Commission Decision no. 2002/657 of 14 August 2002 implementing Council Directive 96/23/EC regarding the performance of analytical methods and the interpretation of results;

● **Government Decision no. 520 of 22.06.2010** harmonizing certain provisions of Commission Regulation (EC) no. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs;

● **EURACHEM Guide** - The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics, Second edition 2014;

● **Government Decision no. 941 of 01.10.2010** harmonizing Commission Regulation EC 333/2007 of 28 March 2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs;

● **EA 4/02 M:2013** - Evaluation of the Uncertainty of Measurement in Calibration;

● **EA 4/14:2013** - Selection and Use of Reference Materials;

● EA 4/16:2013 - Guidelines on the Expression of Uncertainty in Quantitative Testing;

● **EA-4/18:2003 -** Guidance on the Level and Frequency of Proficiency Testing Participation;

● **ILAC-P9/06:2014 -** ILAC Policy for Participation in Proficiency Testing Activities.

Validation and/or Verification of Test Methods

Laboratories can use both standardized (national and international) and non-standardized methods, issued by relevant technical organizations and scientific publications, even those containing sufficient and concise information about the procedure for carrrying out tests and which are compulsorily validated to demonstrate reliability and suitability for purpose or to determine how the laboratory manages to achieve the indicated performance.

The laboratory must confirm that it can apply these methods correctly through recovery tests, repeatability and reproducibility tests, control charts, estimation of measurement uncertainty.

Deviations from standardized methods must be identified and justified by validation.

The validation of test methods is performed according to an annual validation plan.

Establishing the extent of validation

The extent of validation and/or verification must take into account:

- the relevance of parameters estimated for the intended use (e.g. to determine the detection limits of the method or selectivity may be relevant for determining residues);

- the relevance of the parameters estimated to satisfy the customer's requirements (e.g. detection limit with maximum allowable concentration);

- the nature of changes produced when improvements of the method are made;

- the nature of differences when comparing different methods, different equipment, etc.

Validation must be a balance between costs, risks and technical possibilities.

Validation must be as comprehensive as necessary to meet the needs related to the given application or scope.

The techniques used to determine the performance of a method should be one of or a combination of the following:

- calibration, using reference standards or reference materials;
- comparing the results obtained by other methods;
- interlaboratory comparisons;
- systematic evaluation of the factors that influence the result;
- assessment of uncertainty on results.
  Validation for the test methods is "approval" for a certain use, for an analyte/matrix combination.
  Validation includes three important segments:
- specific use (analytical requirement which is to be fulfilled);
- objective evidence (performance parameters of the method are calculated by statistical processing of data from the experiences planned);
- confirmation (suitability for the purpose of the method).

Technical conditions for method validation

In order to be sure that the data provided as a result of validation process are correct, the following requirements must be fulfilled:

- person in charge of validation must be competent;
- the environmental conditions must correspond to the requirements of the method specifications (e.g. specifications given by the manufacturer of equipment, reagents, specialist literature);
- the measuring equipment must be in good operation condition, must be calibrated before validation, the interval between two calibrations is established according to ILAC G-24 requirements, after each re-calibration, the laboratory must revalidate the parameters affected (e.g. measurement uncertainty);
- the reagents used must be suitable for working with the equipment to be used and must have unexpired service life;
- the reference materials used in the validation process must be appropriate to the method to be validated (e.g. matrix and concentration level) and must have unexpired service life.

Means for determining the performance parameters include:

- reference standards,
- certified reference materials;
- blank samples (matrices that do not contain the analyte of interest);
- samples with added analyte or fortified samples;
- samples with analyte content;
- statistics.

Defining the performance parameters of analytical methods

1. Selectivity/specificity – ability of a method to distinguish between the analyzed substance being measured and other related substances (isomers, metabolites, degradation products, matrix constituents, etc.) or ability of a method to separate the signal of interest from interferences.

Specificity is the perfect state of selectivity.

Note: *It is important to identify the most likely interferences and to check their effect. Interferences may occur where the method responds significantly to other species present, or sometimes where the analyte signal is increased or reduced by other species, whether observed or not.*

$$SE = \frac{PC}{PC+NF} x100$$

SE = selectivity

RC = number of consistent results

NF = number of inconsistent results

$$SP = \frac{NC}{PF+NC} x100\%$$

SP = specificity

NC = number of inconsistent results

PF = number of falsely consistent results

2. Repeatability*(r)* – degree of concordance between the results of successive measurements of the same measurand carried out in the same measurement conditions (same measurement procedure, same analyst, same measuring device used in the same conditions, same place, within a short period).

Date	**Calculated values**		
	a_1	**a_2**	**a_{med}**
14.07	0,408	0,385	0,3975
14.07	0,385	0,386	0,3855
Standard deviation: S_r = 0,0084 Standard deviation: $S_r = \sqrt{\frac{\sum_{i=1}^{n}\left(a_i - \bar{a}\right)^2}{n-1}}$ **r= S_r•2,77**			

3. Reproducibility – degree of concordance between the results of measurements of the same measurand carried out in different measurement conditions (analyst, measuring device used in different conditions, different locations, different reagents, within a longer period).

Note: *In order to obtain a representative estimate of the standard deviation of repeatability and reproducibility, it is recommended to perform 6 to 15 parallel samples.*

4. Range/linearity

The range of a method defines the range of values for which the method can be applied. In order to determine the suitability for the purpose, it is necessary to evaluate the range and to confirm that it corresponds to the concentration range of analytical requirements.

To determine the range, the response/concentration is observed. At the lower limit of the range of concentrations, the range is related to the limit of detection (LOD[1]), at which it is difficult to be sure that any signal that appears is from the analyte and not from the background, and the limit of quantification (LOQ[2]), which is the lowest concentration that can be determined with acceptable uncertainty. The top end of the range is defined by concentrations at which significant anomalies of sensitivity are observed.

Between these boundaries, there is an area in which the response appears directly proportional to the change in concentration (linear domain).

5.Limit of detection (LOD) is the lowest concentration of a compound that can be detected and identified using the relevant equipment and methodology.

LOD is numerically equal to: three times the standard deviation of the mean of blank determinations (n> 20).

The LOD study is applied to the repeated integral method including any correction of the line due to the baseline or blank:

LOD = 3s + response to blank or baseline

(where: s – standard deviation)

6. Limit of quantification (LOQ) is the lowest analyte content that can be analyzed with reasonable statistical certainty. If both precision and accuracy are constant for a range of concentrations around the limit

[1]LOD =limit of detection

[2] LOQ =limit of quantification

of detection, then the limit of quantification (LOQ) is numerically equal to six to ten times the standard deviation of the mean of blank determinations (n> 20).

7. Decision limit is the limit to which and until which it can be concluded with a minimum probability of error that a sample is not consistent.

8. Stability/robustness is the sensitivity of an analytical method to the variation of experimental conditions that can be expressed as a list of materials to be analyzed, substances analyzed, storage conditions, environment and/or conditions of preparation of samples, based on which it can be applied as such or with minor changes specified.

The robustness test is a set of experiments that allow the analyst to identify the experimental parameters that have a significant effect on the method performance. Variations of these parameters produce changes in the method performance.

It is important to know which parameters are critical for the method performance. These are the parameters that have to be controlled in the routine application of the method. For critical parameters, control limits are set to ensure the desired method performance in the future.

The typical key parameters that are aimed at in case of the robustness test are concentration and volumes of reagents; pH; extraction time; extraction temperature; combination ratio between the mobile phase components for LC, flow rates in chromatography systems; age of the chromatographic column.

9. Recovery isthe actual percentage of concentration of a recovered substance during the analytical procedure. It is determined either by using certified reference material or by fortifying the sample with a known amount of the compound of interest.

$$\mathbf{R\%= C_d/C_{ad} x100\%}$$

C_d- Concentration determined;

C_{ad}- Concentration added

10. Accuracy/precision – approaching an agreement between the average value obtained on a large series of test results and the accepted reference value.

11. Measurement uncertainty is aparameter associated with the measurement result, which characterizes the dispersion of values that could reasonably be attributed to the measurement.

Standard uncertainty (u): uncertainty of the result of a measurement expressed by a standard deviation.

Combined standard uncertainty (uc) – standard uncertainty of the result of a measurement, when that result is obtained from the values of different quantities, equal to the positive square root of a sum of terms, respective terms being the variance or covariance of those quantities, weighted according to how the measurement result varies with changes in those quantities.

Expanded uncertainty (U) is the quantity defining an interval about the result of a measurement, expected to encompass an interval in which a high fraction of the distribution of values could reasonably be attributed to the measurand. Expanded uncertainty is calculated from the combined standard uncertainty and an expansion factor

Expansion factor (k) is a numerical factor used as a multiplier of the combined standard uncertainty to obtain the expanded uncertainty.

The standard uncertainty (u_c) of the independent test result, which characterizes the random errors of measurements, is calculated using the formula:

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

where:

x_i - second test result

$\overline{x_i}$ - arithmetic mean for a series of *n* TESTS

The combined standard uncertainty (u_c) is calculated according to the formula:

$$u_c(y) = \sqrt{\sum_{i=1}^{m} u_i^2} = \sqrt{u_A^2 + u_B^2},$$

where:

u_i - TYPE *A* AND *B*uncertainties estimated;

m - number of estimated factors

To obtain an expanded uncertainty *U,* the final step consists of multiplying the combined standard uncertainty by a chosen expansion factor. The expanded uncertainty is required to provide an interval, in which it can be considered that a large part of the dispersion of values is included, which can responsibly be attributed to the measurand:

$$U = k \cdot u_c,$$

Presentation of results

The expanded uncertainty determines an interval within which the measurand is assumed to fall within a particular level of confidence. U is obtained by multiplying u_c (y), combined standard uncertainty, with a coverage factor k. The choice of factor k is based on the desired confidence level. For a 95% confidence level, k is 2.

In the absence of other requirements, the test result ***y*** must be declared together with the expanded uncertainty ***U***, calculated using an expansion factor *k* = 2 (which has a confidence level of about 95%). The following presentation is recommended:

y =x ± U (UNITS)

Selection of performance parameters

The validation criteria are differentiated according to the level of action applied for each group of residues.

For contaminants, there is a number of guides and specific legislative specifications for validation or performance requirements, such as Regulation 333/2007 for heavy metals (Pb, Cd, Hg, Inorganic Sn), Regulation 401/2006 for mycotoxins, Regulation 1883/2006 for dioxins, dioxin-like PCBs, Regulation 396/2005 – procedures for establishing the technical validation guide.

For residues of veterinary medicinal products, Commission Decision 657/2002 applies.

For microbiological criteria, Government Decision no. 221 of 16.03.2009 applies.

The performance parameters generally targeted must take into account the type of analytical method used (screening/confirmation, qualitative/quantitative).

The analyst responsible for validation of a method shall prepare a Validation Report establishing the analytical requirements and the performance parameters that will be experimented.

The data obtained from experiments for establishing the performance parameters of the method are interpreted to determine if the method is suitable for the intended use, taking into account the performance limits imposed by working standards, the Horwitz equation or provisions of the Regulations regarding the performance of test methods and the interpretation of results.

Revision of the method validation report shall be carried out whenever major changes occur (equipment change, replacement of the responsible analyst, extension of the scope of method, etc.).

4.2.1 Using proficiency testing and other interlaboratory comparisons in the process of accreditation

Proficiency testing/interlaboratory comparisons are one of the reliable and efficient mechanisms to prove the proficiency of the laboratory/inspection body (where the testing activities, which directly affect and determine the result of the inspection, are available and justified or when this is required by law or authorities).

Participation in PT schemes provides information on the performance of the measurement/ activity system (equipment, method, personnel, etc.) and shows aspects of the management system (request analysis, sample acceptance and preparation, data processing, reporting of results, etc.), representing adequacy of risk management and identifying the training needs of the personnel of the laboratory/inspection body.

4.2.2 Selection and use of reference materials

Reference materials are an important tool in achieving a number of aspects of measurement quality and are used for method validation, calibration, estimation of measurement uncertainty, training as well as for internal QC and external QA.

1. **Types of reference materials**

RMs are used to support measurements related to chemical composition, biological, clinical, physical, engineering properties and other particularities such as taste and odor. They can be characterized by "identity" (e.g. chemical structure, fiber type, microbiological species, etc.) or by "property values" (e.g. amount of substance of specified chemical entity, hardness, etc.). Some of the most common types of reference materials are:

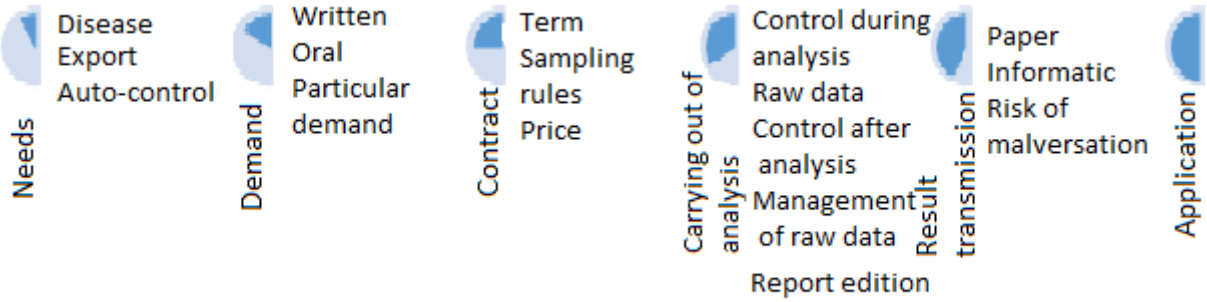
1. **Pure substances** characterized in terms of chemical purity and/or traces of impurity.
2. **Standard solutions and gas mixtures**, often prepared by gravimetric method from pure substances and used for calibration purposes.
3. **Matrix reference materials,** characterized in terms of composition of major, minor or trace chemical constituents. These materials can be prepared from matrices containing the components of interest or by preparing synthetic mixtures.
4. **Physico-chemical reference materials,** characterized in terms of properties such as melting point, viscosity and optical density.
5. **Reference objects or artifacts,** characterized in terms of functional properties such as taste, smell, octane number, flash point and hardness. This type also includes microscopic species characterized in terms of properties that vary from fiber type to microbiological species.

Classification of reference materials

Two classes of reference materials are recognized by ISO, "certified reference materials" (CRM) and "reference materials" (RM). By definition, CRMs must be traceable to an accurate realization of the unit in which the property values are expressed. Each property value must be accompanied by an uncertainty at a declared level of confidence. RMs are materials whose property values are sufficiently homogeneous and well established to be used in calibrating a measuring instrument, evaluating a measurement method or assigning material values.

4.3 The performance of analyses

Introduction



4.3.1 Traceability

The traceability means the managements of documents manualy written or/and informatic, produced during analysis. The objective of the traceability is to allow to control different phases of analysis, to verify if all these phases are properly caried out and to allow the validation a posteriori of analysis results. Th traceability must allow to follow the integrity of analysis including errors occuring due to some operations (calculation errors, equipment occurrences etc.).

There are different means allowing to carry out the traceability :

Paper traceability : The collect of documents produced at different phases (cards related to sampling such as storage / stock reduction before analysis, preparing of samples, preparing of reagents, consumables, equipment etc.). These documents are places physically into dossier during analysis.

These documents should be produced in order to allow :

- An easy reading : the documents should readable. The erasures if they are inevitable shoud be readable as well. : e.g. : erasure accaptable – er~~asure~~ inacceptable. [redacted]
- The writing means producing texts that can be erased should be excluded.

Advantages : Easy use in time as it doesn't need a special software

Desadvantages : Voluminous storage, breakable, less ecologic

Digital traceability: The digital traceability can take different forms such as files with texts, Excel tables, video and sound files. Today, the venue of the information systems such as leptops, tablets allows to insert the information during analysis.

Advantages : different means according to desired type of saving.

Disadvantages : reading of files is less easy because of the evolution of software. Lost of data because of « re-writing » of date (we erase and we re-write).

4.3.2 Incertainity of results

Every measurement of result is clouded by one « error » that is a sum of systematic and random incertainities. These incertainities should be known and calculated by laboratory.

Example of incertainities occuring during analysis :

- Homogenity of samples
- Reagents
- Equipment
- Staff
- Etc.

Advantages : The « limits » of analysis are known.

Disadvantages : Time and cost of incertainities calculation, confusion for certain clients if incertainities are given in the report of analysis.

NB : The calculation of incertainities is a long work cariied out by a team of laboratory. It is not possible to list here all actions necessary for this process but the minimus of knowdge needed for launching the calculation should be remembered:

- *Mathematics and more particularly statistics*
- *Knowlege of Excel*
- *Research and implementation of the calculation plan taken from the scientific litterature and/or national and international norms specifically related to the work field (for example : chemistry, metrology etc.)*
- *In certain cases, use of specific informatic tools.*

4.3.3 Control during analysis

The analyses that we carry out have an important impact on the client that commanded them. A result of analysis can confirm or confute a serious disease where the treatment can be hard (cancer, diabetes etc.) for a person, anoth result can impact production mood, suspect an important contamnation (heavy metals, radionucleudes etc.). For all these reasons the control as severe as possible should be applied during analysis : internal controls and external controls if they exist.

The internal controls will take different aspects such as samples voluntary contaminated and added in the beginning of analysis or during its different phases, ranges of samplings for chemical analyses. The analyses are carried out two in parallel in order to smooth the problems of sample homogeneity that can't be controlled by technician in cases of complex matrices (muscles, live, kidneys etc.).

Advantages : The results of series of analyses are « garanteed » by the internal control.Overcosts are alleviated by big serries of samples.

Disadvantages : Grouth of costs in case of small series. The time needed for analyses is increased.

The external controls are applied to samples where the of contaminants is given with related uncertainty. These certified reference material are sold with known results of contamination, the result itself is given with un uncertainty.

Advantages : Results garanteed and validated by the use of certified refernce materials.

Disadvantages : Sometimes the cost of refernce materials is high.

4.3.4 Raw data

The data presenting all data produced during analysis before their treatment. These data can be written manually, can have the form of cards, can be written in the work-book that is filled in during analysis, be presented as graphs or numeric documents. These data will be treated (calculated, interpreted etc.) in order to allow the edition of the analysis result.

Examples of raw data :

- Ammount of samples taken for analysis
- Making standard solutions (in case of chemical analyses)
- Graphs made by equipments or operators
- Etc.

These data should remain readable ; the calculations and treatment of these data should be explained and be traceable.

Advantages : The raw data should allow to verify analysis or even to re-make it theoretically through these data.

Disadvantages : The storage is voluminous, fragil. Management of archives.

4.3.5 Control after analysis

Before editing report of analysis, the control should be carried out by a competent person having a critical view of the result. This control should make sure that the analysis was carried out in proper conditions. This control should allow to have another view of the analysis and possible ecarts committed and traced.

These controls concern mainly the following :

- The calculations and the risks of errors related to the unities
- The cards of non conformity, the causes of non conformities and their elimination
- The re-transcription of velues given by equipment
- Carrying out of maintenance planning and metrological control

Advantages : Raw data should allow to verify analysis or even redo it theoretically using them.

Disadvantage : Storage voluminous, fragile.

4.3.6 Editing of analysis report

The analysis report should be edited after all analyses carried out. Nevertheless all precautions should be taken in order to avoid the errors. We can mind around the impact of an erratic result in the cancerology or diabetology etc**.**

The analysis report should correspond to the requirements of national legislation as for its form, provided information, and to the requirements of national accreditation bodies.

The verification procedures should be written and applied in order to minimize the risk of transcription errors.

Advantage : « Sure » results for clients.

Disadvantage : It takes more time for the delivery of results.

4.4 Obtained results and their coherence. Quality of Analytical Results

General aspects of quality assurance are covered in ISO/IEC 17025. The goal of any regulated laboratory is to provide reliable and valid data suitable for its intended purpose. Analysts use validated methods, system suitability tests, and in-process quality control checks to ensure that the data they acquire is reliable, and there are specific guidance and procedures available to ensure compliance. When methods have been validated and demonstrated to be fit for purpose for a single matrix, these methods should not be applied to similar or other matrices prior to establishing the fitness for purpose within these additional matrices. Results from all test portions shall be consistent. When at least one test portion gives a positive result and at least one gives a negative result, the analysis shall be repeated.

Quality — the realization of specific requirements (which include the standards established by the quality control system in addition to accepted in-house requirements). Analytical quality — consistency of the obtained results with the accepted assumptions. The quality of information can be divided into components: quality of results, quality of the process, quality of the instruments, and quality of the work and organization.

Quality control — a complex system of actions to obtain measurement (determination results) with the required quality level. A program of quality control includes: Assuring a suitable l • level of staff qualifications • Assuring the proper calibration of instruments and laboratory equipment • Good laboratory practice (GLP) • Standard procedures.

The laboratory shall monitor its performance by comparison with results of other laboratories, where available and appropriate. This monitoring shall be planned and reviewed and shall include the following: participation in proficiency testing, participation in interlaboratory comparisons other than proficiency testing.

4.5 Discussion with client

Types of clients

Three categories of relations with client can be identified. These categories are the results of supplier's manner to consider and to treat his clients.

Client – king: Client is considered incorrectly as someone whose all demands should be honored. The reasons of the supplier's motivation can be different (internal dogmas, financial etc.). Laboratory acting by this way makes a double mistake: first, it doesn't allow client to refine his demand and thus it can't know if in reality his demand is justified or not, too exhausting or not enough.

Second mistake: supplier becomes a « slave » of his client, he does his best to satisfy client, so further this client will demand a lower price and finally will leave the supplier for finding someone « less expensive ».

Client - partner : With this client supplier created the relations of confidence, allowing him to make specified his demand, his exact needs for short and middle term, to be sure that the demand corresponds to procedure that is necessary and sufficient for the client. The price conform to demanded and provided service is agreed by both parts (supplier and client). Due to good negotiations both parts are satisfied with the result

Client - prisoner: who hasn't feel it with some suppliers of internet or telephone access? In the beginning of the contract the price is low, and it increases when the client if attached. If he asks for some explanations no answer is clearly given, the formulated demands stay without answer.

If the client asks for some additional services, he has to pay a high price. Services proposed by supplier are provided without preliminary agreement of client and are invoiced with high price. Finally, the client will leave for another laboratory even if its prices are a little more expensive.

For an efficient communication the demand of client should be:

Precise: what does he need? What is deadline? What is the number? Is his demand compatible with techniques applied at the laboratory? Will results be exposed according to acting normative or regulated requirements? The precision should be determined in the partnership with laboratory. The financial part will be treated with the same precisions.

Written: Traceability of demands should be conserved by laboratory. It can be referred if needed.

The "problems" that may give credence to the results issued during the analysis must be clarified to the client, with their effects on the results. This communication should be carried out without waiting.

Result and its expertise: results should be clear, precise, without the possibility to lead to mistakes of comprehension. On the other hand, the analysis of the result must be carried out by an expert external to the laboratory or at least, to the analytical unit, in order to avoid "jaundice".

4.6 The ethic at laboratory

4.6.1. General statements

Following the ethical principles in laboratory is intended to ensure that laboratory staff considers and manages variety of potential risks and ethical conflicts when designing and conducting activities with respect for persons, beneficence, and fairness. Before undertaken any kind of activity within the laboratory, the lab staff should consult the specific rules of their laboratory/research unit/ institution and applicable state/national/international regulations.

Commonly the codes of ethics among others support the "nondiscrimination policy", which states no discrimination of persons with any kind of involvement in lab activity on the basis of race, color, sex, ethnicity, national origin, religion, marital status, pregnancy, disabilities, gender identity, sexual orientation, genetic information, etc.

The ethical rules and principles to be followed depend on the specificity of the work/research conducted at the laboratory, though speaking in general, good laboratory practice means that laboratory reports only sample results that meet all compliance criteria and properly qualifies all results not meeting any of those criteria. With this regard the laboratories are encouraged to develop an ethics policy and add it to the laboratory's quality assurance manual. The laboratory analysts should undergo ethics training. The fraud detection and deterrence program (e.g. data validation and verification techniques; analyst notation) should also be developed and widely implemented into practice.

Lab fraud, usually associated with a perceived harm, is defined as "the deliberate falsification during reporting of analytical and quality assurance results that failed method and contractual requirements to make them appear to have passed requirements" (2006). Top vulnerabilities according to EPA OIG (2006) include: censoring of information based on reporting limits; data manipulation; failure to follow SOPs/reference methods; falsifying existing data; improper calibration; inappropriate manual integrations; overwriting files: peak shaving, juicing, deleting; inadequate training; inappropriate sample collection process; incomplete record keeping; mislabeled sample; no demonstration of competency; no requirement for collector; reporting data for samples not analyzed (dry lab); retention times not assured; sample integrity unknown; selective use of QC data; sequencing analysis; spiking samples after preparation; time travel (warping). Measurement deceptions include the deletion/adding, manipulation or direct fabrication of data. The management reason for that usually gets into three categories, which are to avoid looking "bad" to upper management, to avoid financial penalties, or simply to please the client. To avoid that one should take in mind that the consequences of fraud could be much more devastating than the "calculated" "benefit" and that "broken reputation" could not be restored easily.

Within respect to said above the lab employee responsibilities should include: upholding of the ethics policy and practices demonstrated in daily conduct; seeking adequate assess when the proper course of action is unclear or undetermined; remaining alert to the situations that could result in improper, illegal actions or in direct violation of the ethics practices; reporting any kind of violations of the ethics policy and practices.

Various laboratory surveys and staff feedback conducted on ethics inspired the following comments on the ethical behavior at the laboratory (cited from "Defensibility and Ethics in the Laboratory" by Jo Ann Boyd, Quality Assurance, Chemistry and Chemical Engineering, Southwest Research Institute, 6220 Culebra, San Antonio, TX 78016, USA):

● Be sure the decisions you make are decisions that will allow you to sleep at night and feel good about yourself.

● Remember the reputation of the organization you represent and their business depends directly on you and client satisfaction.

One of the most important issues in laboratory working is the **defensibility** of the data, which is essentially provided by documented evidence fro the first till the very last step of the process. The documentation must be comprehensive and detailed enough to enable external analysts and laboratories to reanalyze data, if necessary, and produce reproducible data by following the original processes and the same documented steps. The analytical testing process and documentation requires continual challenging of ethics to assure that even tests that are performed incorrectly are documented and there is evidence of proper corrective action. These efforts are all necessary to provide legally defensible data to the client and to provide them with the confidence that the laboratory has performed quality work.

4.6.2. Ethics when dealing with animals

Numerous species of animals are used in scientific research during drug testing and education in the field of biomedical sciences and agriculture, as well as in veterinarian laboratories, for the understanding of basic biological processes and the improvement of the quality of life of man and animals.

Realizing the weight of the problems, numerous protocols and guidelines were established. One of the examples is the publication by Council for International Organizations of Medical Science or CIOMS titled "The International Guiding Principles for Biomedical Research Involving Animals" (1985).

The ethical guidelines have facilitated the accuracy of results and many developments in various aspects of laboratory animal science, such as:

- The development of various animal models for human diseases;
- The development of the genetic engineering for the production of transgenic animals for tackling various human diseases which no animal models have ever been successfully used.
- The better care and management of animals, decreasing degree of animal suffering, refinement of techniques used in animal experimentation.
- The search for alternatives to replace or to reduce the number of animals used (Computer simulation, mathematical application and *in vitro* biological systems have proved successful to a certain extend but cannot yet be generalized).

According to guidelines developed by the American Psychological Association (APA) Researchers working with nonhuman animals (acquisition, care, housing, use, and disposition) must conduct their activities in compliance with applicable federal, state, and local, laws and regulations, institutional policies, and with international conventions (Section 8.09 of the *Ethical Principles of Psychologists and Code of Conduct* (APA, 2010)).

Ethical Principles and Guidelines for the Use of Animals underline the value of life of animals and establish the best practice in using animal models. The issues to be considered are the usage of minimal number of animals while achieving the accuracy of the research; the proper usage of wild animals not to violate laws or policies for wildlife conservation; considerations of animals as living beings, which include the guideline on animal transportation, environment at the animal facility, animal care, management, techniques and records in animal experimentation.

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4.6.3. Ethics when dealing with humans

Research with human subjects is to be distinguished from the practice of medicine, public health and other forms of health care, which is designed to contribute directly to the health of individuals or communities.

Research involving human subjects includes:

- studies of a physiological, biochemical or pathological process, or of the response to a specific intervention in healthy subjects or patients;
- controlled trials of diagnostic, preventive or therapeutic measures in larger groups of persons, designed to demonstrate a specific generalizable response to these measures against a background of individual biological variation;
- studies designed to determine the patterns for individuals and communities of specific preventive or therapeutic measures;
- studies concerning human health-related behavior/responses in a variety of circumstances and environments.

Patients, health professionals, researchers, pharmaceutical companies and others rely on the results of Lab research for activities and decisions that impact individual and public health, welfare. Therefore, they must ensure that proposed studies are scientifically sound, build on an adequate prior knowledge base, and are likely to generate valuable information. Research involving human subjects may employ either observation or physical, chemical or psychological intervention; it may also generate records/make use of existing data containing biomedical or other information about individuals who may or may not be identifiable from the records or information. The use of such records and the protection of the confidentiality of data obtained from those records are discussed in International Guidelines for Ethical Review of Epidemiological Studies (CIOMS, 1991) and in International Ethical Guidelines for Health-related Research Involving Humans (CIOMS, 2016),

Research with human subjects should be carried out and/strictly supervised only by qualified and experienced persons, in accordance with a protocol that clearly states: the aim of the research; the reasons for involvement of human subjects; the nature and degree of any known risks to the subjects; the sources of subjects recruitment; and the means proposed for ensuring that subjects' consent will be adequately informed and voluntary.

New vaccines and medicinal drugs, before being approved for general use, must be tested on human subjects in clinical trials; such trials constitute a substantial part of all research involving human subjects.

Research involving human subjects (including identifiable human tissue or data) can be ethically justifiable only if it is carried in accordance with **three basic ethical principles**, namely ***respect for persons,beneficence*** and ***justice.***

- ***Respect for persons*** incorporates at least two main ethical considerations, namely: a) respect for autonomy and b) protection of persons with impaired or diminished autonomy.
- ***Beneficence refers*** to the ethical obligation to maximize benefits and to minimize harms.
- ***Justice*** refers to the ethical obligation to treat each person in accordance with what is morally right and proper, to give each person what is due to him or her.

The guidelines are directed at the application of these principles to laboratory research/studies involving human subjects. According to guidelines, lab staff and research sponsors must ensure that proposed studies involving human subjects conform to generally accepted ethical principles and are based on adequate knowledge of the pertinent scientific literature. These considerations should be reflected in the research protocol. The protocol should be scientifically and ethically appraised by one or more suitably constituted, independent of the research team review bodies in the country of the sponsoring organization (International

Ethical Guidelines for Health-related Research, CIOMS, 2016, *Guidelines 1 and 23*).

For all studies involving humans, the special information in language/another form of communication that the individual can understand should be provided before requesting to participate in research and the voluntary informed consent of the prospective subject should be obtained. In the case of an individual not capable of giving informed consent, the study needs the permission of a legally authorized representative in accordance with guidelines and applicable law. Waiver of informed consent is to be regarded as uncommon and exceptional, and must in all cases be approved by an ethical review committee. (CIOMS, 2016, *Guidelines 9,10*). The guidelines define the obligations of lab investigators/researchers, assessment of potential benefits and risks, including the minimization of risks in the cases, when there is ethical and scientific justification to conduct research with individuals incapable of giving informed consent (CIOMS, 2016, *Guidelines 4,16*) and reimbursements for lost earnings, travel costs and other expenses incurred in taking part in a study, including free medical services as well. For all research involving human subjects, the investigator must ensure that burdens and benefits are equitable distributed, reasonably balanced and risks are minimized(CIOMS, 2016, *Guidelines 3,4,13,)*.

There are special issues for the research *in populations and communities with low-resource limits,* research *involving children, women (including pregnant and breastfeeding), vulnerable persons* discussed in guidelines (CIOMS, 2016, *Guidelines 18,19*). Before undertaking study in a population or community with limited resources, the researcher must ensure that the all procedures is responsive to the health needs and the priorities of the population or community in which it is to be carried out (CIOMS, 2016, *Guideline 2*). The children involvement needs permission ofparent or legal representative and the agreement (assent) and justification that the research might not equally well be carried out with adults. The special justification is required for involvement of vulnerable individuals as well and, if they are selected, the means of protecting their rights and welfare must be strictly applied (CIOMS, 2016, *Guidelines 15,17*).

As a general rule, research subjects in the control group of a trial of a diagnostic, therapeutic, or preventive intervention should receive an established effective intervention. In some circumstances it may be ethically acceptable to use an alternative comparator, such as placebo or "no treatment" *(Guideline 5: Choice of control in clinical trials).* Placebo may be used: 1. when there is no established effective intervention; 2. when withholding an established effective intervention would expose subjects to, at most, temporary discomfort or delay in relief of symptoms; 3. when use of an established effective intervention as comparator would not yield scientifically reliable results and use of placebo would not add any risk of serious or irreversible harm to the subjects.

The secure safeguards of the confidentiality of subjects' research data should be established. Subjects should be told the limits, legal/other, to safeguard confidentiality and the possible consequences of breaches of confidentiality. Investigators should ensure that research subjects who suffer injury as a result of their participation are entitled to free medical treatment and financial/other assistance to compensate them for any resultant impairment, disability or handicap (CIOMS, 2016, *Guidelines 12,14*).

Research involving human biological materials (tissues, organs, blood, DNA, RNA, proteins etc.) may be collected/stored for a specific (research, medical or diagnostic) purposes and also be used in future. In the guidelines, the term 'biobank' is used for the collection of stored biological materials and associated data. An individual whose biological materials and related data are used in research is a study participant and the same ethical guidelines are applicable. Since the precise nature of the future studies is typically unknown and it is impossible to obtain specific informed consent at the time the material is collected, broad informed consent is an acceptable alternative to specific informed consent. The guidelines regulate rules of storage, use and final fate of biological materials and to which other sources of personal information the results of analyses may be linked (CIOMS, 2016, *Guidelines 11*).

Many countries lack the capacity to assess/ensure the scientific quality/ethical acceptability of research dealing with humans, proposed or carried out in their jurisdictions. In externally sponsored collaborative research, sponsors and investigators have an ethical obligation to ensure that research projects for which they are responsible contribute effectively to national or local capacity to design and conduct biomedical research, and to provide scientific and ethical review and monitoring of such research.

References

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4.6.4. Laboratory Safety and Lab Ethics

The enforcement and control of safety regulations and laws in the scientific/diagnostic laboratories are the responsibility of all of stuff and represent an integral part of the planning, preparation, and implementation of any laboratory activities. These controls consist of, but aren't limited to, policies, guidelines, training requirements, standard operating procedures, personal protective equipment, and laboratory audits. It is obligatory for all laboratory associates and students to attend lab specific trainings to be aware of safety procedures and restrictions defined by detailed laboratory safety manuals (GLSM) for the safe handling of hazardous materials and other common laboratory hazards. The GLSM describes a minimum level of safe practices that are expected from all individuals involved in the laboratory operations.

In 1990, OSHA issued the Occupational Exposure to Hazardous Chemicals in Laboratories standard (29 CFR 1910.1450). The OSHA Bloodborne Pathogens (BBP) standard (29 CFR 1910.1030) is designed to protect workers from the health hazards of exposure to bloodborne pathogens. As laboratory workers may be exposed to a variety of hazardous on the job, other OSHA standards provide procedures how to prevent or reduce exposure to some of the more common chemicals/hazardous: The Personal Protective Equipment (PPE) standard (29 CFR 1910.132), The Hand Protection standard (29 CFR 1910.138), Recordkeeping standard (29 CFR 1904) etc. U.S. Centers for Disease Control and Prevention (CDC) jointly with National Institutes of Health (NIH) published manual for *Biosafety in Microbiological and Biomedical Laboratories* (5th Ed., 2009) which has become the code of practice for biosafety and an authoritative reference for the risk assessment in microbiological and biomedical laboratories.

General Laboratory Safety Principles defined by standards and guidelines are regarded as universal and should be read and interpreted as a whole as they have cross references to each other. These documents with can be used by an individual researcher or laboratories for identification or recognition of hazards and the evaluation of the risks and to formulate a plan to minimize/manage the risks prior to the start of work. The general principles for the safe laboratory practice are:

1. Knowledge of materials (a. minimize all chemical exposures; b. approach all chemicals as hazardous and use caution; c. avoid underestimating the risk; b. observe the Threshold Limit Values).
2. Follow safe practices and use Standard Operating Procedures (SOPs).
3. Knowledge emergency procedures and location of safety equipment.

The laboratory safety and security status assessment strategies are provided in safety manuals and imply managing and control of different lab systems using lab safety checklists:

- ✓ Personal Protective (PPE), laboratory equipment (fume hoods, instruments, ventilation, water protection, temperature etc.) and engineering safety
- ✓ Administrative safety (SOPs, procedural controls, access to laboratories, lab design and construction, ventilation rates, area inspection and laboratory security)
- ✓ Biological Safety (biological safety levels, tissue cultures and cell lines, safety data sheets for infectious substances, decontamination, rooms (gel, dark, radioisotope and cold) safety, transportation of biological materials)
- ✓ Chemical Safety (general chemical procedures and exposure monitoring, chemical spills and highly toxic chemicals, chemical storage guidelines)
- ✓ Cryogenic, electrical, mechanical, radiation, laser and nanomaterial safety
- ✓ Cleanroom safety, waste management and emergency procedures

The standards and guidelines provide internationally vetted ethical principles and detailed commentary on how universal ethical principles should be applied focusing primarily on rules and principles for reliably safeguarding. A number of laboratory professional organizations have code of ethics based on common principles, defined by International Federation of Biomedical Laboratory Science (IFBLS) and applied to

Biomedical Laboratory Scientists (BLS) worldwide. BLS have the responsibility to contribute from their sphere of professional competence to the general well-being of the community:

1. Maintain strict confidentiality of patient information and test results
2. Safeguard the dignity and privacy of patients
3. Be accountable for the quality and integrity of clinical laboratory services.
4. Take personal responsibility
5. Treat patients and colleagues with respect, care and thoughtfulness
6. Perform duties in an accurate, precise, timely and responsible manner
7. Safeguard patient information as confidential, within the limits of the law
8. Prudently use laboratory resources
9. Advocate the delivery of quality laboratory services in a cost-effective manner
10. Render quality services and care regardless of patients' age, gender, race, religion, national origin, disability, marital status, sexual orientation, political, social, health, or economic status.

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Chapter 5. The references

5.1 The laboratory standards

First, let's put the problem and some definitions: for the laboratory, the problem is the result, its obtaining and its reliability.

In the documentation, these problems are called results, processes and uncertainties.

• The result can be quantitative and display a value. In this case, the laboratory must take into account, and therefore determine the uncertainty linked to the result. The uncertainty is determined internally according to standards (ISO8655, GUM, etc.)

• The result can be qualitative by only reflecting a state of the sample (consumable, non-consumable, etc.). In this case, the state must be obtained by comparison with figures, for example (number of colonies of bacteria per gram, etc.). This comparison remains within the competence of the analytical expert.

• The process also called process or protocol describes the phases for obtaining the result. This process must be selective, repeatable, reproducible, fair, etc. This is different criteria will be defined and validated by the laboratory. This validation will be internal and external.

We can "classify" the laboratories according to the type of results sought and thus determine what types of documentation is linked to them:

• Basic research laboratories: in this type of laboratory, the protocols are being developed. Researchers are developing them in every way. The standard most used in this type of laboratory is often the ISO 9001 standard which is organizational recognition. The analytical processes are only very little "validated" because they are completely new (therefore not used by other laboratories). Some public bodies choose to create their own repository. The "rules of the art" of the profession of laboratory analyst are also a good basis.

• Applied research laboratories: in this case, the problem is the development of detection / quantification methods or preclinical studies. The standards used are good practices (GLP, GCP, etc.), ISO 9001 standards

as well as certain benchmarks that may have been developed by companies or institutions. But these documents remain documents of organization, protection of works, dissemination of works, etc. Internal validation is carried out according to the standards in force (ISO8655 for example)

• Analysis laboratories: in this case, the result emitted is of great importance because it directly impacts the product analyzed (case of agro-food, manufactured products, etc.) or the patient (human medicine, veterinarian). An error is or can be catastrophic in terms of impact on health (illness not discovered, unnecessary or inappropriate treatment, etc.) or financial (destruction of the product, loss of brand image, user, etc.). These laboratories therefore have a complete Quality management system as well as national and international recognition of their techniques obtained by accreditation. ISO17025 is the most used standard in this case.

The protocols are validated using standards such as ISO8655 and the maintenance of the technical competence of the laboratory is ensured during participation in Inter-laboratory Tests.

5.2 The national and international recognition (accreditations, GLP, GMP, etc.)

Good Laboratory Practice (GLP) is a set of measures, requirements and methods aimed to ensure the highest quality of laboratory research. Principles of Good Laboratory Practice (GLP) are eleborated to improve the quality and reliability of test results. Observance of GLP general principles in conducting research will facilitate the process of information exchange, promote the protection of human health, animals and the environment.

Good Manufacturing Practice (GMP) is part of a quality assurance system that ensures that the lab is controlled and compliant with the applicable standards. GMP rules are designed to reduce the risk of laboratory activities.

Quality management system is a set of basic processes in the organization of activities (managerial, executive, reporting, controlling), aimed at achieving coherence of action to reach the goals.

The quality management system in the laboratory necessarily requires cooperation and joint efforts for proper work, confirmation and meeting the current requirements of regulations. The implementation of a quality management system allows the laboratory to work efficiently and fully use the possibilities of ISO 17025 taking into account the requirements of international standards of ISO.

The main principles of the quality management system (according to ISO 9001: 2000 Quality management systems - Requirements):

1. ***Client orientation.*** The laboratory depends on its customers and therefore needs to understand current and future needs, fulfill their requirements and strive to exceed their expectations.
2. ***Leadership.*** The leadership establishes the unity of purpose and directions of development; creates and maintains conditions for stuff actions to ensure fulfillment of tasks; constantly cares about training of the personnel, provides the necessary resources. regular feedback on the effectiveness and efficiency of the quality management system is important thing in the implementation of the principle of leadership; development of corrective and preventive actions.

3. Staff involvement. **Competent, empowered and involved staff is essential for improving the laboratory's ability to deliver effective results. The quality system and its mechanisms should encourage employees to improve the quality of the laboratory's activity, take responsibility for solving the quality problems, deepen their knowledge actively and transfer it on to colleagues.**

4.Process and system approach. Concertedand predictable results are achieved more effectively if people understand the activity and manage it as interconnected processes (integral system). The quality system consists of interconnected processes. The company must have processes of internal exchange of information and a system for responding to requests and wishes of consumers. The system approach involves continuous improvement of the system through analysis and evaluation, adoption of corrective and preventive actions.

5. Constant improvement. **It is necessary to strive for improvement of activity efficiency, but not to expect occurrence of a problem.**

6. Decision making based on facts. **Effective solutions are based on the analysis of reliable and accurate data and information. This principle applies not only to the management of the functioning and technical functioning of the operation, but also to the management of the testing itself by the methods that make up the laboratory accreditation area.**

7. Relationship management. **The laboratory manages its relationships with relevant stakeholders, such as suppliers, to achieve sustained success. The implementation of this principle requires the identification of major suppliers, the organization of clear and open relationships, information exchange, cooperation with clearly understandingof needs of consumers.**

Elements of the quality control system in the laboratory

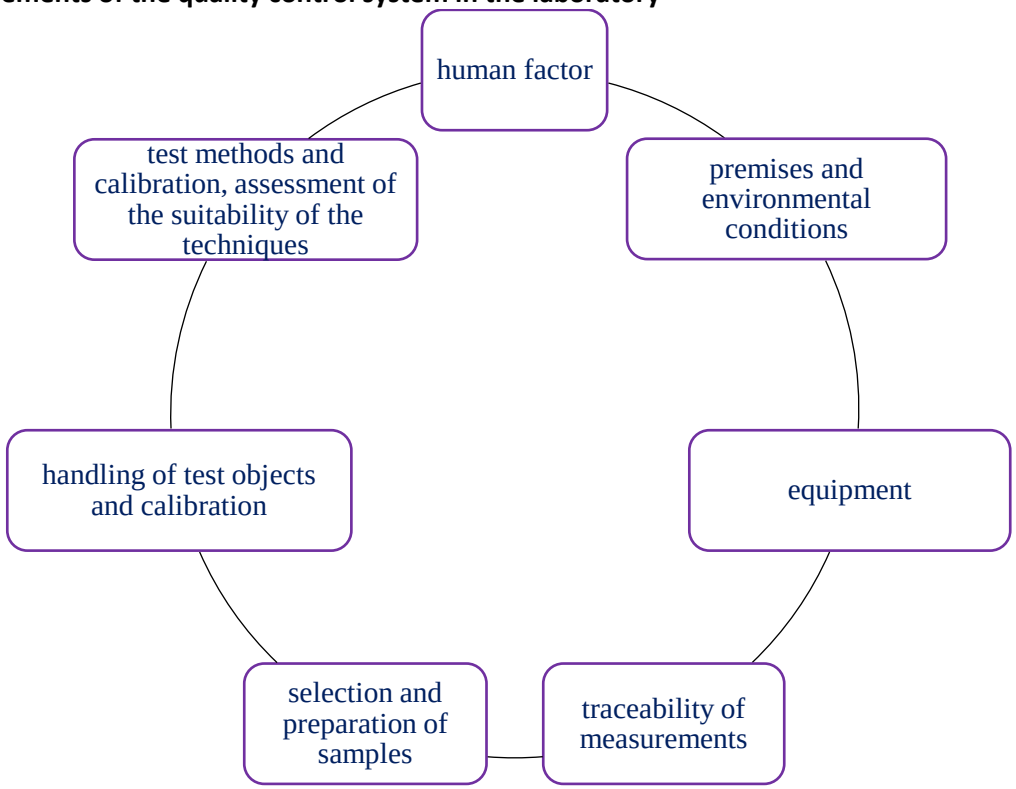


Fig. 1.1 – **Factors which define the objectivity and reliability of laboratory research**

The rational organization of the laboratory work must include all its activities:

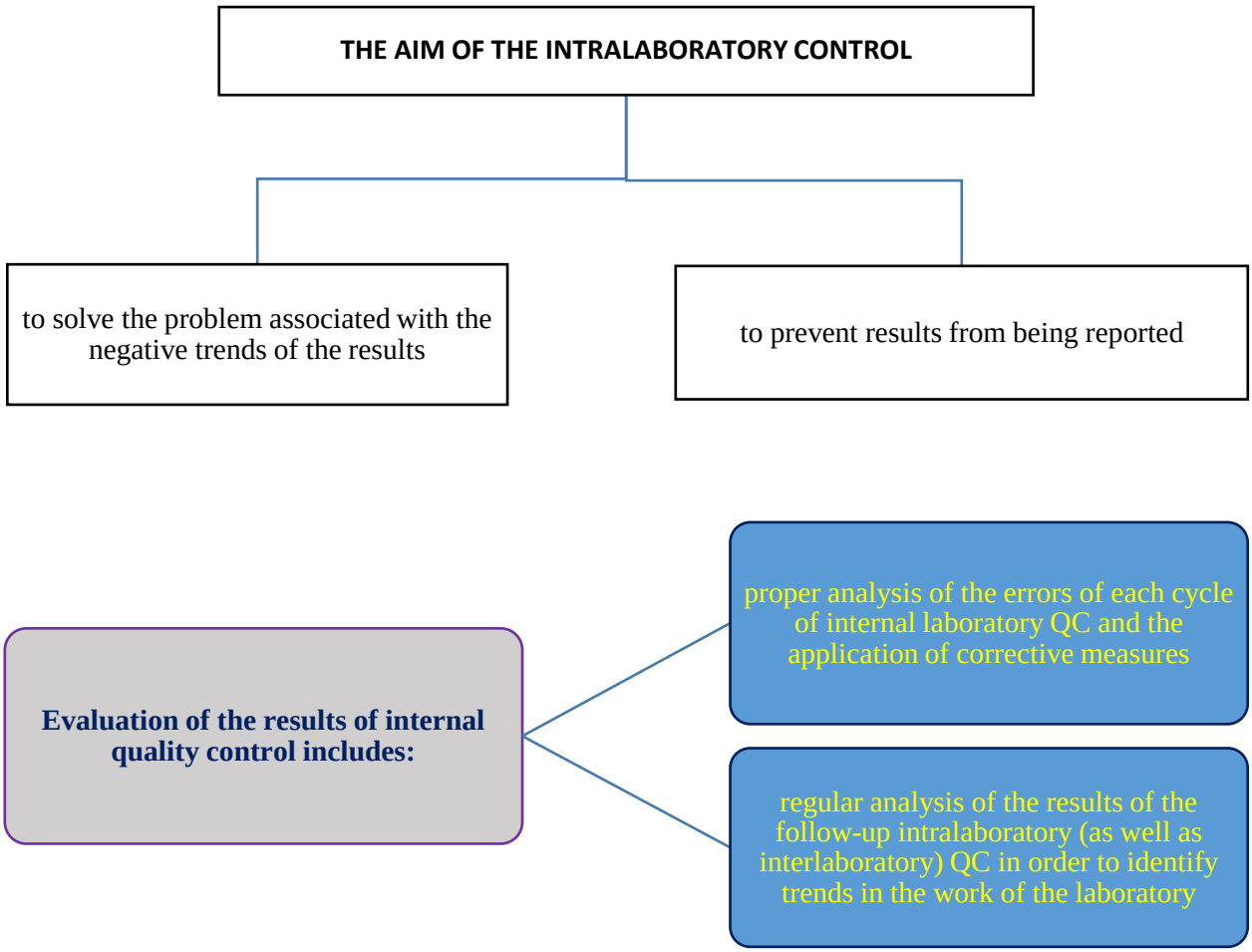
- clear planning of activities;
- availability of appropriate equipment, premises, equipment;
- metrological support in exact accordance with rules and norms;
- convenient organization of workplaces (certification of workplaces);
- availability of instructions, methods of conducting research at the workplace
- knowledge and compliance with safety regulations and occupational safety;
- rational organization of the process of laboratory research;
- provision of necessary reagents and control materials;
- proper observance by each laboratory member of his responsibilities;
- theoretical and practical competence and further training of laboratory staff;
- compliance with the biosafety rules in accordance with the requirements of regulations.

External and internal control of the quality system

Two basic approaches to quality control (QC - Quality Control) to be implemented in the laboratory:

- Intralaboratory QC, which is a daily monitoring of the characteristics of the research results and / or performed in the established order for the sampling during the series of studies;
- External QC, which is based on the participation of the laboratory in professional testing programs.

Intralaboratory control is planned systematic activity, which is obligatory for any laboratory. It is necessary to plan intralaboratory testing by periodic duplication of tests by another operator for instance.



One of the main criteria for assessing the technical competence of laboratories during accreditation according to ISO/IEC 17025 is the results of their participation in interlaboratory comparative tests (ICT).

Interlaboratory comparative tests are the organization, conducting and evaluation of tests on identical or similar control samples by two or more laboratories according to specified conditions (ISO / IEC 17043 Conformity Assessment - General Requirements).

The EAL-P7 "Interlaboratory comparative tests" document sets out the main criteria for the ICT and complements the ISO / IEC 43-1 ... 2 Guideline. During the ICT of the accredited laboratories, ISO 33 "*Uses of Certified Reference Materials*" and ISO 35 "Certification of Standard Samples. General and Statistical Principles " are used.

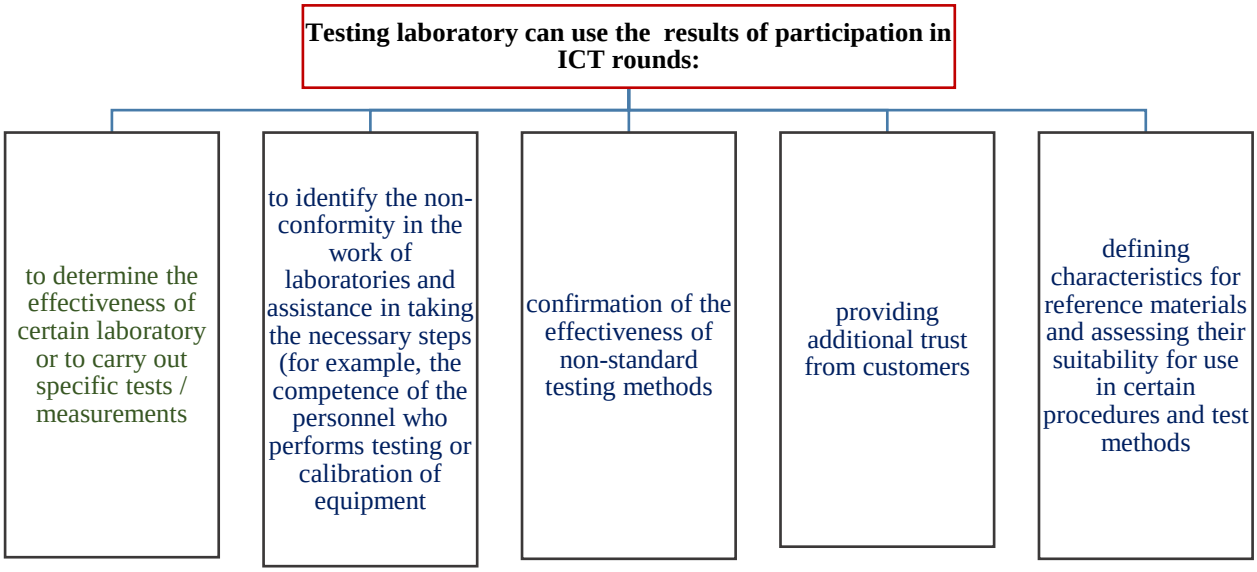
The main task which is solved by means of ICT is an experimental verification of the technical competence of the laboratories in identification of the parameters of the content and properties of substances and materials in the declared (approved) accreditation area, which is carried out both at the stage of accreditation and at the next inspection control. An analysis of the experience of various organizations shows that if there is a group of several laboratories that perform the tests of the same substances and materials, ICT is the most rational method of verification of technical competence. Participation in the ICT increases confidence in the results of the testing laboratory during accreditation and inspection. As a rule, a properly planned interlaboratory experiment allows not only to estimate the test validity of each laboratory, but also to solve a set of other tasks related to the metrological assurance of tests.

In particular, the ICT is conducted:

- To determine metrological characteristics of the methods for tests, measurement, control (TMC);
- To verify the unity and reliability of measurements for a specific TMC in a group of laboratories;
- assessment of the level of acquisition of several laboratories of a specific TMC
- Verification of the statistical control of TMC in a group of laboratories
- refinement of the prescribed terms of the characteristics of the bias of methods;
- attestation of standard samples of content and properties, as well as samples for control.

Participation of the testing laboratory in the ICT rounds is one of the main methods for determining the effectiveness of the test method along with calibration using standard samples, comparison of the results obtained with other methods, systematical assessment of the factors that influence the result, assessing the uncertainty of the results on the basis of scientific understanding of the theoretical principles and practical experience.

Depending on the ICTs coordinators, they are divided into national and international ones. Coordinators for the international rounds are internationally recognized organization or centers and they are authorized to carry out the relevant work. The rounds carried out by national coordinators within Ukraine are also defined as professional testing.



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http://www.european-accredition.org

5.3 Documents and their management

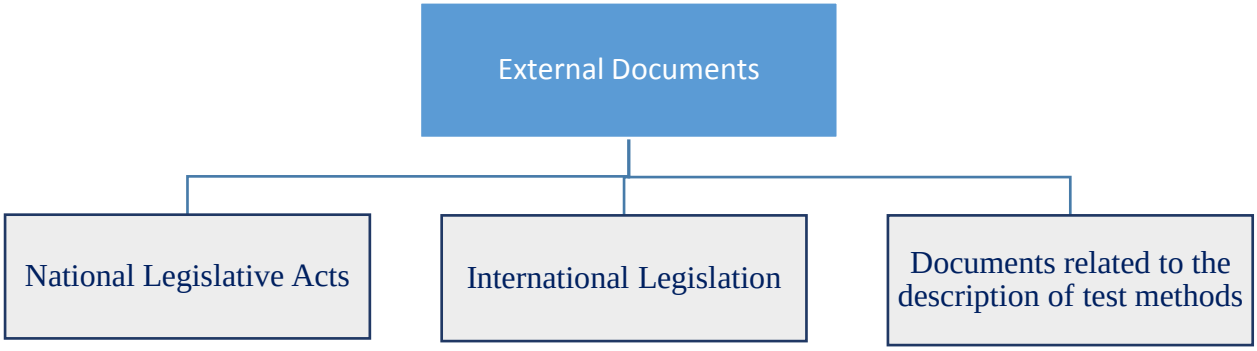
Each laboratory elaborates standard operating procedures (SOPs) for document management in order to ensure compliance with the requirements of the Document Management Standard ISO/IEC 17025: during its elaboration, identification, approval, publication, modification and desamination in departments (sectors) of the laboratory, as well as under time of storage and archiving.

The main tasks of the documentation of the quality control (management) system is a description of all necessary processes of the control and analytical laboratory sufficiently and the reporting of information to the staff, simplifying data registration and monitoring the work of the control and analytical laboratory. The quality management system documents should meet the internal needs of the executives and laboratory managers in their daily activities, as well as provide evidence for verifications that the relevant requirements of the external regulations are taken into account and put into practice.

Document managementis the process of elaboration of documentation, its identification, approval, publication, variation and desamination in departments (laboratories), as well as storage and archiving.

Document is the presentation of the required information on paper or in any other medium (electronic, photographic, analogue and digital).

The external documentation affects the internal documentation of the quality management system.



The internal documentation of the laboratory must include:

– Quality Instructions

– Standard operating procedures (SOP);

– operational instructions;

– job description;

– Test procedures;

– Documentation on data on the operation of the quality management system and technical data (logs, cards, forms, protocols, etc.).

Quality manual is a basic document that describes the various elements of the quality management system to ensure the quality of the research results obtained by the laboratory. The Quality manual should describe the policy of the quality, program, system, procedure, and instruction which is necessary for a brief description of the processes of the laboratory and confirmation of compliance of these processes with the requirements of the external regulatory act.

Standard Operating Procedures (SOPs)are written manuals for managing/conducting activities in all areas of production or quality control of products approved by managers.

The objective of the SOP is to provide staff with instructions on what to do and when to do.

SOPs should describe all the basic processes that are listed in the sections of the Quality Manual, and cover more narrow range of activities: internal marking, quarantine and storage of materials; installation, adjustment of tools and equipment; installation and validation of equipment; research materials, description of methods and equipment used in this process; sampling; exploitation, cleaning of premises, sanitary measures, technical safety; calibration of analytical instruments; control of environmental indicators; qualification requirements, training, hygiene of the staff; preparation and control of standard samples, etc. SOPs are step-by-step instructions, they have a specific purpose, scope, responsibilities, execution procedures, and necessary links. SOPs provide a sustainable, identical, correct and reproducible performance of work.

Benefits of SOPs:

1. Ability to ensure uniformity, conformity and process control and to ensure that the procedure is carried out in the exact same way each time, regardless of the operator.

2. A standard and general base for staff training is created; adapting new employees to the workplace becomes faster and more efficiently.

3. A negative impact of the "personal" factor is minimized (experience, knowledge, skills and abilities of a certain employee are not of much importance); the probability of false actions and behavior is minimized as well).

4. The unchanged and stable level of service provision is maintained.

5. Conditions for implementation of quality management systems are created in the laboratory.

Preparation of the SOP is assigned to the most competent laboratory staff which is directly involved in the carrying out of the described procedures, using regulations, methodological support, monographs, scientific publications.

Recommended list of SOPs in the testing laboratory

- Procedures for document management in the laboratory;
- Procedure for drafting SOPs;
- Documentation (registration) of laboratory activities;

- Occupational safety operations;
- Disposal of biological waste;
- Environment monitoring;
- Cleaning the premises of the laboratory;
- Control for laboratory equipment;
- Maintenance of equipment;
- Order, receipt, identification, marking, processing and storage of reagents, equipment and devices;
- Conducting the internal audit of laboratory work
- Maintain the confidentialityof results;
- Measures in case of non-conformity;
- Personnel actions, including further training, clothing and hygiene;
- Work with the laboratory information system (if there's any)
- Validation / verification of equipment and research methods;
- SOP for a certain study method.

The current documents of the laboratory may be amended or supplemented due to the following reasons:

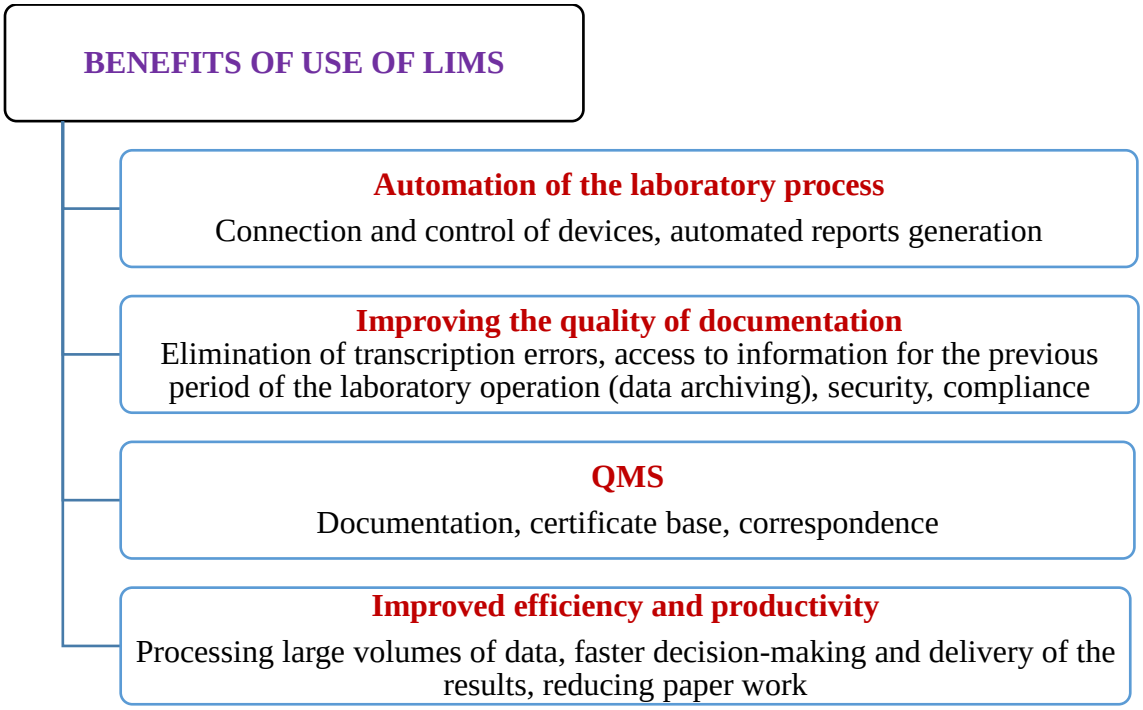
- on the results of internal audit;
- on the results of audit;
- on the results of the analysis made by the management;
- customer complain;
- the employee's initiative to further develop the quality management system

Laboratory Information Management System (LIMS) is a professional software for managing of the laboratory work flows and documents, providing reliable information about test results and optimization of management (input, tracebility, documentation) of this information in order to use it to make on-time management decisions.

Laboratory Information Management Systems must have functional capabilities to meet the requirements of Good Laboratory Practice (GLP), Good Automated Laboratory Practice (GALP), Good Manufacturing Practice (GMP), ISO 17025, ISO 2859, ISO 3951 standards and other regulatory acts.

Typical software includes a set of features:

1. Laboratory data (methods, procedures, personnel, work processes, SOPs).
2. Samples (registration, testing, processing, login, archiving, standards, reference).
3. Tools (maintenance, calibration, service, workload, planning).
4. Resources (work with equipment and staff load, time, analysis costs).
5. Quality (specifications, limitations, audit, verification, etc.).
6. Communications (infrastructure, suppliers).
7. Security (secure systems - users, passwords, groups, authorization, logins).
8. Validation (integrity and sequence of data, regulatory compliance, validation).



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5.4 Metrology at the laboratory

5.4.1 Structure of the metrology service, objectives and basic principles of the work of the services

The central executive body (hereinafter as CEB), which ensures the formation of the state policy in the field of metrology and metrological activity, carries out the state administration of ensuring the unity of measurements.

The powers of the CEB are:

- providing regulatory and legal regulation in the field of metrology and metrological activity;
- organization of basic research in the field of metrology;
- ensuring the functioning and improvement of the national reference base;
- developing or participating in the development of national scientific and technical programs related to ensuring uniformity of measurements;
- representation and participation in the international, European and other regional metrology organizations;
- exercise of other powers, defined by the laws and assigned to it by acts of the Cabinet of Ministers of Ukraine.

b. CEB which implements the state policy in the field of metrology and metrological activity, the powers of which include:

- coordination of activities to ensure the functioning of the metrological system of Ukraine;
- organization of functioning and preparation of proposals for improvement of the national reference base;
- authorization to carry out verification of legally regulated measuring equipment in use;
- exercise of other powers, defined by the laws and assigned to it by acts of the Cabinet of Ministers of Ukraine.

c. CEB that implements the state policy in the field of metrological supervision;

d. Scientific metrological centers are determined by the Cabinet of Ministers of Ukraine among state enterprises, institutions and organizations that implements state policy in the field of metrology and metrological activity and create, improve, keep and apply national standards.

The regulations on scientific metrology centers are approved by the CEB, which implements the state policy in the field of metrology and metrological activity. Scientific metrological centers defined by the regulations and legal acts on them:

1. carry out basic scientific research in the field of metrology, as well as perform works related to the development and implementation of state programs in metrology and the concept of development of the metrological system of Ukraine;
2. carry out applied scientific researches and carry out research works related to the creation, improvement, storage, verification, application of national standards, creation of systems of transfer of size of units of measurement;
3. participate in the drafting of technical regulations, other legal acts, as well as normative documents in the field of metrology and metrological activity;
4. carry out coordination and scientific and methodological support of works on ensuring the uniformity of measurements in the relevant fields of activity;
5. carry out the assessment of conformity of measuring equipment;
6. carry out calibration and verification of measuring equipment;
7. carry out measurements in the field of legislatively regulated metrology;
8. keep an information fund in the directions of their activity;
9. carry out international cooperation on issues within their competence.

Scientific metrology centers under contracts with legal and individual entities may perform other works (provide other services) related to ensuring uniformity of measurements.

e. state enterprises belonging to the sphere of management of the central executive body implementing state policy in the field of metrology and metrological activity and conducting metrological activities in regions and Kyiv (hereinafter metrological centers);

f. The Single Time and Reference Frequency Service performs cross-sectoral coordination and execution of works aimed to ensure the unity of time and frequency measurements and determining the Earth's rotation parameters and providing time-frequency information to consumers in the sphere of economy, science and defense, as well as legal and individual entities, including the provision of information to ensure the use of a single accounting time.

Reference Standards of Composition and Properties of Substances and Materials Services performs cross-sectoral coordination and performs work related to the development and implementation of standards of composition and properties of substances and materials.

The Standard Reference Data on Physical Constant and Properties of Substances and Materials performs cross-sectoral coordination and provides work related to the development and implementation of standard reference data on physical constant and properties of substances and materials.

Objectives and basic principles of the activities of the above mentioned services are determined by the provisions of the Cabinet of Ministers of Ukraine.

g. metrological services of central executive bodies, other state bodies, enterprises and organizations can create metrological services for carrying out works (providing services) for ensuring uniformity of measurements in certain areas of activity. The structure, functions, rights and responsibilities of these services are governed by the provisions for such services, which are approved by the heads of these bodies, enterprises and organizations. The typical provision is approved by the CEB, which ensures the formation of state policy in the field of metrology and metrological activity. Enterprises and organizations working in the field of regulatory metrology are required to create metrological services or appoint persons responsible for ensuring uniformity of measurements.

h. Bodies for conformity assessment of measuring instruments and calibration laboratories.

i. metrological support of the activity in the sphere of defense of Ukraine is carried out with the attention to the peculiarities determined by the Cabinet of Ministers of Ukraine.

5.4.2 Providing competence of metrological laboratories of enterprises in modern conditions

There are no requirements for certification of measuring and calibration laboratories in the Law of Ukraine "On metrology", as regulated by the requirements of the previous Law of Ukraine" On metrology and metrological activity"dated 11.02.1998 № 113/98-BP. The procedure for attestation of these laboratories was regulated by the "Procedure for attestation of the main and basic organizations of metrological services of central executive bodies" (hereinafter - "Procedure "). All the issues of checking the competence of laboratories, the form and content of the required documents were identifiedIn this "Procedure". Ukrainian measurement laboratories actively used a certification procedure that helped them to ensure the quality of measurements and confirmed the competence of the laboratory. Mainly recognized as a means of confirming the competence of laboratories which perform metrological activity (hereinafter - metrology laboratories) at the international and national levels is compliance with the requirements of DSTU ISO / IEC17025: 2017. Metrological activity refers to activities related to ensuring uniformity of measurements (Article 1 of the Law on Metrology). Therefore, according to modern requirements, metrology laboratories include reference, calibration and measuring laboratories. They can have specific names at the enterprise, but the main thing is metrological activity they carry out.

According to the Law "On metrology", reference laboratory is an enterprise or organization or their separate subdivision, which performs verification of measuring equipment; calibration laboratory is an enterprise/organization or their separate unit that performs calibration of measuring equipment. A measurement laboratory is an enterprise/organization, their separate divisions, and individual entrepreneurs who carry out specific measurements that are not related to the measurement of the conformity of products, processes, and services.

Competence of metrology laboratories is an officially confirmed ability of a laboratory to perform metrological activities in a specific field on the basis of compliance with the requirements of the measurement procedures and on the basis of appropriate resources. Regardless of the name of the laboratory, its competence is always determined by appropriate criteria and is ensured the following resources in accordance with the field of activity of the laboratory:

- competent staff;
- premises and environmental conditions;
- technical equipment;
- methods of work;
- quality management system.

Specific requirements for the criteria for competence are set out in those documents that specify the requirements directly for the competence of the laboratories or for the work performed, and for which competence is evaluated. Basic requirements for the competence of laboratories are regulated by DSTU ISO/IEC17025: 2017. Confirmation or recognition of competence may be exercised by the first, second and third parties. The first party is the enterprise that ensures the quality of the laboratory's performance of testing or calibration of the measurement equipment on the basis of providing it with the necessary resources and conducting an internal audit of its activities (internal audit). The other party is the customer of the products, which may set up requirements for conducting metrological control of the measurement equipment, as well as requirements for checking the competence of the laboratory. A third party is an independent organization that verifies and confirms the competence of a laboratory to test or calibrate measurement equipment.

No matter which party inspects the laboratory, the criteria are the same as those mentioned above. And they should always meet the requirements of the test/calibration techniques used for metrological control of specific measurement equipment. First of all, there must be qualified staff whose competence is confirmed by education, readiness for work and skills. Personnel charged with product testing, equipment calibration must have experience, know and comply with test/calibration requirements, be able to carry out and process test/calibration results. Each 5 years of training for the staff is required, as well as a periodic internal audits of the quality of work performed is needed.The requirements for the premises and environmental conditions in the laboratories must also meet the requirements of test/calibration techniques.

The premises must also meet fire safety, sanitary and labour safety requirements. First of all, environmental conditions include the ambient temperature requirements. Deviation of temperature beyond the set limits during measurement is not allowed, as it can lead to incorrect results of determining the suitability of the equipment due to the appearance of additional components of uncertainty and affect the reliability of the results. It applies to all influential factors identified in the methods.

One aspect of measuring / testing quality assurance is the participation in rounds of interlaboratory comparative testing (hereinafter referred to as ICT). ICTs are widely used for a variety of tasks and they are use all around the world. Typical tasks of the ICT are:

- evaluation of the characteristics of laboratories for carrying out specific tests or measurements and constant monitoring of them;
- identification of problems in laboratories related to the use of incorrect measurement /test procedures, insufficient effectiveness of training and personnel management and their elimination;
- establishment of efficiency and comparability of test/measurement methods;
- ensuring maximum confidence of the customers;
- identification of differences between laboratories;
- training of metrology laboratories participating in rounds of ICT based on the results of reconciliation check;
- confirmation of the declared uncertainty;
- evaluation of the characteristics of the method;
- assigning values to standard samples and assessing their suitability for use in specific measurement/testing procedures;
- support for the established equivalence of measurements performed by national metrology institutes through key reconciliation checks and supplementary reconciliation checks, conducted on behalf of the International Bureau of Weights and Measures (BIPM), and by regional metrology organizations cooperating with them.

Laboratory tests are conducted in the form of:

- tests which are technical operation intended to determine or control one or more of the characteristics or parameters of a test object, which are performed in accordance with the established procedure of interlaboratory testing;

Interlaboratory tests are conducted in the form of:

- interlaboratory comparisons of measurement results;
- mutual interlaboratory experiments to establish reference values;
- interlaboratory reconciliation checks
- interlaboratory comparative tests.

Participation in interlaboratory testing promotes the improving the technical level of the laboratory and the competence of its staff.

In international practice, during measurements and tests considerable attention is paid to ensuring the uniformity of measurements and to such components as traceability of measurements, calibration of measuring equipment, estimation of accuracy of measurements and validation and verification of measurement methods.

The general competence criteria for calibration laboratories are the same as for other laboratories.

Technical equipment which is use for testing/measurement are standard samples, standard reference and quality control materials, calibration standards, and ancillary equipment, and must be in the test/calibration format. The equipment calibration can be found in the article 6.4 of DSTU ISO \ IEC 17025: 2017 General requirements for the competence of testing and calibration laboratories. Also in Ukraine documents such as DSTU OIML D23: 2008 Metrology. Principles of metrological control of calibration equipment (OIML D23: 1993, IDT) and DSTU OIML D8: 2008 Metrology. Standards. Selection, recognition, application, storage and documentation (OIMLD8: 2004, IDT) concerning working standards are harmonized with the international ones are in force. Working standards are not related to the objects of legally regulated metrology, so the enterprise/laboratory determines he calibration of standards and as the intercalibration interval for working standards is recommended, so the enterprise/laboratory independently determines this interval on the basis, for example, standards of the International Organization of Legal Metrology systems implemented in Ukraine as national standards DSTU OIML D23: 2008 Metrology. Principles of metrological control of calibration equipment (OIML D23: 1993, IDT) and DSTU ILAC-G 24 / OIML D 10: 2013 Metrology.

Guidelines for determining the intercalibration intervals of measuring equipment (ILAC-G 24 / OIML D 10: 200, IDT). An enterprise/laboratory decides whether verify or calibrate standards based on the following:

- during verification the conformity of the metrological characteristics of the working standard to its operational documents is established. You can use state calibration schemes to establish the discharge of a working standard for its selection by calibration method. The refusal of the metrology center to calibrate the standard is not justified, because the enterprise/laboratory as the customer sets out its requirements and the requirements of the customer must be fulfilled. Issuance of a certificate of calibration of the standard as a working measuring equipment is also not an obstacle for the application of the standard, but in this case a verification protocol should be ordered to establish the traceability of the working standard to the primary standard;

- the calibration sets the deviation of the working standard of the enterprise from the values realized by the standard used during the calibration, as the deviation and uncertainty of the measurement of these deviations. The enterprise must directly (precisely) determine the suitability of the calibration standard for verification on the measuring equipment.

If an enterprise intends to calibrate measurement equipment, it should decide whether the first, second, or third party must have accreditation or evaluation of the competence of the calibration laboratory. In this case, it is obligatory to introduce accreditation in the DSTU calibration laboratory for accreditation and to apply for this procedure to the National Accreditation Agency of Ukraine.

In case when accreditation is not envisaged, it is mandatory to have a documented traceability of its standards to national standards. This means that working standards must be calibrated in accredited calibration laboratories with a relevant field of activity.

Thus, metrology laboratories should make every effort to ensure their competence. Modern requirements for quality assurance of metrology work imply that there are different approaches to establishing the competence of laboratories, which gives freedom to enterprises to solve this issue, at their discretion and/or at the request of customers of products.

5.4.3 Quality management system of the laboratory, documents

The purpose of implementation of the quality management system (QMS) in the organization is to confirm the activities in accordance with the requirements of regulatory documentation (standards, norms, etc.), which gives confidence to customers in obtaining quality appropriate testing services.

In our opinion, the most effective in creating and implementing QMS in any organization are the requirements set out in the ISO 9000 standards "Quality management systems", the application of which allows to achieve the following advantages:

- better understanding and consistency of quality activities in an organization;
- guarantees of continuous use of the quality management system in general;
- improving the documentation system
- raising staff's understanding of quality aspects;
- increase of labor productivity, reduction of costs;
- creation of a basis for continuous improvement of activity.

However, compliance of the metrology laboratory (measuring, calibration, analytical) with the requirements of ISO 9001 "Quality management systems - Requirements" does not yet guarantee it to obtain reliable results. A laboratory is a complex system that involves many elements: staff, infrastructure, measurement techniques, processes, equipment, working methods, documentation. System complexity requires all elements to work properly and in a coordinated way. The main objective of the laboratory quality system is to guarantee the accuracy, reliability and timeliness of presentation of test results, analysis. Today there are many developments in the field of laboratory quality management. The main international standard is ISO/IEC 17025 "General requirements for the competence of testing and calibration laboratories", which set requirements for both the management system and its technical competence. The requirements for the quality system in the two above mentioned standards are similar, but their formulations in ISO/IEC 17025 are more specific regarding the work of the laboratory and therefore differ from ISO 9001.

The compliance of the laboratory with the requirements of ISO / IEC 17025 means that the laboratory complies with the requirements of both technical competence and management system and thus guarantees customers compliance with the conditions of obtaining reliable test results.

Besides the above mentioned international normative documents of the laboratory quality management system the most well-known are:

- ISO/IEC 17043 "Conformity assessment – general requirements for proficiency testing";
- ISO 13528 "Statistical methods for use in proficiency testing by interlaboratory comparison"
- ISO Guide 34 "General requirement for the competence of reference materialproducers"
- ISO 10013"Guidelines for quality management system documentation".

These documents are accepted as national standards of Ukraine:

- DSTU ISO 9001 "Quality Management Systems. Requirements";
- DSTU ISO/IEC 17025 "General requirements for the competence of testing and calibration laboratories"
- DSTU ENISO / IEC 17043 "Conformity assessment. General requirements for professional level verification";
- DSTU ISO 13528 "Statistical methods for professional-level verification through interlaboratory comparisons";
- DSTU N ISO/IECGuide 34 "General requirements for developers of standard models";
- DSTU ISO/TR 10013 "Guidelines for the development of a quality management system".

If a laboratory develops and implements QMS in accordance with its field of concern, it may be accredited in an international or national system. Criteria that are developed for most accreditation systems include quality system requirements and are in line with international standards.

However, accreditation should not be the ultimate goal of a laboratory, even in terms of competition in the laboratory services market. The QMS of the laboratory should be constantly maintained and upgraded to guarantee the quality of services and to confirm

5.4.4 Laboratory documentation

The main "product" of the laboratory's activity is the information presented in the form of data and documents that are an element of QMS. In order to ensure the availability, reliability, timeliness of the laboratory documentation, it must be constantly monitored by the documentation management system, either electronically or in paper form, or their combination. Documentation management is the systematic application of established rules to both a single document and a group of documents.

The documentation that regulates the work of the laboratory consists of various types of documents: organizational, administrative, correspondence, legal, personnel, accounting, forms of data registration. Most of them are regulatory and technical documents, which mat be external (laws, technical regulations, technical requirements, standards of technical requirements for the product to be tested and its test and measurement methods, equipment passports, etc.) and internal (instructions, rules, SOPs, methods, test procedures) that are developed in the laboratory.

Documents belonging to the same type in most cases have the same management scheme.

Main steps in managing the documentation of the laboratory:

- Development. Before development of a document, you need to define the purpose, content requirements, involved people.
- Identification. Each document must be tied to a place and time. Identification rules allow you to identify definitely the document and track its movement.
- Analysis. Before a document kicks in, it needs to be analyzed for compliance with the goals of the creation, the full coverage of the problem.
- Coordination. Required to verify the justification of the information presented in the document.
- Dessimination. It is necessary to make sure that the document is delivered to stakeholders.
- Accessibility. It means that staff can use the document at the right time and place to perform their duties.
- Storage (electronically or in hard copy). It is important to ensure that the document is secure and can be restored.
- Review. It is performed to keep the documentation up to date at specified intervals.

According to the level of the hierarchy, the structure of documents repeats the levels of laboratory management: strategic, tactical, operational. The level of the hierarchy determines the status of the documents, the scope of their activities and allows to share responsibility for the actions with the document.

Laboratory Documentation is a QMS element with a help of which communication which occurs inside and outside the laboratory must always be relevant, sufficient and accessible for staff.

5.5 The audit and the accreditation bodies

Internal audit. Objectives of the audit

Independently of standards applied by laboratory / enterprise, the internal audit corresponds to the following objectives:

1. To verify the conformity to requirements applied by laboratory / enterprise (norms, regulatory texts, terms of references, specifications, clients …).
2. To verify if organizational provisions (processes) and operational provisions (procedures, instructions...) are established, known, understood and applied.
3. To verify the efficiency of audited area, it means its capacity to achieve the objectives.
4. To identify the ways for the improvement and the recommendations for guiding the laboratory / enterprise to the progress.
5. To enhance the observed good practices in order to encourage the team and to capitalize its practices at the laboratory / enterprise.

Perimeter of the audit.
The internal audit concerns all activities carried out at the laboratory / enterprise (organization, finances, human resources, products....).

In addition to five objectives mentioned above some objectives of the verification of costs efficiency, good social practices, environment respect, respect of laws related to labor hygiene and security can be added to the internal audit upon the requirement of the general administration.

Method
In order to achieve these objectives some basic rules should be respected during the audit :

- To adopt an objective attitude without personal extrapolation.
- To avoid to be impacted by your own technical origins.
- Stay natural and attentive.
- To ask questions exhaustively.
- To distinguish exceptional and current facts.
- To observe the functioning of audited unit.
- Think about results not about means.
- To listen to interlocutor, don’t outstrip him.
- To observe well the interlocutor (voice, emotion, gests).
- To distinguish opinions.

Finally the audit is a practice based on risk approach. It is convenient to identify potential or proved risks that can be conditioned by the situation or its performance. It will allow to put a priority on the improvements.

Auditors should be trained as for the audit techniques, norms applied by the laboratory / enterprise, editing of the audit report and constated deviations.

The internal audit should be planned regularly (minimum once per year) and cover all standards. So, it is always preferable to divide the audit in several parts.

Certification

This the provision by an independent body of written assurance (a certificate) that the product, service or system meets specific requirements.

Certification is the confirmation that a client (also called a Certificate Holder) operates in line with a set of requirements, which are defined by a standard setter. **Conformity Assessment Bodies** (CABs), also commonly called **Certification Bodies (CBs),** perform these certification activities. To perform certification, **CB** must be accredited by an accreditation body.

Certification can be done according to following standards and norms such as: ISO 9001, ISO 22 000, ISO 14 000, BRC, IFS

Accreditation is the formal recognition by an independent body, generally known as an **accreditation body**, that a certification body operates according to international standards.

But also some standards can be accredited: **e.g laboratory standard ISO 17025.**

There is **usually only one** accreditation body for each country (e.g., UKAS for the United Kingdom, PCA for Poland, etc), while there are several certification bodies operating in each country – ranging from small local certification bodies to large multinational corporations like SGS, BSI, DNV, BV, Dekra, TUV, etc.

Accreditation is a third-party evaluation and demonstration of competence.

It is the assessment of independence, objectivity and competence for the performance of defined activities.

Accreditation means increased confidence in the observance of required level of quality of the provided services.

Accreditation is a public authority activity. It is the last level of public authority control.

It is designed to ensurethat conformity assessment bodies (e.g. laboratories, inspection or certification bodies) have the technical capacity to perform their duties.

Accreditation can be used in regulated sectors and voluntary areas. Accreditation increases trustin conformity assessment. It also reinforces the mutual recognition of products, services, systems, and certification bodies across the EU.

Accreditation in EU:

- Regulation **(EC) No 765/2008** of the European Parliament and of the Council of 9 July 2008 setting out the requirements for accreditation and market surveillance relating to the marketing of products and repealing Regulation (EEC) No 339/93
- **EN ISO/IEC 17011:2006** standard *Conformity assessment- General requirements for accreditation bodies accrediting conformity assessment bodies*, as well as requirements resulting from the signed multilateral agreements, such as: International Accreditation Forum, International Laboratory Accreditation Cooperation, European Co-operation for Accreditation

Requirements for accreditation are set in Regulation 765/2008.

The main principles of accreditation are:

-One accreditation body per EU country (it is possible however to use another country's national accreditation body)

-Accreditation is a public sector activity and a not-for-profit activity

-There is no competition between national accreditation bodies

-Stakeholders are represented

-Accreditation is the preferred mean of demonstrating technical capacity of notified bodies in the regulated area.

The national accreditation bodies are obliged periodically undergo peer evaluation by EA – European co-operation for Accreditation (as required by Article 64 of Commission Regulation EU No 600/2012) and therefore, Member States shall accept the accreditation certificates of verifiers accredited by these national accreditation bodies, e.g :

FRANCE : Comité français d'accréditation (COFRAC)

GERMANY: Deutsche Akkreditierungsstelle GmbH (DAkkS)

POLAND: Polskie Centrum Akredytacji – Polish Centre for Accreditation (PCA)

UKRAINE: National Accreditation Agency of Ukraine (NAAU)

Laboratory accreditation

Laboratories can be accredited in follwing areas according special norms:

- Testing -EN ISO/IEC 17025
- Medical examinations -EN ISO 15189
- Calibration -EN ISO/IEC 17025

Laboratory accreditation is the formal recognition of an organisation's competency to perform certain specific tests, examinations or instrument calibrations.

To be accredited, quality control and quality assurance programs must be in place for all aspects of the laboratory operations.

All facilities and equipment should assure adequacy for intended applications.

The lab also must be participating in a proficiency analytical testing program.

Without accreditation, it is unclear what standards and quality systems are actually employed by the laboratory.

To maintain this recognition, laboratories are re-evaluated periodically by the accreditation body to ensure their continued compliance with requirements, and to check that their standard of operation is being maintained.

Clients check for what specific tests or measurements labs are accredited.

This information is usually specified in the laboratory's scope of accreditation, issued by the accreditation body.

The description in the scope of accreditation also enables the customers to find the appropriate laboratory or testing service.

Laboratory accreditation bodies publish the scopes of accreditation for their accredited laboratories in either hardcopy directories or on the internet.

A regular assessment by an accreditation body checks all aspects of lab's operations.

Areas for improvement are identified and discussed, and a detailed report is provided at the end of each visit.

Where necessary, corrective actions are monitored by the accreditation body so the facility is confident that it has taken the appropriate action.

Accreditation is an effective marketing tool for testing, calibration and measurement organisations.

Through a system of international agreements accredited laboratories receive a form of international recognition, which allows their data to be more readily accepted on overseas markets.

This recognition helps to reduce costs for manufacturers and exporters that have their products or materials tested in accredited laboratories, by reducing or eliminating the need for retesting in another country.

5.6 The International Organizations in the field of laboratory practice: Association of Official Analytical Chemists, Microbiologists, ISO, European Norms

AOAC INTERNATIONAL brings together government, industry, and academia to establish standard methods of analysis that ensure the safety and integrity of foods and other products that impact public health around the world.

As a leader of analytical excellence, AOAC INTERNATIONAL advances food safety, food integrity, and public health, by bringing together members, organizations, and experts dedicated to developing and validating standards, methods and technologies, of global relevance.

AOAC International was established in 1884 as the Association of Official Agricultural Chemists. Later, the Association of Official Analytical Chemists name was adopted to better reflect additional safety interest areas. Today, the organization's legal name is AOAC INTERNATIONAL in an effort to reflect the global nature of impact.

The Official Methods of AnalysisSM (OMA) program is AOAC INTERNATIONAL's premier methods program. This methods program is distinct from the annual publication, Official Methods of Analysis of AOAC INTERNATIONAL. Approved methods undergo rigorous, systematic scientific scrutiny to ensure they are highly credible and defensible—and can be used with confidence by industry, regulatory agencies, research organizations, testing laboratories, and academic institutions.

Official Methods of Analysis℠ (OMA) is the most comprehensive and reliable collection of chemical and microbiological methods and consensus standards available. Many Official Methods have been adopted as harmonized international reference methods by the International Organization for Standardization (ISO), International Dairy Federation (IDF), International Union of Pure and Applied Chemistry (IUPAC), and the Codex Alimentarius Commission.

ISO (International Organization for Standardization)

ISO is the world's largest developer and publisher of international standards, and ISO standards are applicable to many kinds of organizations, including clinical and public health laboratories.

ISO is a network of the national standard institutes of 157 countries, one member per country, with a Central Secretariat in Geneva, Switzerland, that coordinates the system. It is a nongovernmental organization and it forms a bridge between the public and private sectors. On the one hand, many of its member institutes are

part of the governmental structure of their countries or have been mandated by government. However, many members have roots uniquely in the private sector, having been set up by national partnerships of industry associations. Therefore, ISO enables a consensus to be reached on solutions that meet both the requirements of business and the broader needs of society.

The work of preparing standards is conducted by ISO technical committees. Each member body has the right to be represented on the committees. International organizations, both governmental and nongovernmental, also take part in the committee activities. Draft international standards adopted by the

technical 1 ISO/IEC Guide 2:1996 (EN 45020:1998) Standardization and related activities—general vocabulary. Geneva, International Organization for Standardization, 1996.

126 Laboratory Quality Management System committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75% of the member bodies casting a vote.

CLSI (Clinical and Laboratory Standards Institute)

CLSI is a global, non-profi t, standards-developing organization that promotes the development and use of voluntary consensus standards and guidelines within the health care community. CLSI documents are developed by experts working on subcommittees or working groups under the direction and supervision of an area committee. Development of CLSI standards is a dynamic process. Each CLSI area committee is committed to producing consensus documents related to a specific discipline, as described in its mission statement.

CEN (European Committee for Standardization)

CEN was founded in 1961 by the national standards bodies in the European Economic Community and associated countries. The general terms include openness, transparency, consensus and integration. Formal adoption of European Standards is decided by a weighted majority vote of the CEN national members and is binding on all of them. The responsibilities are shared between 30 national members from each country, 7 associate members and 2 counsellors, as well as the CEN Management Centre in Brussels.

WHO (World Health Organization)

WHO has developed several standards for disease-specifi c diagnostic laboratories. One example is polio, where accreditation is required in order for a laboratory to participate in the Polio Network for Eradication of Poliomyelitis. Seven criteria have been selected, including a minimum activity of 150 samples annually, successful participation in proficiency testing, and accuracy and timeliness of reports of cases to the network

The EPTIS database is a joint publication of a worldwide consortium of organizations. The members of this consortium are all involved in PT in one way or another and play a prominent role in national or international quality infrastructures. There are coordinating members, who play a more active role in EPTIS, and supporting members who provide a more intangible backup. Overall coordinator of EPTIS is the Federal Institute for Materials Research and Testing (BAM) in Germany.

The European PT Information System or EPTIS was the name of an EU project that aimed at making an inventory of regularly operated PT schemes in the participating countries. The project partners were major national laboratories and institutes.

EPTIS is a database established in 2000 in Europe, which initially brought together proficiency testing providers from 16 countries in the region and currently has a registry of more than 800 programs. Since then, EPTIS coordinators have strived to widen their field of action to include other countries and regions. Said initiative has the support of important international institutions which represent the testing and calibration laboratories, as well as the International Laboratory Accreditation Cooperation (ILAC) and other accreditation cooperations.

Through an IAAC project, financed with resources of the Physikalisch-TechnischeBundesanstalt (PTB) and under the leadership of the IAAC Laboratories Subcommittee, the proficiency testing providers of Canada, Mexico, Caribbean, Central America and South America shall be integrated into EPTIS, thus helping laboratories to have access to information regarding the programs available in the region.

Chapter 6. Genetic analysis

6.1 Polymeraze chain reaction (PCR) as a method of genetic – molecular analysis

PCR is an experimental method based on the principle of exponential increase of copies number of a parcel of ascendant DNA – matrix by means of enzymes in vitro. Herewith the only parcel limited by primers is copied, it happens only in the case if it is present in the analysed sample of biological material. The PCR method was elaborated by Carry Mullis in 1983; ten years late he received the Nobel prime in the field of chemistry of this discovery [Glazko V.I., 2001; 2003].

The polymeraze chain reaction passes three stages with the corresponded temperature and time parameters that are automatically chained in programmed thermostat – amplificator.

The first stage is the thermic denaturation: destruction of double chain DNA molecule at the temperature 93-95 °C DNA creation of two single chains. For the complete denaturation of DNA with the purpose to increase the outcome of PCR product the primary denaturation is done at the temperature of 95 °C ducircular 2-5 minutes.

The second stage is hybridization: joining of primers to complementary succession of DNA on opposite DNA chains in the limits of specific parcel. The hybridization of primers occurs according to the complementaruty rules of Chargaff, where it is indicated that in DNA bichain molecule opposite to adenine (A) there is always tymine (T), DNA opposite to hyanine (H) is always cytosine (C). One of the most important parameters of PCR is the temperature of primers hybridization (T_A), that, as a rule, depends on primers structure DNA is averagely 55–65 °C that is 3–5 °C lower that the temperature of their melting.

The third stage is elongation – the systesis of complementary DNA chain by *Taq*–polymeraze at the temperature 72 °C. The temperature cycle of amplification repeates many times DNA ducircular 25 – 40 cycles mixture accumulates specific short synthesized DNA fragments – amplocanes in a quantity sufficient for their visualization [Mullis K, 1986].

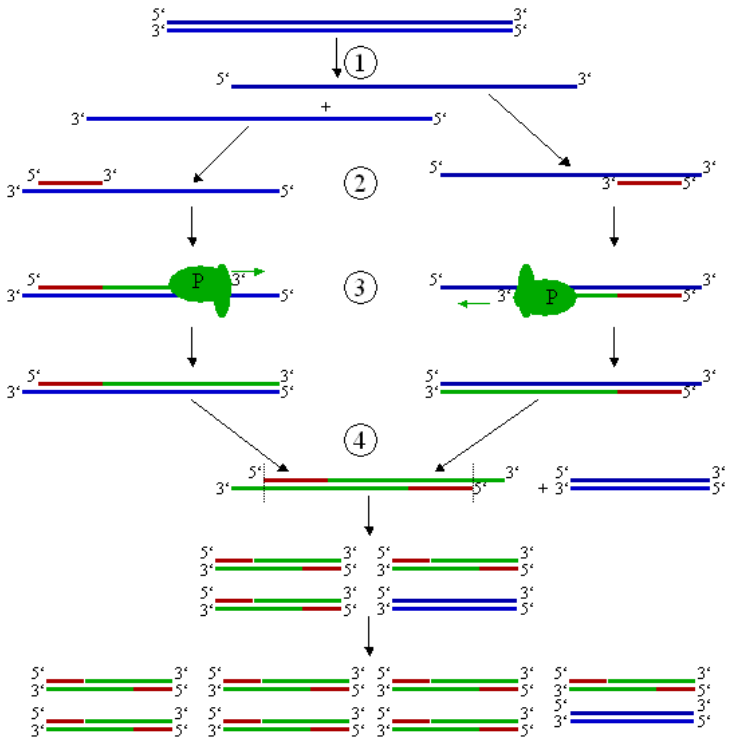


Figure 6.1. Scheme of PCR cycle

(1) denaturation at 94-96 °C; (2) hybridization at 56-65 °C; (3) elomgation at 72 °C; (4) end of first cycle. Two obtained DNA chains are the matrix for next cycle, that is why the quantity of matrix DNA doubls ducircular every cycle.

Specific fragments limited by primers at their ends, appear in the end of second cycle for the first time, accumulate in the geometric progression DNA very soon begin to dominate among other amplification products [Глазко, 2006].

The growing of necessary product in the geometrical proression (expotentially) is limited by reagents qualtity, imhibitors presence, formation of auxiliary products. The accumulation of specific PCR products in mixture is shown by the following formula:

$A = M (2^n – n – 1) \sim 2^n$,

where A is a quantity of specific (limited by primers) products of amplicons reaction; M is an initial number od DNA-targets; n is a number of amplification cycles; ~ is a sign of mathematical equivalence of some process (in our case it is an amplification process).

At last cycles of reaction the formation slows, it is called “plateau effect». The term “plateau effect” is used for describing the process of accumulation of PCR products at last amplification cycles when amlificons number reaches 0,3-1 picomoles.

Depending on conditions DNA number of amplification reaction cycles, the moment of “plateau effect” obtaining is impacted by: substrats utilization (dNTP DNA primers); reagents stability (dNTP DNA enzyme); inhibitors number, including pirophosphates DNA DNA-duplexes; non-specific products or primer-dimers compiting for promers, dNTP DNA polymeraze; concentration of specific product DNA incomplete denaturation at high concentration of amplification products. The lower is the initial concentration of DNA target, the higher is the risk of reaction yield on plateau. This moment can happen before the quantity of amplification specific products become sufficient to be analyzed. It can be avoided by the optimization of conditions for polymeraze chain reaction carrying out, namely composition DNA concentration of reaction mixture components DNA amplification mode [McPherson M.J, 2000].

The stDNAard components of PCR-mixture are:

- DNA - matrix;
- mixture of forward DNA reverse oligonucleotide primers complementary to the ends of target fragment;
- thermostable DNA-polymeraze – enzyme catalysing the reaction of DNA polymerization. Polymerase used in PCR should keep its activity at a high temperature ducircular a long time, that is why the enzymes extracted from thermophile bacteria Thermus aquaticus (Taq-polymeraze), Pyrococcus furiosus (Pfu-pilymeraze), Pyrococcus woesei (Pwo-polymeraze) DNA others are used [Arezi B, 2003].
- Deoxynucleotidetriphosphates (dATP, dGTP, dCTP, dTTP);
- Іони Mg^{2+}, necessary for the work of polymeraze;
- Buffer solution providing necessary conditions for the reaction - pH, ion force of solution. It contains salts DNA bovine serum albumin.

The interpretation of PCR analysis results DNA the evaluation of PCR efficiency can be carried out by the separation of amplification products in agar gel by the horizontal electrophoresis method, by the capilar electrophoresis method or on computer monitor with the application of fluorescent (PCR – real time) sondes [Rebrykov D.V., 2009].

6.2 Requirements to the tools and the equipment

6.2.1 Description to the tools and the equipment with their characteristic and destination for laboratory works

A special set of necessdary laboratory equipment is defined according to functional destination of laboratory. The molecular-biological methods and the test – systems that will be used for reseaches should be taken into account as well as volumes of works that will be done.

A number of devices and consumables are used for analyses, they include also test-tubes and arrow-heads excluding the possibility of cross-contamination (pollution) of outcomes, of extracted nucleic acids (DNA/RNA) and products of polymeraze chain reaction (PCR).

For these purposes it is necessary to use:

- Thermostats with solid block;
- Single-use plastic test-tubes free of DNAses and RNAsas ;
- Arrow-heads with filetr for micro-pipettes free of DNAses and RNAsas ;
- Special containers for throwing used arrow-heads and test-tubes.

Pipettes, working and external surface of devices should be resistant to washing products, desinfectants and ultra-violet radiation.

When applying PCR method with hybridization and fluoriscent detection « according to fibnal point » a special detector of « Dzhyn » or « Ala1/4 » is used. It is helpful to install it in amplification room having connecte dit to computer with a special operational system according to installed equipment [МУ 1.3.2569-09.М., 2009].

Genetic analyser used for sequenation and fragment analysis is installed in a separate room for the detection and interpretation of results

Minimum lists of main equipment for genetic analysis laboratory

For material treatment and DNA/RNA extraction

According to the automatization level of nucleic acids extraction, the following methods are defined: manual methods, semi-automatic and completely automatic.

For the manual method of nucleic acids extraction we need:

1. Biological security box of class II and higher;
2. Centrifuge – Wortex;
3. Micro-centrifuge for 12 – 16 thousand rpm for test tubes with volume of 1,5 ml.
4. Solid thermostat with a range of work temperatures from 25 to 100 °C;
5. Vacuum aspirator with flask-trap ;
6. Spectrophotometer;
7. Set of automatic pipettes with changeable volume (5 mm^3, 200 mm^3 and 1000 mm^3).
8. Refrigerator for 2 – 8 °C with freezing compartment for – 20 °C;

Biological security box. It was elaborated for the protection of laboratory staff, environment and working materials against the impact of infectious aerosols and against the entecircular of particles that can appear at the work with materials containing infrctious components. Depending on the classification different levels of the protection against contamination (pollution) are proposed.

		
Picture 6.2 Biological security box (BSB) provides protection of staff and environment but doesn't protect products (samples …) against contamination.	**Picture 6.3 Biological security box (BSB) of class II** provides protection of staff, environment and protection against samples contamination due to recirculation of air through HEPA filter in laminar vertical flux inside the box.	**Picture 6.4 Biological security box (BSB) of class III** provides an absolute protection level that can't be reached in boxes of classes I and II. The box is hermetic and maintains low pressure providing additional guarantee of safe work.

Centrifuge - wortex

The centrifuge – wortex provides reproduction of the "Spin – Mix – spin" technology, destined "to drop" micro-volumes of reagents to bottom of test-tube (first centrifugalization – spin), further mixture (mix) and second collection of reagents (seconf spin) from walls and caps of micro-test-tubes. The maximum centrifugalization speed with centrifuge – wortex is 6 000 rotations per minute. It is destined to work with 12 test-tubes simultaneously that allows economizing the time for samples prepacircular and is a necessary tool for PCR-analysis.

Figure 6.5 Centrifuge – wortex "Multispin" "MSK-6000", *Biosan*

Micro-centrifuge

The micro-centrifuge is a laboratory device for high speed sedimentation of particles? That allows maintaining such methods of samples micro-volumes treatment as, for example, extraction of nucleic acids and albumines. It provides high maximum values of relative centrifugal force, depending of model, up to 13 000 rotations per minute, 17 000 rotations per minute or 21 000 rotations per minute and allows working with complex and simple methods. It is destined to work with test-tubes whose volume if from 1,5 to 2,0 ml. These centrifuges are equipped by hermetic covecirculars, that steadely pressurize biological samples inside rotor. There are modern models with changeable volume rotors, e.g. 24 x 2 ml, there are also rotors for PCR-strips 8 x 8 centifugalization, allowing to treat lage number of samples ducircular one cycle. Solid materials of constructions and accessories are resistant to impact of aggressive chemical matters, allow carrying out scrupulous cleaning of devices and autoclaving of equipment ducircular many years, guaranteeing their safe and robust exploitation.

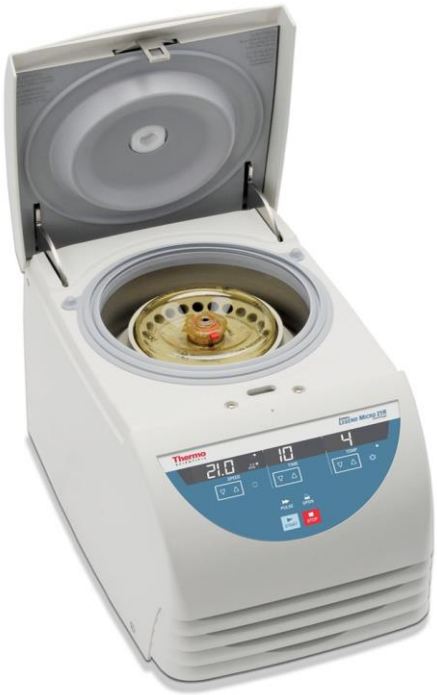


Figure 6.6 Mirco-centrifuge «MicroCL 17», *Thermo fisher*

Solid thermostat

Solid thermostats or Dry-bloks, with thermoblocks made from different metals, are use for maintaining programmed temperature of samples in test-tubes, that are put into holes of thermoblok. Thermostats differs according to diapason of maintained temperatures, according to kind of blocks for different volumes of test-tubes and according the accurancy of maintaining programmes temperature. The accurancy of ±0,1 °C from the temperature programmed by user is considered as a high one. Depending on model and manufacturer, thermostats have the diapason of temperatures from +5 to +130°C. Modern thermostats contain thermoblocks for test-tubes with different combinations of tolerable volumes, e.g. one thermoblock can contain holes for micro-test-tubes of 1,5 ml and 0,5 ml, or the combination of micro-tubes of 1,5 ml, 0,5 ml and 0,2 ml.

Solid thermostats contain integrated fan that allows to cool thermoblock in a short time. LCD cristal display facilitates the work with the device, allows to establish with precision and to survey all parameters of thermostat work. The device allows to apply methods requicircular different incubation temperatures at different stages of one process, and also when heated at high temperatures.

Digital thermoblock are largely usedat scientific and medical laboratories for samples prepacircular, enzimes storage, enzymatic reactions, DNA amplification, serum coagulation, destruction of gel for gel-electrophoresis etc.

Actually, different leading companies, such as Biosan, DNA-technologies, Thermo Scientific, Eppendorf and others propose solid thermostats with different technical characteristics.

		
Picture 6.7 Digital solid thermostat «TDB-120», *Biosan* Diapason of work temperatures +25 - +120 °C, accurancy ±0,1°C, with aluminum block A-103 for micro-test-tubes of 1,5 ml (32 places), 0,5 ml (21 places) and 0,2 ml (50 places)	**Picture 6.8 Digital thermostat «Compact Dry Bath S», *Thermo Fisher*** Diapason of work temperatures +2 - +100°C, accurancy ≤ ±0,5°C, different combinations for changeable blocks for test-tubes of different volumes from 0,2 ml to 50 ml.	**Picture 6.9 Solid thermostat « Gnom », DNA-Technology** Diapason of work temperatures fromambiant temperature to 99 °C, accurancy ±0,5°C, format of the block: for test-tubes of 1,5 ml (40 places), 5 ml (28 places). Equipped with heat-insulating clamping cap for even heating and preventing the opening of test-tubes.

Vacuum aspirator with trapping flask

Vacuum aspirator with trapping flask is destines for the aspiration (elimination) of alcohol traces (or buffer) from walls of Eppendorf test-tubes at the purification of DNA / RNA or for other technologies of macro-molecules redeposition.

The device can be also used for rutine operations of cells washing from nutritive milieu and resuspension in buffer. The principle of aspirator work consists in the creation of negative pressure in trapping flask with microcompressor, intergrated into the body. The trapping flask is connected by a plastic tube to the tip. The

liquid is removed from the test-tubes into the trapping flask at the contact of the tip with the solution surface. For convenience, a mini-tripod organizer designed for two test-tubes required for washing and storage of the tip for its reuse can be placed on the right side of the device.

The aspirator contains a microbiological filter precluding risk of coming out of bacteries, viruses and infectous particles for trapping flask. Tha aspiration microbiological filter – hydrophoben : retains particles of more than 0,027 μm, that are smaller that viruses of hepatitis A,B and C with the efficiency till 99,9%.

The specifications of aspirators from different manufacturers differ depending of trapping flask volume, vacuum pressure, size, available additional accessories etc (picture 6.9, picture 6.10).

	
Picture 6.9 Aspirator with trappig flask «FTA-1», *Biosan* Maximum vaccum – 0,05 Mpag, volume of trapping flask – 1000 ml, compact sizes 160 x 210 x 340 mm	**Picture 6.10 Electric vacuum aspirator «7A-23B», *Biomed*** Maximum vacuum – 0,09 Mpag (685 Mmhg), volume of trapping flask – 2500 ml × 2 volumes, sizes 350×305×795 mm

Spectrophotometer

The spectrophotometer is a laboratory device that provides data about spectral characteristics of analyzed components and is deseigned for:

- measucircular of nucleic acids concentration : double-stranded DNA, single-stranded DNA, RNA, oligonucleotides at 260 nm ;
- determination of protein concentration of the absorption at a wavelength of 280 nm ;
- determination of sample purity by ration 260/280 nm;
- identification of matters and analysis of microparticles ;
- determination of cell cultures density;
- quantitative analysis of proteins, conjugates, metalloproteins ;
- calculation of fluorescence dyes labeling intersity.

The measurement of nucleic acids concentration is a routine practice of biochemical, molecular and genetic, pharmacological, immunological, microbiological and other laboratories.

The determination of concentration and quality of nucleic acids preparation is one on key initial stages in experiments, that use PCR, qualtitative PCR, fragment analysis and sequencing of new generation. It is necessary to note that the evaluation of source material before experiment with expensive reagents is crucial.

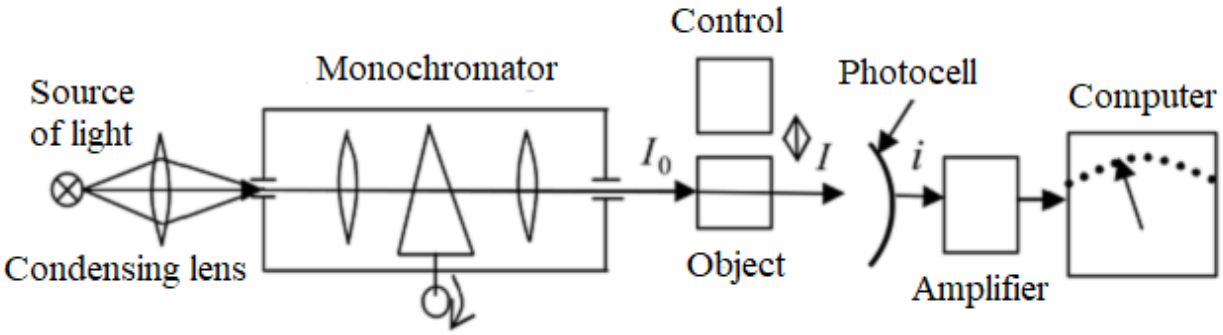
Spectrophotometric analysis using cuvettes. Spectrophotometerdevices and their characteristics can vary greatly depending on manufacturer and tasks for with the device is designed. But the main contruction elements are similar in all devices. They are light source, monochromator, cuvette compartment with a sample and registrating detector.

The monochromator is a device for the isolation of separate narrow part (1-2 nm) from a total radiative spectrum.

The spectrophotometer has two sources of the light: for visиду part of spectrгь (VIS) and ultraviolet source (UV) from 100 to 390 nm. Also, sets og light filters can be additionally used at some devices.

There is a cuvette compartment of stectrophotometer containing cuvette with analysed sample that can be additionally equipped with mechanisms for thermal regulation, mixing, and additing of substances directly ducircular measurement.

The device scheme can be presented by the following way **(Picture 6.11).**



Picture 6.11 Scheme of spectrophotometer structure

This scheme of spectrophotometer is called signe-wave. To measure absorption the same monochromatic ray of light should alternately pass through cuvette with sample and through cuvette with solvent which serves as a control.

The modern models of spectrophotometers are designed accordint to the long way principle. In this type of soectrophotometers the monochromatic ray is periodically directed by rotating mirror on two channels; the cuvette with sample is placed into one channel, the cuvette with solvent in placed into another one. The rays pass the sample and control in the antiphase, at the same time the difference in intensity is recorded by the photometric system with following interpretation of the result.

Various photometric functions, namely single and multiple wave analysis, combined with kinetic methods, provide scanning over a wide wavelength range from 190 nm to 1100 nm.

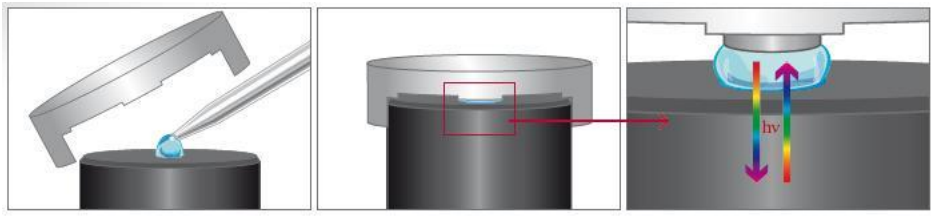
The example of a cuvette spectrophotometer is shown in picture 6.12.

Picture 6.12 Spectrophotometer with cuvette compartment and sensor screen «DS-C»,*DeNovix*

Spectrophotometric analysis without cuvettes. For analysis of substances micro-volumes, a technology without cuvette is used, in this case the sample is held by the surface tension forces of the

fluid.The innovative technologies allowto carry out accurate measucircular of sample micro-volumes from 0,3 mm^3.

For spectrophotometric analysis, a drop of sample is placed on the fixed module of the device. The movable module of the device is lowered on the drop, resulting the creation of a liquid column from the sample between the movable and fixed modules. The height of the column is automatically adjusted. The device measures the light absorption in VIS / UV rays in the sample column (picture 6.13, picture 6.14).



Picture 6.13 Scheme of spectrophotometric analysis without cuvettes

Picture 6.14 Spectrophotometer for analysis without cuvettes «NanoDrop 2000/2000c», *Thermo Scientific*

Automatic pipettes

Automatic pipettes are pipette devices of piston type. Pipettes must comply with ISO 9001:2000 for measurement accuracy and reproducibility.

The operation principle of pipettes is based on air displacement of liquid. The dosage is due to the movement of piston in measucircular cylinder. In this case, the piston either reduces the volume of air below, or increases it. If the pipette tip is placed into liquid, as the volume between the liquid and the piston increases, the liquid will tend to fill the space released under the piston. So, the volume of liquid equivalent to the volume released under the piston when moving it, will come into the pipette tip. When it is necessary to remove the withdrawn liquid from the pipette, the piston moves in the direction of the tip and reduces the volume of air between the liquid and the piston, so that the liquid is completely pushed from the tip by the air.

There are variable or fixed volume pipettes, electronic and manual mechanical, single and multi-channel.

Fixed volume laboratory pipettes are designed to take and to dispense a specific volume of substance. Variable-volume pipettes allow to take a wide range of fluid micro-volumes. Manual mechanical pipettes provide the dosage of taken fluid by rotating a single plunger head (picture 6.15).

Picture 6.15 Manual mechanical single- and eight-channel variable volume pipettes, *Eppendorf*

When using electronic pipettes, the discharge volume is set and adjusted by controlling the readings on the pipette screen (picture 6.16). These pipettes allow to pre-programme multi-step processes to solve specific tasks and allow users to set the dosage volume much faster than standard manual mechanical pipettes.

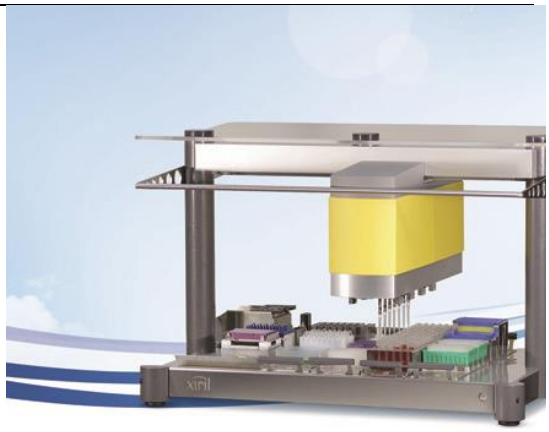
Picture 6.16 Electronic single and eight-channel variable volume pipettes, Picus® DNA Picus® NxT

Automatic pipettes are made of modern high-quality plastics, resistant to chemically active milieus, which allows user to work with different liquids, so with solutions as with concentrated liquids.

Automation of DNA / RNA isolation processes

The efficiency of DNA/RNA isolation will be significantly increased if special devises are used for these purposes. Reducing the number of manual operations at the stage of nucleic acid isolation leads to increased productivity, reduced time, reduced risk of cross-contamination, standardization of the isolation process, and reduced chance of operator' errors.

Devices for automation of nucleic acid isolation process have been developed, that increased the rapidity and productivity of isolation. The use of automated systems is particularly useful for RNA isolation, which is rapidly inactivated by various ribonucleases. So these purposes the following devices were developed: QIAsymphony SP/AS (Qiagen) (picture 6.17), Neon-100 (Xiril) (picrture 6.18), MagMAX™ Express-96 (Applied Biosystems) (picture 6.19) [І.В. Дзюблик, 2012].

	
Picture 6.17 Device QIAsymphony SP/AS, *Qiagen*	**Picture 6.18 Neon-100, *Xiril***

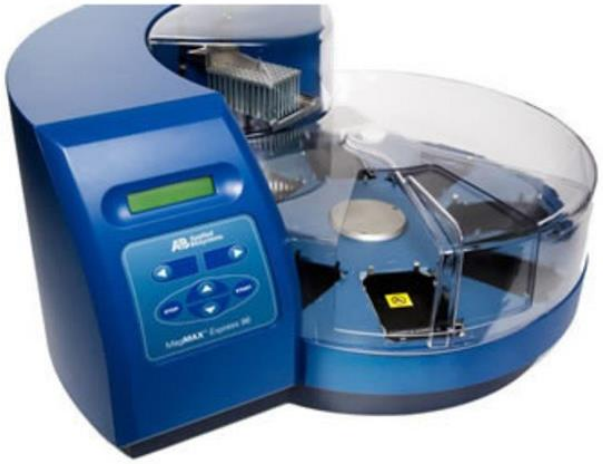


Рис. 6.19 MagMAX™ Express-96, Applied Biosystems

Equipment required for PCR amplification prepacircular:

1. PCR-box;
2. Thermal cycler or amplifier for PCR-real time;
3. Centrifuge Wortex;
4. Set of automatic pipettes with variable volume;
5. Common refrigerator;
6. Freezing chamber;

PCR-box:

The PCR-box destined for steril works UVC/T-M-AR is used for clean work with DNA-samples. It provides the protection against contamination. All boxing models are desktop, made of metal frame, glass and have stainless steel work surface (Picture).

The oxes are equipped with one open ultra-violet (UV) lamp installed in upper part of box. UV radiation disinfects the work surface, inactivating DNA / RNA fragments for 15-30 minutes. The available digital timer controls the duration of direct ultraviolet irradiation. The daylight lamp inside the box illuminates the workplace.

In addition, the boxes are equipped with a bactericid flowing UV recirculator of air (AR), which ensures permanent disinfection inside the box ducircular operation. It is recommended for the work with DNA / RNA amplicons.

Picture 6.20 UV-box for PCR UVC/T, *Biosan*

Thermal cycler

The DNA amplifier, thermal cycler is a device used for PCR. The devoce automatically maintains needed number of reaction cycles and optimal time and temperature parameters for every cycle.

There are classic amplifiers and amplifiers for PCR – real time.

The classic amplifiers are destined for PCR carrying out with the following interpretation of results using additional methods and equipment for visualization of PCR products and interpretation of results by horizontal electrophoresis, capillary electrophoresis and other methods. Some examplesof classic amplifiers are the following: VeritiWell (Applied Biosystem) (Picture 6.21), MiniAmp™ (Applied Biosystem) (Picture 6.22), Mastercycler (Eppendorf) (Picture 6.23) etc.

		
Picture 6.21 Amplifier VeritiWell, *Applied Biosystem*	**Picture 6.22 Amplifier MiniAmp™, *Applied Biosystem***	**Picture 6.23 Amplifier Mastercycler, *Eppendorf***

The amplifiers for PCR – real time are multifunctional devices that comprise the functions of standard amplifier and fluorescent detector. The PCR system is used for carrying out the polymeraze chain reaction in real time that differs from the classic PCR by the detection and the calculation of obtained DNA quantity [S. A. Bustin, 2004; Ball, 2003].

Tasks that are solved using Real Time Amplifiers:

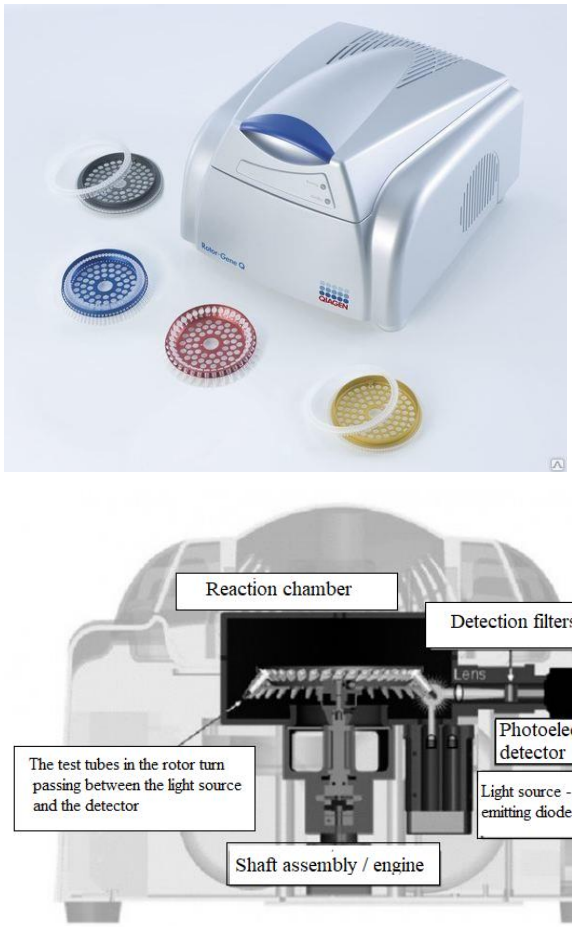
- qualitative PCR analysis that provides:
  a) identification of pathogens of a wide range of infectious diseases;
  b) simultaneous identification of several pathogens in the test material for genotyping of microorganisms;

- quantitative PCR analysis:
  a) determination of viral load in infections;
  b) determination of genetically modified ingredients concentration;
  c) evaluation of genes expression level;
  d) application of alternative amplification technology to validate the results and to identify viable pathogens (NASBA-analysis);
- endpoint fluorescence detection;
- for melting high resolution PCR products (HRM), analysis of DNA / RNA point mutations, HLA screening, epigenetic methylation analysis.

Real-Time Amplifiers are divided into plate and rotor type amplifiers with a number of channels for detecting fluorescent dyes from 4 to 6 channels. In terms of their functional and technical characteristics, the devices are quite similar, but it may be possible to use different technologies of heated air flow to avoid demerits associated with the appearance of a boundary effect when using plate type amplifier.

Plate type amplifier is a real-time multifunctional PCR system that has a plate format of thermal block. The warming up to operating temperatures and changing of temperature gradient occur by supplying heated air through the amplifier cover.

Rotor type amplifier is an amplifier that has a circular (rotary) design of the reaction block (Picture 6.24). The technology of heating reaction mixtures in test tubes involves a uniform distribution of hot air over the surface of the tubes with the reaction mixture.



Picture 6.24 Rotor type amplifier Rotor-Gene Q, *Qiagene*

The equipment needed for the detection of PCR analyses results by the horizontal electrophoresis method:

1. Electrophoresis chamber with power supply unit (DC source), cuvette for forming agarose gel, combs for forming wells in the gel;
2. Transluminator;
3. Gel-documenting station;

When using PCR in the classic format, the detection of amplicons is carried out by electrophoresis in agarose gel. In addition, this method allows to check the quality of the isolated DNA for further research.

The electrophoresis is an electrokinetic phenomenon of the movement of particles of a dispersed phase (colloidal or protein solutions) in a liquid or gaseous medium under the action of an external electric field.

The horizontal electrophoresis in agarose gel is a standard method used to separate, to identify and to purify DNA and RNA.

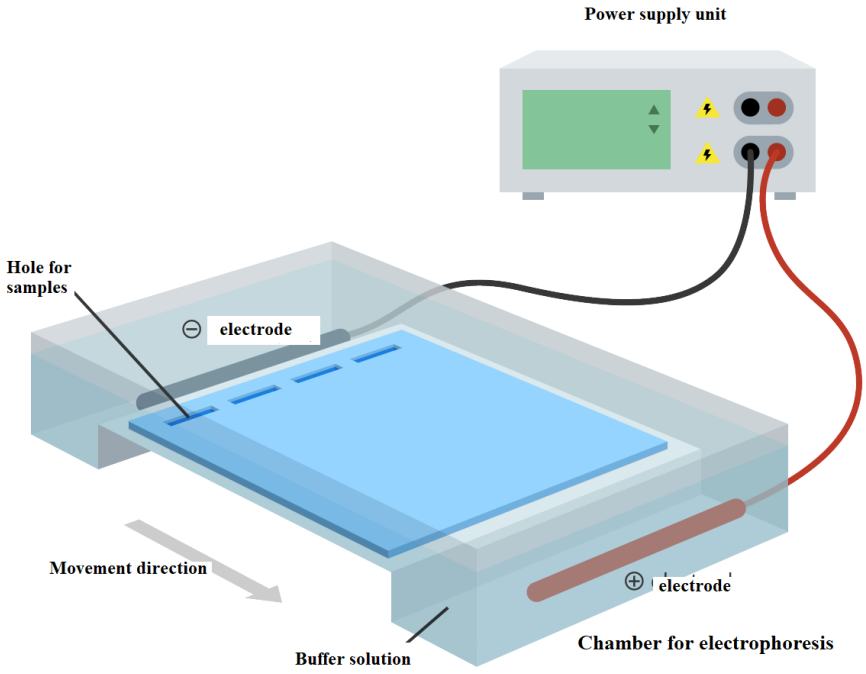
The detection on special gel-documenting systems allows to visualise the results with the help of fluorescent and intercalating dye ethidium bromide. By using the ethidium bromide, about 0.1 µg of nucleic acids can be visually detected in ultraviolet rays. The method does not foresee the quantification.

Chamber for horizontal electrophoresis

The chamber for horizontal electrophoresis is a device related to laboratory equipment, namely, to devices for separating charged molecules by electrophoresis in horizontal gel, and is destined for electrophoretic analysis of biological macromolecules. The device can be used for laboratory analysis of proteins and nucleic acids, including the detection of PCR products.

Nowadays, devices for horizontal gel-electrophoresis, as a rule, are modifications of the devices proposed by Schaeffner (1), Laurel (2) or Vime (3), where the agarose gel is placed on a separate glass or plastic plate or in a special cup. The plate is placed on the platform of the device so that the gel was directly under the surface of buffer solution for electrophoresis. The resistance of the gel slightly differs from the resistance of the buffer solution for electrophoresis, so, a significant part of the current passes through the gel.

The chamber contains a protective cover and cords for connecting to the power supply unit that is a direct current source (Picture 6.25).



Picture 6.25 Chamber for horizontal electrophoresis in agarose gel

Transluminator and gel-documenting station

Depending on the method for the detection of PCR amplification products, it may be necessary to have electrophoresis equipment with a documentation system, such as a gel-documenting station or transilluminator, to visualize the separation of PCR products in UV rays.

	
Picture 6.26 Gel-documenting station, *BioRad*	**Picture 6.27 Transluminator,*Coleparmer***

An alternative replacement for the application of PCR method with "endpoint" hybridization-fluorescence detection is the use of a special detector of type "Djyn" (Picture 6.28) or "Ala ¼" (Picture 6.29), that could be installed in the amplification part, and connected to the computer with a specific operating system according to the equipment used.

	
Picture 6.28 Fluorescent detector "Djyn-4", DNA-technology	**Picture 6.29 Fluorescent detector «Ala ¼», *Biosan***

Main equipment required for the detection of PCR results by capillary electrophoresis:

1. Genetic analyser;
2. Thermal cycler;
3. Centrifuge - wortex;
4. Common refrigerator;
5. Freezing chamber;

Genetic analyser:

The genetic analyser is a device for analysis of fluorescently-labeled DNA fragments using the method of capillary electrophoresis and can be widely used for various types of genotyping that are applied in different fields:

– in the forensic science, in creating genomic databases for identification of person, in establishing affinity and in resolving issues of paternity;

– for DNA analyses in the agriculture for the selection and pasportization of valuable animal breeds, plant varieties etc;

– for diagnostics of oncological and infectious diseases and for monitocircular of diseases progression;

– for the diagnosis of hereditary diseases and the predisposition to them;

– for decoding the genome of humans, animals and plants;

– for searching and study of new genes responsible for important physiological processes (cancer, aging, etc.).

The work principle of a capillary genetic analyzer foresees a system for excitation and detection of the radiation from multiple capillary channels located on a cross-scanning table. The ends of the capillaries placed in the cathode and anode trays are connected to a high voltage source. The laser source irradiates the capillaries in the detection area, and the optical signal is transformed by an optical system including a lens that focuses the laser radiation and collects a fluorescent signal; a dichroic mirror that reflects laser radiation and directs it to the lens, and also allows the fluorescent signal; two light-filters, one of which cuts the laser radiation reflected from the capillary, and the other is an interference light-filter. Having passed through this optical system, the signal is registered by a photoelectron multiplier (PEM). In one of the versions of the device realization the fluorescent signal can be divided into four equivalent beams, each of them is passed through a corresponding interference filter and registered by the PEM. The signal from the PEM after amplification is sent to the computer for the appropriate processing (US patent N 5274240, IPC 6 G 01 N 21/64, published in 1993).

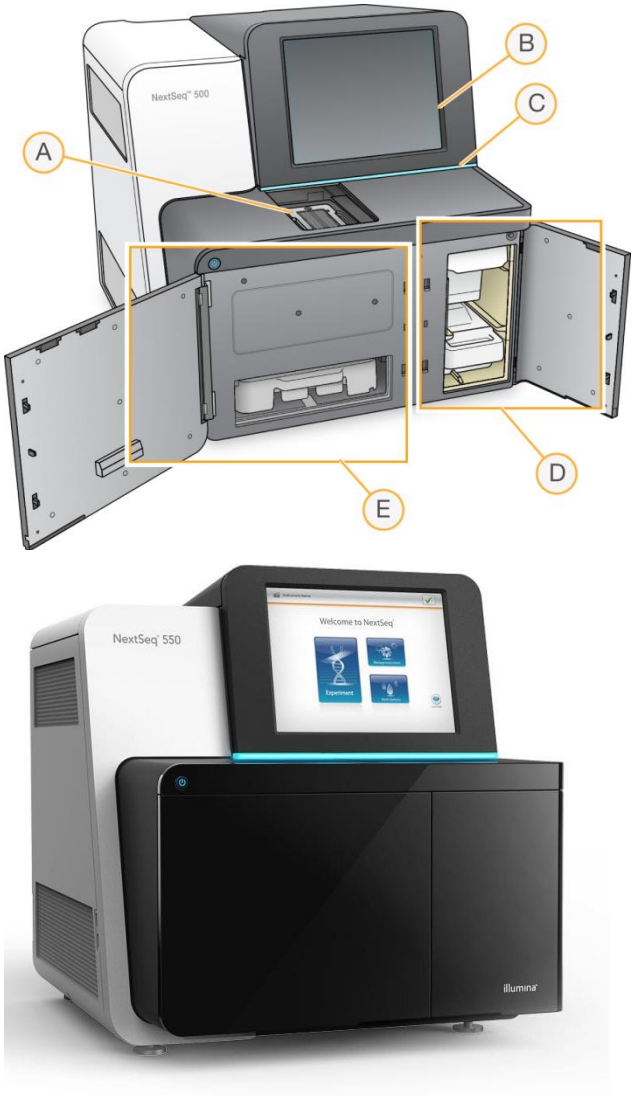
Nowadays, genetic analyzers (sequencers) from *Applied Biosystems* society (*Life Technologies*) represent the gold standard of capillary electrophoresis. The models have from 4 to 8 capillaries and offer complete automation of researches.They are used for sequencing *de novo* and for re-sequencing, as well as for fragment analysis: LOH, AFLP, single nucleotide polymorphisms studies (SNPs), microsatellite analysis, HLA-typing, MLPA studies, and others.

Picture 6.30 Genetic analyzer (sequencer)«ABP 3500», *Applied Biosystems*

Today's the most effective technology is the use of microchips on the base of Single-nucleotide polymorphism (SNP). This methos allows to make genetic analysis as specialized as possible, to study differences of tissues belonging to one organism. But today this method is quite expensive, so its availability is limited.

Ducircular the last decade, researches in molecular genetics has been devoted to the study of single nucleotides substitutions (SNPs). Their huge number in genomes of every species of farm animals and the latest technologies for genotyping according to this type of markers have been developed in leading scientific laboratories and replace other types of markers from studies of species and breeds genetic diversity.

The NextSeq 550 (Picture 6.31) is a performant sequencing system that provides a high quality of dates and a flexible power required to sequence the entire genome, transcriptome and target redistribution, as well as the ability to scan microchips, including Infinium MethylationEPIC BeadChip and other selected BeadChip.



Picture 6.31 NextSeq 550 DNA / RNA Microchip Scanner, Illumina

A - samples or microchips compartment, B - monitor, C - instrument status line - indicates the position of the instrument: working (blue), requicircular attention (orange), ready for sequencing (green) or switching off in the next 24 hours (yellow) , D - compartment for cartridges with buffer solution, E - compartment for cartridges with reagents.

Separate refrigerators for the storage of reagents and samples should be provided for each stage of the study.

A refrigeration chamber plus 4 ± 2 ° C and the freezer minus 18 ± 2 ° C shall be installed in the room for reception, registration, analysis and initial processing of material. A nucleic acid isolation room is fitted with a refrigerating chamber plus 4 ± 2 ° C to store reagents set and a freezer to minus 18 ± 2 ° C for a long (up to one year) storage of DNA / RNA. For a longer (one year and more) DNA / RNA storage, a freezer to minus 70 ° C should be installed.

Refrigeration chambers plus 4 ± 2 ° C and a freezer minus 18 ± 2 ° C are installed the room for PCR reaction mixtures preparation to store reagent kits needed for PCR mixtures.

In the detection room of the PCR amplification products a refrigeration chamber plus 4 ± 2 ° C is placed for the storage of reagents for electrophoretic detection [I.V. Dzublyk, 2012].

6.3 Main technical requirements for laboratories, organization of work areas, requirements for premises

The general layout of the PCR laboratory premises, the placement of working and auxiliary zones in it ahould be carried out according to the list of molecular biological techniques that will be used in that laboratory.

The PCR laboratory should have two clean and dirty zones and should include the following minimum set of work spaces:

- “clean” zone:
- premise for reception, registration, analysis and initial processing of material;
- premise for nucleinic acid isolation;
- premise for preparation of reaction mixtures and amplification
- "dirty" zone:
- premise for detection of amplification products (when applied the electrophoresis methods);
- premise for sequencing.

The schematic diagram of the organization PCR laboratory is shown in the picture.

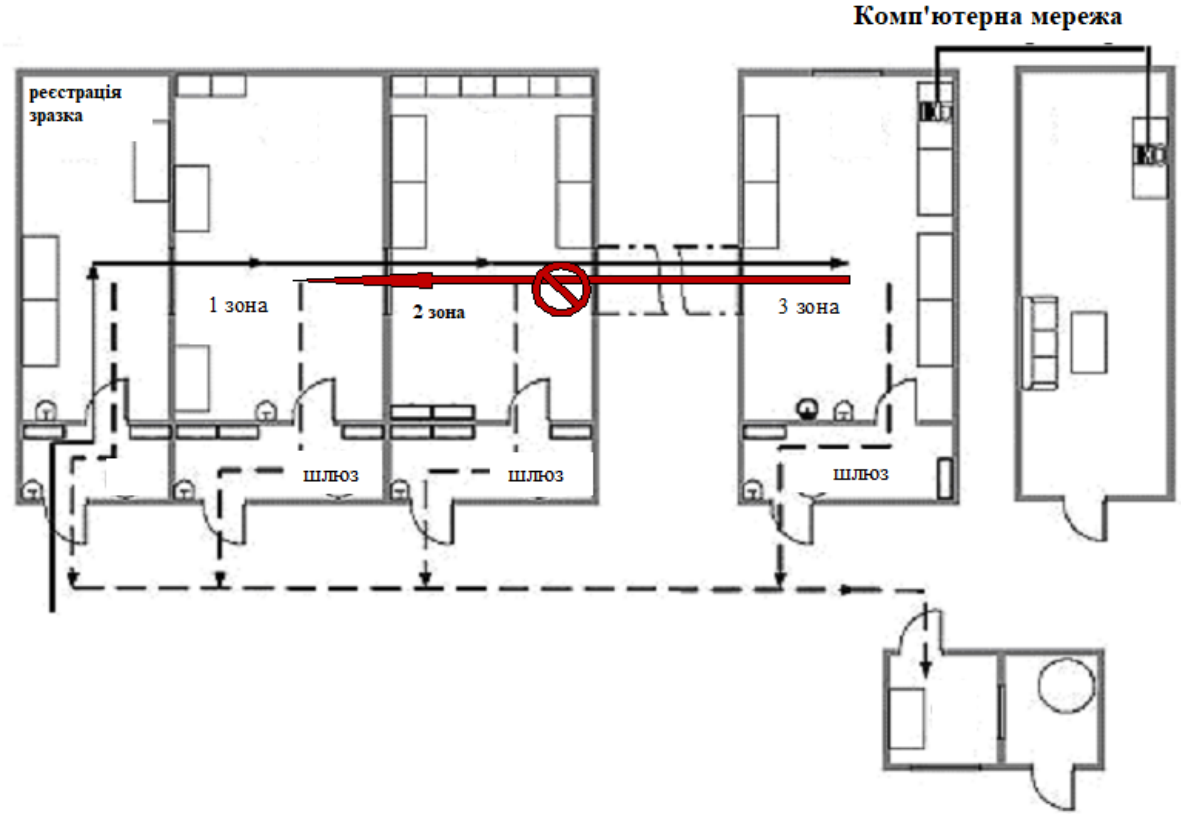


Figure 6.32 Schematic diagram of the organization PCR laboratory

Legend


- sink


- device for drainage of water after wet cleaning of the room


- autoclave


- cabinets in pre-boxes (for clothes) and boxes


- window-gateway


- movement of investigated material


- movement of waste material.

Work premises of the PCR laboratory should be impassable and designed in the type of boxes with pre-boxes. The surface of each PCR laboratory work premise shall be not less than 12 square meters per workplace, including a pre-box not less than 2 square meters. When increasing jobs, the area should be increased by 6 square meters for every workplace, so S = 12 + 6 x (n-1) square meters, where n is the number of jobs.

The PCR laboratory, that functions as an independent structure, should additionally have the following premises: document room (staff room), laboratory manager's office (can be combined with the staff room); changing rooms for the staff; dinning room; toilet; showers for the "clean" and "dirty" areas of the PCR laboratory separately, utilities rooms, premises for decontamination of material and autoclave room.

In the premises of reception, registration, disassembling and initial treatment of the material the following operations take place: preliminary sample preparation (sorting, labelling, centrifugation, etc.), storage and primary inactivation of residues of biological material by disinfectants. These premises can also serve for the reception and treatment of samples for analyses by other methods (bacteriological, virological, immunological, etc.) if a separate equipped workplace for samples preparation for PCR analysis is provided.

All manipulations that are accompanied by the risk of aerosols formation (shaking, centrifugation etc.), when treating the material, are performed in safety boxes of class II or III (depending on the pathogenicity group of the microorganism suspected of being present in the material under study).

The zone of nucleinic acid isolation is placed in a separate room. When organizing a PCR laboratory inside of an active microbiological laboratory, it is allowed to isolate nucleonic acid in rooms where serological studies are carried out, and in laboratories working with pathogens of I-II groups this operation can be done in rooms destined to contamination and autopsy of animals. In these cases, a work area is organized in the room to isolate the nucleonic acid, in which the biosecurity box of class II or III, depending on pathogenicity is set. Only equipment and items that are needed for the isolation of nucleonic acid are located in the work area. No other works are allowed in the biosecurity box destined to the isolation of nucleonic acid.

The isolation of nucleonic acid from clinical material and samples taken from environment is performed in a different safety box than in food testing for safety indicators or for the presence of genetically modified organisms. In addition, it is advisable to differentiate the processes of nucleonic acid isolation from blood / serum and other types of clinical material (in separate security boxes or at different time, after pre-treatment).

The amplification room must be separate. It is used to prepare the reaction mixture, to insert into the test tube for PCR isolated DNA or complementary DNA (hereinafter - cDNA), to carry out the reverse transcription (hereinafter - RT) of RNA and the amplification of DNA or cDNA.

To prepare a reaction mixture and to introduce nucleonic acid specimens into it, separate PCR boxes are set.

In PCR laboratories with a large volume of the same type of researches, a separate boxed room is equipped for the preparation of the reaction mixtures; it is functionally connected through a gateway, with the room for introduction of isolated nucleonic acid specimens and carrying out the amplification.

The room for the detection of amplification products is located in a separate premise, as far as possible from the "clean" zone of PCR laboratory. All conditions for the separation of staff working in the "clean" zone from the staff working in the "dirty" zone are created. Pipettes and utensils intended for electrophoresis should not be used for other research methods.

When used in PCR laboratory, the PCR method with fluorescence detection, as the only method, a separate room for the detection of amplification products is not set **(Picture 6.33).**

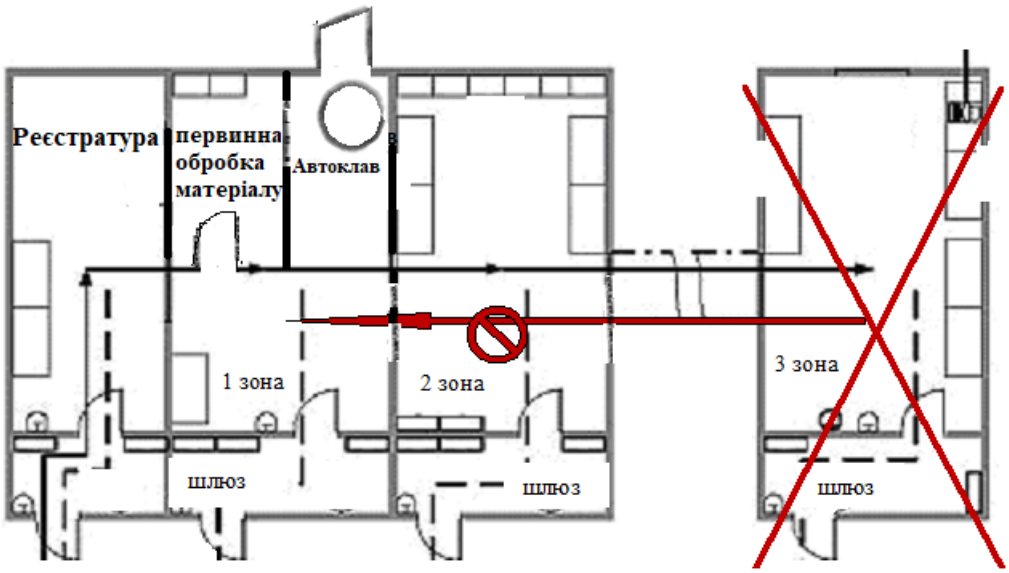


Figure 6.33 Schematic diagram of a PCR laboratory using hybridization-fluorescence detection

The autoclave room may be common to PCR laboratory and other units of laboratory where the PCR laboratory is located, and may function if the biosecurity requirements are respected.

To study the sequence of nucleotides in DNA (sequencing), it is necessary to allocate a separate room - a sequencer in the "dirty" area of the PCR laboratory, which should be located near the premise of the detection of amplification products. The enlargement of the detection area of the amplification products should be foreseen, as here the following additional equipment for sequencing should be placed: table centrifuge with cooling and rotary rotors, table for cleaning and quality control of the obtained PCR product.

The premise of the sequencer is arranged according to the type of box with a pre-box, its total areashould not be less than 12 sq.m, including 10 sq.m of a working room. The pre-box is equipped with water supply and sewerage.

The preparation of a sample for loading into a sequencer consists of several basic steps (and may vary depending on technique used):

- the extraction of nucleinic acid from clinical material is performed in the room for nucleinic acid isolation as for PCR in the classic format;

- the amplification of the section of nucleinic acid, which covers the area of interest (if it is RNA the stage of Reverse transcription to obtain cDNA is necessary) ST stage to obtain cDNA), is carried out in the premises of amplification;

- the purification of the obtained amplified product of the PCR reaction (from primers, residual deoxynucleotides, enzymes, matrix nucleinic acid, non-specific PCR products) is carried out in a phoresis room. The following purification methods are used: by electrophoresis in agarose gel; on columns; enzymatic purification;

– the assessment of quality / quantity of DNA (followed by dilution if necessary) by agarose electrophoresis or by spectrophotometry is carried out in a phoresis room;

– the PCR analysis with the use of terminators is carried out in a sequencer;

– the additional purification of the obtained amplified product after reaction with the terminators (from salts, excessive dideoxynucleotides) is carried out in a phoresis room. The following purification methods are used: ethanol purification, isopropanol purification, gel filtration, membrane filtration;

– the resuspension of the sample (in formamide or water) is carried out in a phoresis room;

– the loading of the sample into the sequencer.

The planning decisions and the placement of equipment should ensure the flow of studied material through thetechnological process. The air exchange between dirty areas and other PCR facilities should be completely excluded.

The movement of the material in the opposite direction is strictly forbidden.

The PCR laboratory must be equipped with water, sewage, electricity and heating in accordance with legislation in power. All laboratory facilities shall be provided with adequate natural and artificial lighting. There should be a hand-wash basin in the pre-box every work room.

When building new or refurbishing existing PCR laboratories, the premises should be equipped with a tidal exhaust or exhaust ventilation. The difference in air pressure in PCR laboratory premises is achieved by different air exchange rates in them.

The rate of air exchange in premises of PCR laboratory must comply with the values given in table 6.1.

Table 6.1.

Rate of air exchange (m^3 / h) in premises of PCR laboratories

Name of premise	Inflow: rate of air exchange, m^3 / h	Exhaust: rate of air exchange m^3 / h
Area of reception, registration, review and initial processing of biomaterial	5	6
Area of nucleinic acids isolation	5	6
Area of reaction mixtures preparation and carrying out of PCR	5	5
Area for the detection of amplification products by the electrophoresis	5	7

Supply and drawing ventilation must be equipped separately for the "clean" and "dirty" (phoresis and ELIZA areas) zones of PCR laboratory.

In the absence of a ventilation system, reducing the risk of samples contamination is achieved by limiting the exchange of air between the PCR laboratory premises (territorial separation).

If necessary, air conditioners may be installed in the PCR laboratory upon condition that they are used ducircular technological breaks. Conditioners must be switched off when working with test materials.

The interior decoration of premises is made according to their functional purpose. The surfaces of walls, floors and ceilings in laboratory rooms should be smooth, crevice-free, easy to handle and resistant to detergents and disinfectants. The floor should not be slippery.

The windows should be tightly closed. For protection of workplaces from the sun rays it is recommended to use light-protective films that are resistant to disinfectants, the use of blinds inside the premises is forbidden.

Laboratory furniture must have coating resistant to detergents and disinfectants. The surface of the tables should be free of cracks and seams.

The premises for all stages of the PCR analysis shall be equipped with bactericidal lamps, which shall be installed at a rate of 2.5 W / m3. It is recommended to use additionally a mobile UV bactericidal irradiator-recirculator.

PCR laboratory should be equiped with telephone, computer and office equipment connected to the local electronic network.

PCR laboratory is equiped with fire extinguishing means.

6.4 ICAR recommendations related to accredited laboratories. World requirements to laboratories, accreditation rules. Work principles of reference laboratories.

The studies in the molecular biology, especially in the genomics, provide new information about industrial animals. On one hand, the use of molecular information can promote consumer confidence to the liability of monitocircular and control of animal production at its stages. On the other hand, the molecular information will have a positive impact on the achievement of genetic improvment of animal traits through the use of genomic selection, markers, gene introgression, heterosis provision, genealogical verifying / provision, and genetic defect status of carrier. In most cases, the benefits of using molecular information through genomic evaluation are due to the increased accuracy of the breeding value of animals, the reduction

of generation intervals, and the increased breeding intensity. Even with these advances, there is still a need for research and development for finding an association between genetic markers and useful traits. In addition, even with this inclusion of genomic information in the selection schemes, the understanding of genes action, genes interaction and differential genes expression is necessary to avoid a negative collateral effect. The cooperation between the livestock industry and science is needed to successfully research of genetic information in commercial livestock populations [ICAR Guidelines].

6.4.1 Genetic markers

Genetic markers are main molecular tools of the genetics. Since the 1960s the blood groups studies have been used as first DNA markers in animal breeding, later the study of microsatellites (MS) was launched, and from the 1990s to the present time the study of single-nucleotide polymorphism (SNP) is used. SNP and MS are polymorphic parts of DNA (allel) for a specific locus of a particular chromosome.

Microsatellites are sections of DNA containing tandem nucleotide repeats, usually dinucleotide or trinucleotide. These segments are spread throughout the genome and are non-coding regions that are not subject to selective pressure. Microsatellites are commonly used to establish the level of genetic affinity, to determine inter- and intraspecific polymorphisms, to establish the origin of different species and populations as a whole.

Single-nucleotide polymorphism (SNP) is the most common type of genetic variability: every SNP represents variability in a single nucleotide. There are many SNPs located throughout the genome of every animal species. For genomics, the most informative SNPs traditionally are located either in (a) a coding region where different alleles change the structure or function of encoded protein; or (b) in non-coding regions involved in the regulatory function of gene. For genomic selection values, SNPs located in other parts of genome are also informative because they may be in an unbalanced relation with alleles that cause phenotypic changes.

One of the advantages consists in the siting of complexs with robust parallel data processing on SNPs, that can test thousands or hundreds of thousands of SNPs, that is a cost-effective and efficient method of checking a large numbers of animals. Currently, under this scheme, the largest animal genotyping laboratories can treat more than 100,000 animals per year. Thus, the presence of large SNP panels facilitates the search for mutations that are the base of genetic variation of simple and complex traits. It also accelerates the detection of traits related to genes or parts of genes and the speed of genomic selection strategies.

Terms and definitions

Table 6.2

Overview of the terms used in molecular genetics, genomics and origin analyzes.

Term	Definition
Animals identification confirmation	It is a process in which genomic markers can be used to prove the identity of a particular tissue sample to a particular animal.
Genomics	Spectrum of technologies that determine the genetic composition of an animal at gene level and DNA sequence of the animal genome.
Haplotype	A group of alleles of genetic markers that are inherited from one parent together. Genetic markers are on the same chromosome and usually contain a certain segment length on this chromosome.
ICAR accreditation	ICAR's recognition that the organization has provided sufficient evidence that it meets all ICAR requirements.

Imputation	Process of filling in missing genotypes of animal based on genealogies and other genetic markers. Usuallythey are used on the basis of SNP and / or microsatellite imputation from SNP.
MAF	Frequency of minor alleles
Forecasting of ancestors on maternal line	Process by which haplotypes inherited from a female are used to predict a feasable male.
Microsatelites	DNA segments containing tandem nucleotide repeats, usually with a dinucleotide or trinucleotide structure. Also they are called STR: short-tDNAem-repeats.
Analysis of paternity	General analysis of genotypes related to origin may include paternity screening, paternity detection, and maternal progeny prediction.
Paternity test	Process allowing to examine prospective parents, usually males and sometimes females, based on genotype matching, it results in identifying the most feasible father and / or mother
Paternity verification	Process allowing to examine genotypes of registered parents (male and / or female) for the genotype of the animal to determine whether one or both of them are parents.
QTL (quantitative traits locus)	Quantitative traits locus. A part of the genome that influences quantitative traits (growth rate, weight, length). This area may have a small <0.01%, or a large effect> 5%, on phenotype. The quantitative trait will have a significant genome-wide QTL prevalence.
Paternity verification of male	Подібно до перевірки батьківства, але заснована на вивченні лише зареєстрованого самця. Similar to a paternity verification, but based on a study of only registered male.
SNP	Single-nucleotide polymorphism: alteration of a single nitrogen base in DNA sequence.

6.4.2. Application of DNA technologies

Definition of paternity and appointment of parents

Before the genotyping by SNP markers, the primary commercial purpose of using genetic markers was to determine paternity. Traditionally, paternity testing was based on the exclusion of relation (namely, male or female) when the genotype of animal did not match the genotype predicted in the pair. The likelihood of being assigned to the right pair of animals will depend on a number of markers used, the number of alleles in the loci, the insignificant frequency of alleles in the population, the number of parents and the number of possible copulations. The International Society for Animal Genetics (www.isag.us) recommends species-specific microsatellite panels and SNP markers, which can be accessed by the link given in Annex 1. For cattle, ICAR has developed the SNP Origin Kit, ICAR554, which includes the recommended ISAG panel and other highly informative SNPs. This panel allows accurate paternity testing and detection without high density assumptions. Thus, the ICAR554 panel may be distributed to countries and competitors for paternity analysis, not being afraid that others may use them to predict genomic selection values. ICAR and Interbull Center are collaborating to promote an international genotype-shacircular service called GenoEx, which is described later in Chapter 5, especially for the exchange of SNP genotypes for the main purpose of paternity analysis.

Traceability and authentication of animal products offered to consumers

Multiple crises, including outbreaks of bovine spongiform encephalopathy, have increased the demand for these studies.

Genetic markers are used for the authentication of products of animal origin for quality labeling related to geographical location, specific breeds or their crosses. This requires the establishment of molecular standards or allele frequencies for each breed within one species. Much information comes from studies devoted to genetic diversity among breeds. Genes that can be intensivly selected in each population are particularly interesting. With a sufficiently large set of SNPs and genotyped purebred reference animals, one can also predict the most likely breed composition of individuals.

Molecular and genetic information for marker schemes in breeding

Quantitative traits are usually controlled by a large number of genes. However, some genes sometimes affect a large number of variant traits. For example, double-muscle in cattle is related to myostatin gene, milk components in dairy cows, fertility of the Burul breed and phenomenon of polyovulation are related to DGAT1 gene. As genotype of animal does not change throughout life, the use of DNA information through the identification of QTL-related markers with influence on productive traits or the identification of gene itself along with the causal variant is of great interest. However, with complex traits, there is a growing need for a sufficiently large set of markers to include molecular information for selection decisions. The inclusion of genomic information is an important selection criterion for traits whose measurement is difficult and expensive and / or time consuming. By 2018,> 116,000 cattle,> 10,000 chickens,> 28,000 pigs, and> 2000 sheep were identified, whose quantitative loci are related to economically important traits such as health, body carcass, milk productivity, fertility and exterior. The AnimalQTLdb database, hosted by the National Animal Genome Research Program, contains up-to-date information on cattle, chicken, horse, pig, trout and sheep QTL data.

For complex and economically important traits, genetic markers and genomic selection provide significant selection opportunities. In general, genetic markers and genomics play a major role in breeding. Genomics can also allow us to increase the intensity of selection, since we can predict the value of genomic selection on a large number of animals and thus have more candidates for selection.

Disease resistence and genetic defects

Another group of features with high potential for molecular data and genomics use are those associated with disease resistance. There are a number of multifactorial or complex diseases that result from the interaction of animal genome with environmental components. Symptoms of disease resistance are some of the most difficult to include in genetic improvement programs, as they require a qualitative assessment of the animal's disease status and systematic control of its content or environmental status to identify environmental effects on the animal's health. Therefore, if the genes or genetic markers associated with resistance are properly selected, resistant animals will be able to be selected based on their genomic information. For many diseases the identification of genes associated with resistance requires the experimental conditions. Currently, genetic analysis is being used to identify heterozygous carriers of genetic diseases caused by single recessive genes. Examples of dairy cattle include: complex spinal curvature (CVM), brachyspin (BY), cholesterol deficiency (CD), and genes or haplotypes that cause embryo loss or stillbirth in various dairy breeds. In 2018, OMIA (Online Mendelian Inheritance in Animals) listed more than 770 signs or genetic defects in livestock. The inclusion of these causative alleles or related haplotypes in the breeding program will allow producers to minimize their risk of genetic defects, maximizing genetic progress with beneficial traits.

6.4.3 Technical aspects

It is recommended to carry out systematic DNA sampling in several animal populations. DNA can be isolated from any nuclear cell in the body. DNA extraction protocols from blood (white blood cells), semen, saliva (epithelial cells), hair follicles, muscles, skin, visceras (such as liver, spleen, etc.) are now available. Erythrocytes can also be used in poultry breeding, because birds retain the nuclearity of cells, but as for most

other species of animals they can not be used. A small amount of tissue is required for routine DNA analysis. However, if there is a need for repeated use of the individual's DNA (complete genome sequencing, traceability (check), check of alleles validations ...), the costs of DNA storage, extraction, quality and quantity, obtained by different protocols should be carefully considered and optimized. The commonly accepted sampling methods are those that include hair follicles, tissue samples (often obtained with a perforator from ear) in a sealed container, blood stains on filter paper, and nasal lavage.

It is possible to organize a centralized database related to the main use of genetic information:

- Checking, appointment and / or detection of paternity;

- Traceability of meat products;

- Breed identification or breed diversity;

- Qualitative and quantitative traits.

Database tables can contain:

- Identification of animal with links to all information about the animal and its relatives;

- Number of genetic markers (n);

- Standard name of each marker i (where i = 1, n);

- Appropriate marker number, such as dbSNP ID;

- Alleles for marker i;

- Genomic location of marker i;

- Impact of non-reference allele on protein

- Phenotypic manifestation of allele

- Association with other signs.

One of the most important parts of a large genome database is ensucircular that the genotype associated with an individual animal really belongs to that animal. Most large animal genome databases deal with SNP data, so this section will focus on genomic quality control. Both the quality control of the samples and the quality control system of the SNP are required, so it is advisable to develop a system for them early on.

For the standardization related the nomenclature of genes or loci, the information is provided in the links: https://www.genenames.org/about/guidelines/#!#genenames and http://varnomen.hgvs.org/

ICAR services related to DNA technologies

Since 2018, ICAR offers three DNA-related services, each is related to paternity analysis in one form or another, as shown below.

ICAR accreditation for Genotyping Laboratories:

- Quality testing of DNA genotyping services using the ISAG (International Society for Animal Genetics) circular test;
- determination of paternity based on microsatellites;
- accreditation for DNA interpretation centers:
- determination of origin and / or paternity;
- cooperation with the Interbull Center;
- exchange of SNP markers for verification and / or determination of origin.
- accreditation of DNA identification centers.

6.4.4 Accreditation of laboratories providing DNA genotyping services

Considecircular the need for high quality standards in all cases of molecular data use, ICAR has offered an accreditation service based on certain minimum requirements for laboratories providing DNA genotyping services for several years. The main requirements of this accreditation include the confirmation of the minimum internal standards of quality assurance management and participation in the international circular test, developed and proposed by the International Society of Animal Genetics (ISAG).

In addition, such laboratories have generally analyzed obtained genotypes to provide microsatellite and / or SNP analyses for paternity definition, including either verification of origin or confirmation of animal identification. ICAR's accreditation service has previously recognized genotyping laboratories as accredited organizations that provide paternity analysis without further testing for technical accuracy. Since 2018, the launch of the Accreditation Service at the DNA Centers for the Interpretation of DNA data using SNP paternity analysis has replaced the previous accreditation for SNP based verification. Similar procedures are planned for the introduction of microsatellite-based paternity analysis accreditation into ICAR, but the current accreditation process for genotyping laboratories will still be in place.

The following accreditation guidelines are foreseen for microsatellite and SNP genotyping of cattle. Minimum requirements for other species and other DNA analyzes may be determined in the future.

Scope

These guidelines relate to ICAR accreditation of genotyping laboratories that analyze biological samples of cattle using microsatellite and / or SNP genotyping, which can then be used further to analyze paternity, genotype imputation, genomic selection values, and other activities related to genomic selection strategies. This accreditation process also includes microsatellite paternity testing. For ICAR accreditation related to SNP paternity verification, genotyping laboratories must submit their application to ICAR and to DNA interpretation centers for the parallel accreditation, as described below.

ICAR rules and recommendations for the accreditation of genotyping laboratories

The accreditation process includes the following steps:

- Application for accreditation

- Payment of appropriate fees

- Review of statement

- Assignment of accreditation

Laboratory accreditation

Currently, ISO17025 or ISO9001 accreditation is required for accreditation of researches based on microsatellite analysis (STR) data. The ICAR standard, acting since 2020, requires the accreditation of genotyping laboratories according to ISO17025 or equivalent norm, to ensure the quality of internal management systems, which is a mandatory requirement for SNP-based accreditation. In addition, ISO9001 certification will bot be valide since 2020, and only ISO17025, or equivalent norm, will be an appropriate level of accreditation to ensure the quality of internal management systems for microsatellite (STR) accreditation.

Participation and presentation of circular test

ISAG organizes an international circular (comparative) test of laboratories for both microsatellite and SNP genotyping. Participation in ISAG and submission to these circular tests must be open and certified, if any. Applicants must also sign an agreement allowing ISAG to disclose the results of their circular tests for ICAR. The participation in at least two ISAG circular tests is a minimum requirement. For the ISAG microsatellite circular test, genotyping laboratories have to provide information on 12 ISAG microsatellite sets. The Committee of experts will determine the limits for each circular test, taking due account of the structure of the test and the average performance of the laboratories in the circular test per year. Only those laboratories that achieve grade 1 in the ISAG Annual Circular Test are automatically awarded ICAR accreditation as genotyping laboratories, and the accreditation of lower-level laboratories is at the discretion of the committee of experts.

Microsatellite markers. The names of all microsatellites sampled from all animals (markers set I) and those with no origin (markers set II) and from all animals typed during the last two years should be indicated. The minimum requirement for international exchange is a complete set of 12 official ISAG microsatellite markers. To ensure sufficient laboratory experience, the analysis of 500 animals per year is set by the minimum certification requirement for microsatellite control.

The probability of exclusion (2 parents or 1 parent) of each marker and complete sets of markers should be calculated and provided in the application. The population type and the number of animals (minimum 150) used for the calculations should be described. ICAR recommends using the Holstein breed as a reference group. But the ICAR Committee of experts will take decision concerning the accreditation based on population analysis.

SNP markers. It is necessary to indicate all SNP genotypes on animals of both marker groups and on all animals genotyped within at least last two year. The minimum requirement is to use at least 95 SNPs from the kit recommended by ISAG for all animal genotypes.

Nomenclature of markers

The nomenclature of the markers should be described. The ISAG nomenclature is required for the official 12 marker sets as well as for the SNP marker sets.

Accreditation of organizations carrying out SNP-based paternity analysis

With the appearance of SNP genotyping, the function of DNA genotyping as a laboratory activity may be separated from the functions of paternity verification and determination. Therefore, ICAR has established separate accreditation for the application of SNP-based genotyping results, which may be conducted by laboratories, breed associations, genetic evaluation centers and any other organizations involved in paternity verification and / or SNP genotype data processing.

Suppliers of services may use different laboratories for different breeds or species. Considering the importance of animal identification and paternity verification for animal registration, ICAR has decided to set minimum requirements for the use of DNA genotyping results and other information in order to:

- Check paternity check,
- Define parents,
- Confirm animal identification.

The purpose of these recommendations is to create a base for accreditation of processes used by organizations that use SNP cattle genotypes. Minimum requirements for other animal species and other DNA tests may be determined in the future.

Scope

These guidelines address the ICAR accreditation of organizations that use SNP based test results to analyze paternity in cattle, including paternity verification, origin detection and / or confirmation of animal identification.

6.5. Methods of sampling medium samples of biological material

6.5.1 Technique of sampling medium samples and methods of their short or long-term storage for quality indices determination

The complete analytical process can be divided into three successive stages: sampling, sample preparation for analysis and analysis of sample, which are described in detail in the literature [Senchenko B.S., 2001; Ermolaev A.P,, 1998; Zhitenko P.V., 2001; Pronin V.V., 2012].

Execution method. A methodically correctly selected average milk sample is one of the most important conditions for the correct determination of its quality indicators. It can be sampled directly on farm, in milkomg room, at milk receiving points, etc. Before sampling average samples from individual

animals or herds, you should find out about their productivity, daily routine on the farm, and prepare a storage space for bottles (containers) during the sampling [Senchenko B.S.].

If on the day of sampling the feeding mode or daily routine are disturbed, the sampling is not carried out. During the determination of quality indices of milk from every cow, they should be milked by the same milkmaid. For sampling average samples, it does not matter from what milking to start; the main thing is to obtain portions of milk from all milkings. Of course, if the tests are to be carried out immediately (2-2,5 hours later) after the sampling, it is more convenient to start with mid-day (at three times milking) or evening (at two times milking) milking. If the herd is large and cannot be sampled in one go, it is divided into several groups and a schedule for their sampling is drawn up.

According to the requirements, a sample volume of about 0.5 dm^3 (l) is taken for complete analysis. If only acidity and fat content are determined, a sample volume of 50 cm^3 is sufficient.

Samples should be planced into clean, dry bottles (containers) with labels and cork stoppers. For microbiological testing, samples are taken into sterile tubes, bottles or flasks and closed with cotton plugs.

When sampling average samples for analysis, the proportionality should be maintained according to the milk yield or quantity of milk in the vessel. The proportionality is regulated by setting the multiplicity (1, 2, 3), which is respected during the sampling from all vessels or milk yeilds during the day from every animal or by calculating the amount of cm^3 of milk that must be sampled from every kilogram (liter) in the vessel.

Since milk fat rises to the surface fairly quickly, the milk should be mixed well before sampling. After opening the flasks or compartments of milk tank, the milk fat that has accumulated on the lids and walls is removed with a spatula and transferred to the same flasks or tanks and carefully, immersed from top to bottom 8-10 times, stirred with a whisk. In cars tanks, milk is stirred for 3-4 minutes.

When sampling vessels of the same shape (Picture 5) (milk meters, buckets, flasks), metal or plastic tube-samplers (samplers) with an inside diameter of 9 mm are used the most frequently. Metal tubes, scoops and whisks used for this purpose shall be covered with an anti-corrosion alloy. It is prohibited to use for sampling rusted, contaminated, and corrupted tools [Ermolaev A.P., 1998].

Before sampling, the sampler tube should be pre-rinsed with the milk from which the sample is taken. To do this, the tube is slowly plenged and removed from the milk without closing its upper end, and the scoops are simply rinsed in milk. After mixing the milk, the tube is slowly pluged to the bottom of the vessel so that the level of milk in the tube and vessel is the same all the time. The tube is filled with milk to the same level as in the vessel (Picture 6.34). The upper opening is then closed with the thumb and the milk is transferred to dry and clean bottles (containers) with lables indicating animal's name or farm name, working team, milkmaid's name and date of the sampling. Bottles are closed with corks and stored in special boxes.

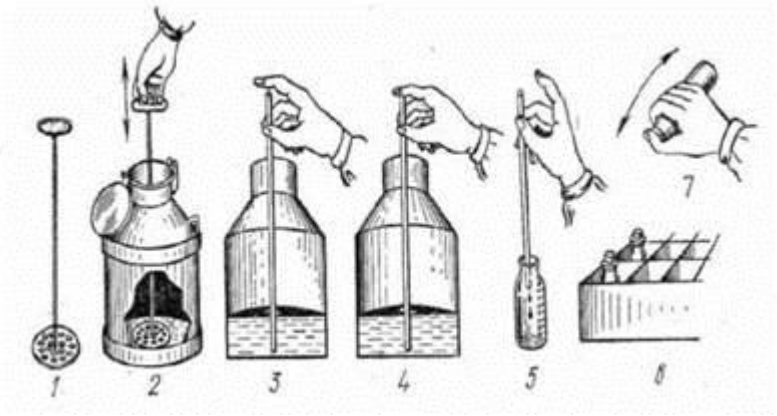


Figure 6.34 Order of sampling medium milk samples

1- milk stirrer; 2 – milk mixer; 3 - pluging the tube into milk for sampling; 4 - position of index finger before transferring sample; 5 - pouring milk into sample bottle; 6 - box for storing milk samples; 7 - mixing average milk sample before carrying out the analyzes.

If there are no tubes, or milk is in different containers, then average sample is taken by measuring with a measuring cylinder or cups (mug), after determining the proportionality of sampling by preliminary calculation. For example, three containers contain 215, 350 and 520 or 1085 kg of milk. For complete analysis it is necessary to sample 0.5 dm^3 of milk, or from each kilogram 500: 1085 = 0.46 cm^3. Therefore, 99 cm^3 should be taken from the first container, 161 cm3 from the second one, and 240 cm^3 from the third one. The small values obtained in the calculations are rounded off for convenience. When sampling milk from each cow under milking conditions, it is possible to use the voltage-check relay 1 or 1A on the milking parlor or in the milk line.

The bottles with samples are stored in a special box with sockets. Periodically, the content of the bottles is shaken in order not to prevent the creation of cream. If the samples are to be transported, the bottles should only be filled by 3/4 of their volume. The bottles should not be completely filled with milk as their content cannot be mixed before analysis.

In the case of simultaneous sampling for microbiological, physico-chemical and organoleptic studies, samples for microbiological studies shall be taken first, using sterile equipment and containers.

Canned samples stored for a long time should also be warmed to 35-40 ° C, mixed well and cooled to 20 ± 2 ° C. For this purpose, the samples are first placed in a bath with water at 46-50 ° C, and cooled in water at 12-15 ° C [Zhitenko P.V., 2001; Pronin V.V.].

6.5.2 Sampling for DNA testing

Sampling of biomaterials for DNA marker testing is carried out by veterinarians working on farms or invited by animal owners.

DNA samples can be obtained from any nuclear cell in body. DNA extraction protocols for blood (leukocytes), semen, saliva (epithelial cells), hair follicles, muscles, skin, viscera (such as liver, spleen, etc.) are now available. Erythrocytes can also be used for birds because they retain a nuclear body, while in most other species it is absent in erythrocyte cells. A small amount of material is required for routine DNA analysis. However, if there is multiple use of human DNA (whole genome sequencing, traceability, probabilities of the pathogen alleles), then DNA storage costs, production costs, quality and quantity obtained by different protocols should be carefully studied and optimized. Common collection methods include hair follicles, tissue specimens (often ears) in a closed container, blood stains on filter paper and nasal swabs.

Blood sampling

It is carried out in an anticoagulant or on a gauze swab. If a gauze swab is used, it is impregnated with sampled blood and air-dried. For DNA analysis, 0.5 ml of liquid blood or blood spot on a 5x5 mm gauze swab are sufficient.

Blood storage conditions:

• liquid - 5-7 days at 4 ° C or several months at -20 ° C;

•dried - in a hermetically sealed dry bag for up to 5 years at room temperature or in a refrigerator at 4 ° C.

The dried blood can be sent by post to the genetic control company (laboratory).

Sperm sampling and storage

0.5-2 ml of native semen are sampled. Sperm for DNA isolation is stored under conditions similar to the storage of liquid blood.

Sampling of biopsy materials

Biopsy materials can be of different origin. For DNA analysis ear pins that remain when tagging animals can be used. The storage life of pieces of 5x5 mm at 4 ° C is 3-4 days, in the frozen state (-20 ° C and below) is unlimited.

During sampling, each sample is labeled with an individual number. The act on sampling is edited in free form; it should contain full name numbers of animals and corresponding numbers of samples.

6.5.3 Basic principles for the formation of biological material banks

Necessity to conserve genetic resources of farm animals

Domestic local breeds of animals cannot compete with classic specialized breeds in terms of basic productive qualities, but they are characterized by extremely valuable features (strength of constitution, duration of productive use, resistance to infectious diseases, high reproducibility, high fat and protein content in milk, high genetic effect when crossing, etc.). So, they remain carriers of valuable hereditary qualities, without which further genetic improvement of animals is impossible. With the disappearance of breeds, the gene pool disappears, that narrows the variety of economically useful traits, and thus restricts breeding. Therefore, the conservation of this national heritage is an important state concern.

Saving genes reserves of populations is a difficult task and related to economic costs. Therefore, it is necessary to determine what breeds should be preserved, to develop criteria for the selection of breeds, forms, methods and numbers of gametes, embryos to be preserved and how to use them for future breeding.

One of the important tasks of conservation and efficient use of the gene pool of breeds is to carry out their inventory and to create on its basis the database of genetic diversity and specific characteristics of breeds, to determine among them genetically valuable breeds and to encoutrange their effective use in breeding programs.

The objective assessment of the diversity of gene pool and genetic status of domestic animals breeds is foreseen to be done with using polymorphism of erythrocyte antigens, blood serum proteins, tissue enzymes, proteins structure, cytogenetic analysis.

Methods of breeds gene pool conservation

The following methods of conservation of breeds gene pool are foreseen:

- pure breeding of collections of "pure" populations of farm animals, both domestic and foreign breeding in the form of closed gene pools, relict farms and animal reserves;
- long-term storage of frozen sperm, ovocytes, embryos in genetic banks;
- conservation of germplasm and DNA as a carrier of encoding genetic variations.

Forms of conservation of local and endangered species and breeds of farm animals

The subjects of conservation of the animal gene pool are gene pools herds, gene pools farms, reserves, collectors, zoos and cryobanks of genetic resources.

A rational form of conservation of breeds gene pool is to conserve livestock in gene pools herds (on at least 2-3 farms).

- gene pool herd: a purebred group of animals allocated for the conservation of the gene pool of a particular breed of animal.
- gene pool farm: a separate farm at an economic unit where purebred animals of all sex and age groups of only one breed are bred.
- gene pool farm, reserve: breeds animals of one or more breeds on different gene pool farms and provides for their purebred breeding.
- cryobank of genetic resources.

The main task of the cryobank of genetic resources of animals, unlike the existing banks at breeding enterprises, is the creation and long-term storage of necessary reserves of semen, embryos, ovocytes of special genetic value animals of local, endangered domestic and small collections of specialized foreign breeds and their use at:

- solving of the most important breeding tasks (breeding new breeds, types, crosses, lines, maintaining and improving the genealogical structure, consolidation, radical improvement of existing ones);
- providing purebred outbred breeding of animals, maintaining genetic diversity in closed populations of gene pools herds, farms, collectors of endangered and local aboriginal breeds and reproducers of specialized breeds;
- for future breeding and reproduction of purebred breeds of animals that have disappeared, if necessary, on the breeding stock of another breed.

The genetic resources cryobank is a research and production unit and is created at research centers that carry out gene pool assessment, determine the need, form and extent of its conservation and use for each breed. The purchase of genetic material is carried out at the expense of the State Budget.

6.6 Methods of laboratory reseaches at cytogenetic analysis

6.6.1 Description of working methods for preparation of specimens and their analysis with microscope

To detect genetic abnormalities, genetic control company (laboratory) provides cytogenetic control and testing of animals by DNA markers.

The cytogenetic control is a control of the state of animal' chromosomal apparatus, its integrity, the presence of structural and quantitative disorders of the karyotype.

The purpose of cytogenetic control is to determine the absence or presence of chromosomal, chromatid, genomic quantitative and qualitative changes and disorders.

The material for the production of chromosomal specimens in cattle, sheep, goats and horses is blood drawn from the jugular vein of the animal; in pigs blood from the ear vein is used for this purpose.

Obtaining of cytogenetic specimens

For blood samling from animals and cells cultivation medical vials with a volume of 30 ml with plastic screw caps, penicillin vials, sterile seals are used.

The preparation of vials is carried out in the following sequence:

1. the vials are washed with hot water using washing powder, trisodium phosphate or a mixture of 1 part of trilon B (hd), 2 parts of sodium hydroxide, and 10 litters of distilled water;
2. they are thoroughly rinsed first with running hot water, then with distilled water and left to soak in distilled water overnight;
3. dried in an oven (110 ° C);
4. boiled for 2 hours;
5. dried;
6. closed with cotton-gauze stoppers;
7. wrapped with parchment;
8. maintained at a temperature of 110 ° C for about 2 hours;
9. the treated vials are closed with clean, sterile penicillin caps, then they are covered with plastic lids with a small opening through which the necessary components are injected with a needle and syringe.

The setup and cultivation of culture are done in a sterile box. In the absence of box, these operations are performed in a normal clean room, which is irradiated with bactericidal wall lamps for 2 hours before starting work.

Setup of peripheral blood lymphocyte culture

0.5 ml of blood and nutrient milieu of the following composition are injected into the vials:

5 ml of milieu 199 (Earle, Eagle) or RPMI 1640;

1 ml of inactivated serum of bovine or human blood of group IV (the serum is inactivated in a water bath at 56 ° C for 1 hour);

0.01 ml PHA "P" or 0.1 ml PHA "M" or concanavalin A;

antibiotics at a concentration of 100 units of penicillinum and 100 μg of streptomycinum or 0.001 ml of gentamicinum per 1 of milieu.

The suspension is mixed, placed in a thermostat at 37 ° C for 48 hours.

Two hours before removal of the culture, colchicine is introduced into each vial at a final concentration of 0.05 μg / ml. The mother liquor of colchicine is prepared in distilled water (10 mg of dry colchicine per 100 ml of water) and stored at 4 ° C for 1-2 months. To obtain specimens enriched with cells in the early meta- and promethaphase stages, 2 hours before fixation (possible simultaneously with colchicine), an ethidium bromide solution is added at a final concentration of 5-10 μg / ml. Since this stage, the work in non-sterile conditions is permitted.

Removal of culture of peripheral blood lymphocytes and preparation of metaphase chromosomes

At the end of cultivation:

1. The contents of the vials are poured into centrifuge tubes and centrifuged for 10-20 min at 1000 turns / minute (the mode of centrifugation at all subsequent treatments is the same);
2. The supernatant fluid is aspirated or gently drained;
3. The precipitate is shaken, hypotonic solution (0.5%) KCl heated to 37 ° C is added to the test tube;
4. The cell suspension is mixed thoroughly using a rubber dropper pasteur pipette;
5. It is incubated for 10 min at 37 ° C;
6. Centrifuged;
7. The supernatant fluid is removed;

8. The cell suspension is fixed in a freshly prepared and cooled retainer (methyl alcohol, glacial acetic acid in a ratio of 3: 1);

9. The fixed cell suspension is left in the refrigerator at 4 ° C for a period of from 20 minutes to 20 hours;

10. The cell suspension is brought through 2-3 changes of fixer;

11. The obtained cell suspension is dissolved in a small amount of fresh fixator to a desired density;

12. 3-4 drops of cell suspension are put on degreased glass, the specimen is ignited or dried with a hot stream of air.

The quality of the obtained specimen is evaluated under a microscope. Chromosomes scatter is monitored in a darkened microscope field. If the scattering of chromosomes is insufficient, the suspension is brought through another change of the fixer, having changed the ratio of components to 2: 1 or adding a few drops of acetic acid into the test tube.

The routine staining of chromosomes specimens is performed using azure and eosin dyes. Both dyes are prepared in distilled water in the form of 0.15% solution and are stored in bottles of dark glass. The solutions are prepared in advance because "mature" solutions are more effective.

Just before staining a working solution is made: 6 parts of 0.15% ("mature") solution of eosin and 9 parts of distilled water. For coloring a ready-made Gimza dye and a ready-made azur-eosin, made according to the Romanovsky method can be used. For obtaining 0.5 ml of original dye, 10 ml of distilled water are needed.

The staining of specimens is carried out in the following order: the finished dye is put to a slide or the slide is placed into a glass with a dye and kept for several minutes. The color intensity is determined under a microscope.

At the visual analysis of cells under the microscope large chromosomal abnormalities are revealed, they are: centric mergers of autosomes, tandem mergers, chimerism of cells by sex chromosomes.

To establish the presence of centric merger, a positive conclusion for 5-10 metaphase plates is sufficient. To determine cell chimerism by sex chromosomes, it is necessary to find 2 X chromosomes in 10 metaphase plates.

To determine the numbers of chromosomes involved in centric fusion, a morphometric analysis on photographic prints is carried out are choosen. The metaphase plates with optimal chromosome helix and no overlays are choosen. The images of chromosomes are cut and glued in pairs.Tthe length of each chromosome is measured on several metaphase plates and its relative length to the total length of the entire haploid female (X-chromosome) chromosome set is determined.

At diagnosis of leucosis, the presence of short-lived hemocultures of mitoses in cytological specimens that are absent in healthy cattle, is the first sign showing the probability of this diseases.

The analysis of cells under the microscope starts with a small magnification of the microscope in 100 times, the location of metaphases is determined by coordinates of the substage. Then they are analyzed under immersion magnification of the microscope in 1000 times and microphotographed.

Registration of chromosomal distortions

The analysis of colored specimens is started under the magnification of the microscope in 100-200 times, with their overall assessment (mitotic index and quality of metaphase plates). Metaphases are best found and selected at low magnification. When selecting metaphase plates for viewing, some of them are excluded because they are not suitable for analysis under the following conditions:

- the chromosomes are superimposed on each other, which hampers their identification and counting of their total number;
- all chromosomes are not within the field of view of the microscope under the magnification in 900-1000 times;
- the chromosome are too long or very short;
- several random chromosomes are noticed in the field of view;
- the chromosomes are inhomogeneously colored, the quality of metaphase plate is poor, etc.

6.7 Methods of molecular – genetic analysis

6.7.1 Description of main methods of DNA and RNA isolation

The isolation of DNA and RNA is a important stage of samples preparing for diagnostic process, without it any of the following methods can't be performed: amplification, detection of amplification products, cloning, sequencing, hybridization etc.

Nucleic acids (NA) are an integral part of complex proteins - nucleoproteins, which are contained in all living cells of animals, bacteria, viruses, plants. Nucleic acids have strongly expressed acidic properties and are negatively charged at physical pH values. This explains one of the important properties of nucleic acids - the ability to interact by type of ionic bonding with major proteins (histones), ions of metal (mainly Mg^{2+}), as well as polyamines (spermine, spermidine) and putrescine.

Therefore, for the isolation of nucleic acids from complexs containing proteins, such strong and electrostatic bonds between positively charged protein molecules and negatively charged nucleic acid molecules must be broken.

If the source of nucleic acids is a sample of blood, semen or bioptate, then it is necessary to find the best isolation methods that allow to obtain maximum amount of product with high purity. When working with cell cultures, it is possible to use simpler methods of isolation, since the yield of NA increases pro rata with the volume of culture [I.V. Dzyublyk, 2012].

6.7.2 Technique of solution purification by organic extraction methods (using phenol, chloroform) with subsequent sedimentation of DNA alcohols and dissolution in water and TE-buffer. Methods of DNA differentiated sorption on solid carrier

Methods of nucleic acids isolation depend on the type of studied material, on its nature, caracter and properties of the object [O.S. Antonova, 2010; S. Herrington 1999].

Modern methods of NA isolation consist of three stages:

- destruction (lysis) of cell
- inactivation of nucleases
- cleaning.

Often, an ideal lysis procedure is a compromise of several techniques, it must be enough strict to destroy the structure of the source material (e.g., tissues) and, at the same time, it must be delicate to prevent NA destruction. The lysis procedure includes:

- Mechanical destruction (grinding, hypotonic lysis);
- Chemical treatment (e.g., lysis by means of detergents, chaotropic substances, or thymol recovery);
- enzymatic breal down of proteins (e.g., by proteinase K).

The processes of cell membranes destruction and intracellular nuclease inactivation can be combined. Nonspecific denaturing agents (ionic detergents, mercury hydroxide, guanindichloride, guanindinisothiocyanate, phenol, chloroform, etc.), as well as specific inhibitors, such as RNAase inhibitor from placenta are used as nuclease inhibitors. After cell lysis and nucleases inactivation, the cell mass is easily removed by filtration or precipitation.

One of the popular methods of DNA isolation is based on the use of cell lysis by a strong chaotropic agent - guanindinisothiocyanate (GuSCN) and subsequent sorption of DNA on a carrier. Guanindinisothiocyanate is a very strong denaturing agent used for the simultaneous lysis and denaturation of all cellular proteins (including RNAse). After a series of washes, DNA remains in the sample, it is sorbed on the carrier, from which it is easily removed by the eluting buffer. The method is convenient, technological and suitable for preparing a nucleic acid sample for amplification. At the same time, DNA loss can occur as a result of irreversible sorption on the carrier, as well as in the process of numerous washes. This fact is particularly important when dealing with small amounts of DNA in a sample. In addition, even small amounts of GuSCN can inhibit amplification reaction.

Nowadays a large number of different advanced techniques are present in molecular biology; they are based on cell lysis by guanindine isothiocyanate or guanidine chloride followed by sorption of nucleic acids on sorbent - particles of silicon dioxide; or use the following acid-phenol extraction of RNA and its landing with isopropanol. These techniques are used in different modifications of DNA or RNA isolation.

Nucleic acid isolation methods can be divided by their basic physical and biochemical characteristics into following classes:

- Liquid-phase methods
- Solid-phase methods

Liquid-phase methods

Classic isolation methods. Classic methods of NA isolation from complex original samples (blood, tissue) include the lysis of biological material by detergents or chaotropic agents, sometimes in the presence of enzymes that destroy proteins. This stage is followed by several steps whre organic solvents such as phenol, chloroform or ethanol are used. Complete separation of proteins from NA can be achieved by the addition of sodium perchlorate. For the separation of RNA from DNA, selective incubation with lithium chloride or specific non-nucleic isolation with guanidine chloride or guanidine thiocyanate combined with phenolic extraction or ethanol precipitation are used.

The given methods increase the probability of NA denaturation, loss of sample or cross-contamination of samples if several samples are processed simultaneously. When RNA is isolated, there is a very high risk of contamination from DNA present in the original sample. The standard method of obtaining a pure specimen is based on the fact that DNA is a polar molecule and is not soluble in organic solvents. Traditionally, phenol-chloroform extraction is used to isolate DNA. With stirring of cell lysate from phenol two phases are formed. DNA is in the upper (aqueous) phase, and denatured proteins are in the lower (organic) phase. The main disadvantage of this method is the lack of automation due to the stage of centrifugation of liquid extraction.

Methods for isolating DNA and RNA simultaneously. There are methods that can be used to isolate both DNA and RNA from a single test object (sample). Most of known methods are modifications of the original procedure proposed by Chirgwin et al. In this case strong chaotropic agents such as guanidine thiocyanate and cesium trifluoroacetate for simultaneous destruction of cell membranes and inactivation of intracellular ribonucleases (RNAse) are used. The factors limiting such techniques are ultracentrifugation and long analysis time I (16-44 hours). Methods for the simultaneous isolation of DNA and RNA, without the operation of centrifugation, also have an advantage due to phenol that acts as an effective deproteinizing agent that destroys cells and denatures proteins. For the efficient separation of high molecular weight DNA from RNA, phenolic extraction is performed at first and then two phenol-chloroform extractions are done to remove simultaneously proteins and lipids from the solution containing NA. In order to increase the yield of NAs, the components of the extracting buffer are optimized. For example, a certain pH of buffer (pH 7.9) in presence of detergent (0.2% sodium dodecyl sulfate) and a relatively low salt concentration (100 mM LiCl) make it possible to separate efficiently NA in the aqueous phase and to dissociate proteins; 10 mM EDTA does not allow formation of protein complexes and forms chelate complex with Mg^{2+}, thus inhibiting the action of magnesium-dependent nucleases.

Solid-phase methods

Basic principles of solid-phase methods. The following processes and principles are used in solid-phase nucleic acid isolation methods:

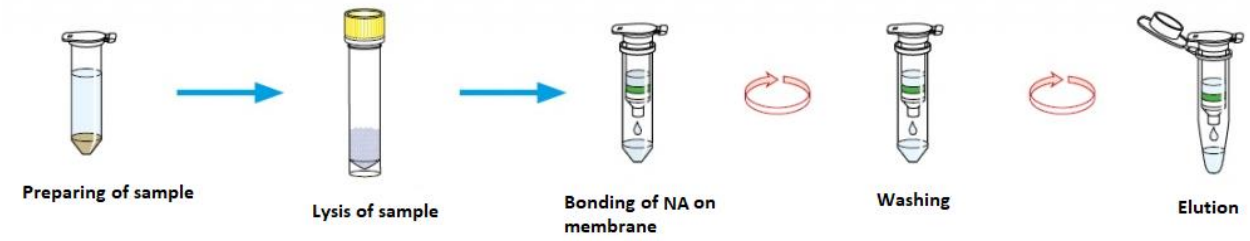
a) hydrogen bonds with an unmodified hydrophilic matrix (quartz) in chaotropic conditions;

b) ion exchange in aqueous solution, usually with the use of anion exchangers;

c) affinity;

d) size exclusion mechanisms.

Solid-phase systems that adsorb nucleic acids are quartz-based particles, glass fibers, anion-exchange carriers used in chromatographic separation columns. As an example, ready-made kits for the isolation of silica-membrane NAs manufactured by Macherey-Nagel (NucleoSpin), Qiagen (QiaAmp) and others can be mentioned.



Picture 6.38. Scheme of DNA isolation on spin columns with silica membrane

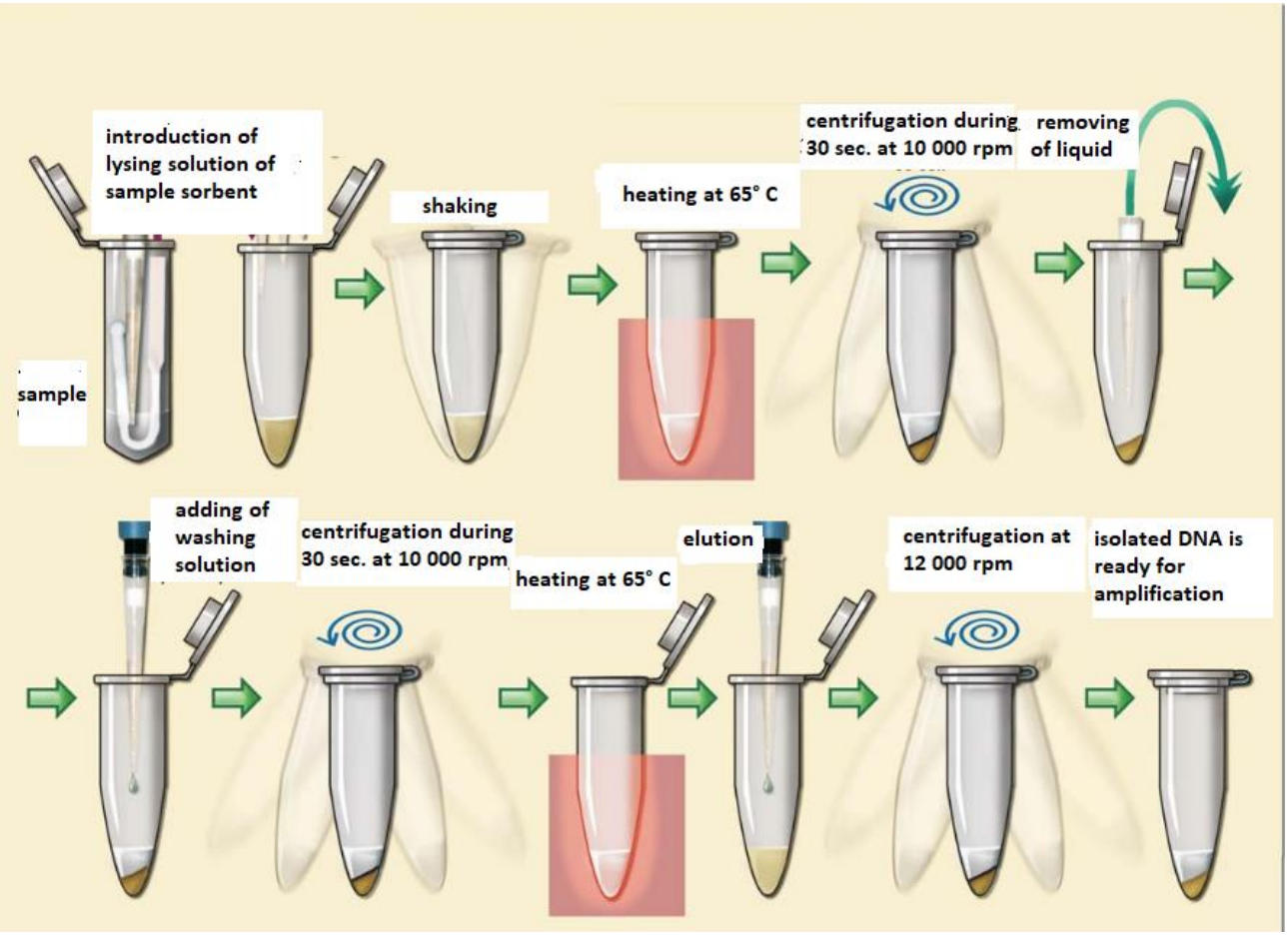
These carriers are used to isolate or purify nucleic acids with highly concentrated solutions of chaotropic salts (sodium iodide, sodium perchlorate, guanidine thiocyanate). The use of diatomaceous earth

as a sorbent is described, in this case the binding also occurs in the presence of chaotropic salt. Other methods are based on the joint detergency with materials binding nucleic acid or on the use of a solid sorbent with functional groups binding nucleic acids in combination with polyethyleneglycans and highly concentrated salts.

A well-known group of universal sample preparation methods is based on the use of Chilex affinity ion exchangers (low sorbent capacity), which, unlike glass, don't adsorb DNA but absorb impurities that impede the reaction. As a rule, this technology consists of two stages: boiling the sample, causing the distruction of cell walls or lysis by non-PCR-inhibiting detergents; and nucleic acids emerge into solution and sorption of impurities take place in ion-exchangers. The method is simple and easy to perform. At the same time, there are clinical specimens with impurities that cannot be removed by ion-exchangers. In addition, cell walls of some microorganisms are not susceptible to simple boiling. In these cases, it is necessary to apply additional stages of sample processing.

The nucleic acids isolation method proposed by Boom et al. is extremely convenient [Boom et al, 1990, Boomet al, 1999]. This method includes the stage of cell lysis by a strong chaotropic agent that destroys cell membranes and inactivates intracellular RNAses by guanidine-cyanate (GuSCN), followed by further sorption of NA onto the carrier (glass beads, diatomaceous earth, glass "milk" etc.) easily removed by eluting buffer. For example, silica-based kits are proposed by AmpliSense, Synthol and others.

The scheme of DNA isolation using such kits is shown in the **picture.**



Picture 6.39. Scheme of nucleic acids isolation based on silica

The nucleic acid binds back to the glass in the presence of a high concentration of chaotropic salts (e.g. guanidine chloride, guanidine thiocyanate). Under these conditions, protein binding to the matrix does not occur. Chaotropic compounds are substances that disrupt the ordered structure of water and thereby cause membranes to chaos (e.g., urea, sodium iodide). In addition to binding, chaotropic agents provide destruction of cell membranes and cell lysis with subsequent release of nucleic acid. The impurities are washed with chaotropic salt and chaotropic salt with 80% ethanol. The purified nucleic acid is removed from the glass with a low ionic strength buffer. Currently, many commercial firms offer for the isolation of nucleic

acids columns with a glass matrix. Methods using these columns include stages of centrifugation or vacuum aspiration.

Method based on magnetic separation. The use of magnetic solid carriers in biochemical and molecular biological processes has many advantages over non-magnetic separation methods. Typically, a magnet is applied to the test tube wall containing the sample to aggregate the particles near the test tube wall and the rest of the sample can be removed. In this way, it is possible to separate the components of the cell lysate that inhibit DNA polymerase and PCR reaction, for example, polysaccharides, phenolic components, humus. Magnetic carriers with immobilized affinity ligands or made of biopolymer, which increases the affinity for desired nucleic acid are used for the isolation process. Magnetic particles are made of various synthetic polymers, biopolymers, porous glass or based on inorganic magnetic materials such as iron oxide with a modified surface. Superparamagnetic particles that do not interact with each other in the absence of a magnetic field are particularly suitable for the isolation. These particles acquire a magnetic moment in a strong magnetic field, but do not retain permanent magnetism when the field is cleared. If the magnetic aggregation and adhesion of the particles are eliminated, then particle suspension and identical extraction of nucleic acids are achieved during the reaction.

Glass-coated magnetic particles are used for the automatic isolation of nucleic acids. The nucleic acid binds to the glass surface, then, bound to the particles, it undergoes the same stages of the extraction process as in the Boom technique: after a series of washes NA, sorbed on the carrier remains in the sample. It is easily removed from the sample using an elution buffer. The kits for isolation of NA on magnetic parts, manufactured by Ambioo, Macherey-Nagel (NucleoMag), Invitrogen (Dynabeads®) and others can be such example.

The method is convenient, technological and suitable for preparation of the sample for amplification, it can be reproduced on robotic pipetting workstations.

Nevetherless the possible lost of product can occur in result of irreversible sorption on carrier in process of numerous washing. This is especially important when dealing with small amounts of DNA in a sample. Gross DNA and RNA are isolated by the same magnetic particles. To separate RNA from DNA, RNA is destroyed by the state of DNA separation. The best option is to add RNAase or alkali. On the contrary, RNA can be isolated during the destruction of DNA by deoxyribonuclease (DNAase).

Typically, binder solutions are added to the magnetic media to bind selectively nucleic acids. For example, both viral proteins and complementary DNA or RNA sequences can be used to bind viral nucleic acids.

There are commercial magnetic particles with oligodeoxythymidines immobilized on their surface for efficient and rapid isolation of highly purified template RNA (mRNA) from eukaryotic cell cultures or gross RNA isolation. The isolation method is based on the hybridization of oligodeoxythymidine sequence by a stable polyadenylated 3-terminal of eukaryotic mRNA. The length of the complementary sequence is 20-30 oligonucleotides. This sequence either directly covalently binds to the surface of the particle, or indirectly, through the biotinylation of oligonucleotides by interaction between streptavidin-coated particles. The number of manufacturers of magnetic particles is constantly increasing, so for this task it is easy to find a convenient method.

Method of differential sorption of impurities by selective DNA precipitation and washing in TE-buffer using a set of Diamond DNA reagents. The technique of DNA isolation using DiamondDNA involves lysis of tissue into lysis buffer, sorption inhibitors of enzymatic reactions from solution and DNA from solution using a highly efficient DNA precipitator. The basic simplicity and low duration of the process of DNA isolation and purification is achieved through the use of selective sorting. A highly efficient selective adsorption of examples is used to provide unique DNA purity indicators (OD260 / OD280 not less than 1.8). Protein residues in purified DNA cannot be identified by differential staining and subsequent fluorimetry. With DiamondDNA, a high degree of purification from such strong PCR inhibitors as polyphenols and polysaccharides is achieved. The DNA content of the solution after purification is directly proportional to the content of nucleic acids in the original object, which increases the accuracy when using quantitative methods of analysis (RealTime PCR and endpoint detection). Unlike widely used methods and kits based on the sorption of DNA molecules on silica particles (in suspension or on columns), DNA purification using DiamondDNA eliminates strong degradation of nucleic acid molecules. As a result, the length of the fragments is more than 20,000 pairs of nucleotides.

Advantages of using a DiamondDNA reagent kit:

- The use of an additional deposition stage allows to obtain a higher quality of cleaning. During the use of DiamondDNA, there is no direct interaction of DNA molecules with the sorbent, which allows to maintain the integrity of the double helix.

- Accidental entry of sorbent into the PCR mixture does not block the amplification.

- The use of proteases is optional and is used to extract DNA from degraded material.

- There are no toxic substances and no components prohibited for free circulation in the kit.

- The isolated DNA using the DiamondDNA kit is ready for further hybrid analysis, PCR staging and sequencing.

It should be noted that nucleic acid isolation methods are continuously improved to reducing the number of manipulations and should ideally consist of one or two procedures. In molecular biology, a number of advanced methods of separating nucleic acids into fractions from total specimen are applied. They can be used to obtain purified nucleic acids specimens and for subsequent amplification reactions. They are chromatographic purification methods: reversed, ion-exchange (as adsorbents DEAE-cellulose, DEAE-Sephadex are used), distributive, gel-permeable chromatography; chromatography of sample on calcium phosphate gel and per affinity on protein carriers; ultracentrifugation in sucrose density gradient, filtration through agarose gels, gel electrophoresis.

6.8 Methods for analysis of obtained genetic dates and their verification

6.8.1 Methods of mathematical processing of obtained dates to confirm working hypothesis.

The main purpose of using markers in breeding is to identify so-called *quantitative trait loci* (QTLs), i.e. regions of genome that statistically significantly affect the variability of quantitative traits. The usual Marker-Asisted Selection (MAS) scheme consists of three steps:

1) detection of one or more QTLs;

2) identification of corresponding gene (causal mutation);

3) increasing the frequency of a favorable allele either by selection method or by introgression (hybridization, penetration of genes of one species into genetic fund of another).

In addition to the marker-assisted selection (MAS), the contrast marker-assisted introgression (MAI) is used. It is based on tandem selection under the backcross program, when the first stage uses donor breed alleles located within or adjacent to the gene that determines the desired trait (mostly resistance to certain diseases), and at the second stage, the backcross is performed in order to have back alleles of the recipient breed.

The examples of genetic tests used commercially are shown in Table 6.3.

Table 6.3 .

Commercial genetic tests used in the animal breeding,

(Dekkers J.C.M.,2004)

Feature category	Marker	Animal species

Hereditary defects	BLAD	dairy cattle
	Citrulinaemia	dairy and meat cattle
	DUMPS	dairy cattle
	CVM	dairy cattle
	Maple syrup urine	dairy and meat cattle
	Mannosidosis	dairy and meat cattle
	RYR	pigs
Exterior	CKIT	pigs
	MC1R/MSHR	pigs, dairy and meat cattle meat cattle
	MGF	
Quality of milk	κ-Casein	dairy cattle
	β-lactoglobulin	dairy cattle
	FMO3	dairy cattle
Quality of meat	RYR	pigs
	RN/PRKAG3	pigs
Feeds intake	MC4R	pigs
Diseases	Prp	sheep
	F18	pigs
Reproduction	Booroola	sheep
	Inverdale	sheep
	Hanna	sheep
Growth and composition of meat	MC4R	pigs
	IGF-2	pigs
	Myostatin	meat cattle
	Callipyge	sheep
Milk yeald and milk composition	DGAT1	dairy cattle
	GRH	dairy cattle
	κ-Casein	dairy cattle

This system includes procedures for collecting and accumulating DNA samples, genotyping and analyzing dates based on the phenotype of animals, as well as daily decision making (which animals should be genotyped or regenotyped in case of errors), which animals should be evaluated by phenotype, etc. (Meuwissen Th., BJHayes DNA ME Goddard 2016).

For the verification of the received dates (from Lat.verum-true, facere-to do), i.e. verification of theoretical positions by comparison of different groups (control, experiment), the variance analysis is often used.

The variance analysis is an independent and important part of the biological statistics. It can be used to establish the role of some factors in the variability of a particular trait, to decompose general variability of trait into components caused by specific studied factors (age of parents, size of parents, breed, level of feeding, etc.), as well as caused by random, uncontrolled factors (temperature, lighting, etc.). Thus, the essence of the variance analysis is to study the statistical effect of one or more factors on resulting trait.

Effective (resulting) trait (e.g. milk yield, multiplicity, multiplicity of piglets), is studied as a result of the influence of two different factors:

1. Organized in researches - different breeds, animals belonging to a specific genetic group (marker group), feeding level, etc. ($\overline{x}$);

2. Not organized, that is, random, not considered in studies *($\overline{z}$)*;

In zootechnics, the term "variance" means the presence of different values of trait at different individuals, grouped in studied animals, as well as the extent that determines the degree of this diversity (in our case, genetic group). The variance is indicated by C. The overall variance is used at variance analysis in one or another group of animals.

The total variance of the sign (C_y) in studied group of animals is broken down into its components:
a) factorial variance (group variance –Cx) caused by organized factors;
b) random variance (residual Cz variance) caused by other factors unorganized in this experiment.

The sum of factorial and random variance is always equal to total variance:
Cx+ Cz=C_y

To obtain reliable dates of variance analysis when constructing dispersion complexs (especially bi- and multifactorial), the following operations must be performed correctly:
1. *Selection of factors*. Factor is any manifestation of a trait whose impact on the productive trait (live weight, milk productivity, multiplicity, etc.) is to be studied
When organizing bi- and multifactor complexs, the free choice of factors for research is limited by the requirement of complete independence between them, i.e. they should not correlate. For example, mass and size of animal, heart girth and weight of animal, multiplicity and size of nest, etc. cannot be taken. The independent factors may be: gender of animals, breed of animals, belonging to marker group, temperature and humidity of room, etc.
2. *Distribution of factors by graduation.* For one-factor and bi- and multi-factor complexs, they can have quantitative (number of stimulants in grams, feeding rate + 20% or - 20% to the norm, etc.) and qualitative gradations (sex - male and female; fattening in pigs: fat, meat, bacon; grease - red-rimmed, light-pale).
3. *Selection of individuals.* The results of variance analysis largely depend on the correct selection of individuals in both quantity and quality. Individuals selected by quality should reflect general population for which the study is being conducted. Therefore, according to the quality (value of resulting (resultant) trait), individuals should be selected on a random basis. Breach of this principle always leads to wrong conclusions.

Quantitatively individuals can be distributed by factors grading in different ways: evenly (1: 1), proportionally (1: 2), which facilitates variance analysis; and unevenly (1: 1,66), which complicates calculations.
*4. Transformation of values of resultant trait.*To facilitate the calculations, inconvenient values of resultant (resulting) trait (multidigit, fractional) can be transformed into convenient ones (lessdigit, expressed in integers).
a) All values of trait can be multiplied by the same number: 0.30 × 100 = 30 or 0.45 × 100 = 45; the end result must be corrected (divide by 100).
b) All the values of gradation trait can be divided by the same number (by 2, 3 or 4) and corrected at the end, i.e. multiplied by the number by which it was divided;
c) You can subtract the same number from all values of resultant trait (it is better to subtract the smallest value of the trait). In the end, the correction should be made only for the arithmetic mean M, adding to them the number previously removed.

5. Technique of calculations at variance analysis. The calculation of dispersion complexs is carried out by special working formulas. However, the calculation technique for small and large groups is different. For small groups (samples) dispersions are calculated more easily, for large groups - a little more difficult. The group of 40-50 variants (multidigit traits) is already considered as large.

Monofactorial complex

It is a complex in which the effect on the resultant (resulting) trait of one factor (the level of protein supply of dams as a factor, multiplicity as resultant trait) is studied.

The technique of calculation of monofactorial complex for small groups can be shown in the following example: to study the effect of a protein additive of animal origin on the multiplicity of sows, if three gradations of this factor are established: first group - 0%, second group - (+ 10%) and third group - (+ 20%) of supplement to the daily norm of digestible protein. The sows' multiplicity during the experimental period was determined as a resultant (resulting) trait. For each gradation of the factor (0%; + 10%; + 20%), two individuals (sows) were retained by the randomness principle, which during the experiment showed different multiplicity, that is expressed in the following numbers: first group 14 and 6; second one - 20 and 16; third one - 10 and 6 piglets. These numbers can be easily converted by subtracting the minimum multiplicity (6 piglets), in result the dates obtained (values of the resultant (resulting) trait), become more convenient for the calculation (first group - 8 and 0, second one - 14 and 10, and third one - 4 and 0).

Table 6.4.

Technique of calculation for variance analysis of monofactorialial for small samples

Symbol	Trait	Gradation of factor			r number of gradations
		0	10	20	
υ	Value of resultant trait, dates	8; 0	14; 10	4; 0	$\Sigma\upsilon = 36$
υ^2	Square of dates	64; 0	196; 100	16; 0	$\Sigma\upsilon^2 = 376$
n	Frequences – number of sows	2	2	2	$n_x=6$
$\Sigma\upsilon$	Sum resultant trait values	8	24	4	
$(\Sigma\upsilon)^2$	Square of sum of productive trait values	64	576	16	
$h=\frac{(\Sigma\upsilon)^2}{n}$	Share of division	32	288	8	Σh=328
$M=\frac{\Sigma\upsilon}{n}$	Share of mean value of resultant trait	4 + (6)	12 + (6)	2 +(6)	M=6+(6)
M	Mean multiplicity of sows (heads)	10	18	8	M=12

In this example, the private mean values are obtained in the converted form: 6 units less than the true ones. To recover the actual value of the resultant (resulting) trait, it is necessary to add to each mean (M) the value 6, - - (4 + 6); (12 + 6); etc. Then we get the actual mean multiplicity by factor gradations: 10.18 and 8 piglets.

Thus, it was found that a protein additive in the amount of 10% of the norm has more positive effect on sows multiplicity than additive of 20% of the norm. For the final conclusion, it is necessary to determine degree or strength of the factor impact on the productive trait.

To determine the degree of organized factor impact it is necessary to find the ratio of factorial variance to total one ($\frac{Cx}{Cy}$). The essence of the calculations made in this table is to calculate the indices ($\Sigma\upsilon^2$ = 376; Σh = 328) used in a special working formulas: general (Cy), factorial (Cx) and random variance (Cz).

For the calculation of general, factorial and random variance it is necessary to determine:

1) the auxiliary value *H* by squaring the total of resultant indices and dividing this value by number of individuals *(n)*

$$H=\frac{(\Sigma\upsilon)^2}{n}=\frac{(36)^2}{6}=\frac{1296}{6}=216$$

2) the value of total variance of resultant (resulting) trait by the following formula:

$Cy = \Sigma v^2$ - H = 376 - 216=160;

3) the value of random variance

$Cz = \Sigma\upsilon^2 - \Sigma h = 376 - 328 = 48$;

4) the value of factorial variance

$Cx = \Sigma h - H = 328 - 216=112$;

5) the impact degree of the organized factor on resultant (resultimg) trait

$$\eta^2=\frac{Cx}{Cy}$$

6) the impact degree of unorganized (random) factors $\eta^2=\frac{Cz}{Cy}$

The results summary of factorial complex of variance analysis for small samples is presented in Table 6.5.

Table 6.5.

Results summary of variance analysis for small samples

Indices		*x*	*z*	*y*
$Cy = \Sigma\upsilon^2$ - H = 376 - 216=160	*C*	112	48	160
$Cz = \Sigma\upsilon^2 - \Sigma h = 376 - 328 = 48$	η^2	0,70	0,30	1,00
$Cx = \Sigma h - H = 328-216=112$ Check: *Cx + Cz = Cy= 112+48=160*	%	70	30	100
Number of degree of freedom	υ	υ_x = l-1 = 3-1=2	$\upsilon_z = n-l = 6-3=2$	$\upsilon_y = n-1 = 6-1=5$
Corrected variance (deviante)	σ^2	$\sigma_x^2=\frac{Cx}{\upsilon_x}=\frac{112}{2}=56$	$\sigma_z^2=\frac{Cx}{\upsilon z}=\frac{48}{3}=16$	
Reliability coefficient F		$F=\frac{\sigma_x^2}{\sigma z^2}=\frac{56}{16}=3,5$		

Table values F 0,99 = 30,8 { at 0,95 = 9,6; 0,999 = 148,5

The summary of this analysis can be expressed as follows:

- impact of organized factor (protein level on sows multiplicity) - 70%;

- impact of unorganized factor (all other factors on dams fertility) - 30%;

– impact of all factors – 100%. Further we define the number of freedom degrees *(v)*. For *Cx* it is equal to the number of classes according to factor l *-1* or $\upsilon_x = l - 1 = 3-1=2$.

The the residual variance Cz the number of freedom degrees is defined by the difference between the number of observations *n* and the number of classes *l*, i.e., $\upsilon_y = \upsilon_x - \upsilon_z = 6 - 3 = 3$.

The nimber of freedom digrees to the general varialbility is equal to the observations number *(n)* without one, or $\upsilon_y = = n - 1 = 6 - 1=5$.

The sum of freedom degrees should give their number to the general varialbility, i.e. $\upsilon_y = \upsilon_x + \upsilon_z$

Next, we calculate the corrected variance (so called deviante – σ^2). For this, every variance (factorial and residual) is divided by correspondent number of freedom degrees

$\sigma_x^2=\frac{Cx}{\upsilon_x}=\frac{112}{2}=56$; $\sigma_z^2=\frac{Cx}{\upsilon_z}=\frac{48}{3}=16$

Then, we determine the reliability of factorial variance. To determine the reliability of factorial variance and the impact of variability factor of trait by dividing the factorial variance by residual one we calculate Fisher's reliability coefficient:

$F=\frac{\sigma_x^2}{\sigma_z^2}=\frac{56}{16}=3,5$

Next, we compare the calculated value of F with its value in the table (Annex II). It should be remembered that in the leftmost column we find value $\upsilon_2=\upsilon_z$ (if it is 3), and in the upper title line we find value $\upsilon_1=\upsilon_x$ (if in the example, it is 2). At the intersection of these row and column we find value of tabulated F at three levels of probability:

0.95 = 9,6;

0,99 = 30,8;

0,999=148,5.

As F is equal to 3, 2, the impact of the studied factor on the resultant (resulting) trait is unreliable.

Task 1. To establish the impact of the breed factor on the digestibility of nutrients (resultant (resulting) trait) if, by the principle of random sampling, for each gradation of the factor three individuals (gilts) were selected. They have the following indices of the coefficient of digestibility of the protein: large white breed - 78.33; 76.70; 76.37%; large white × landrace - 81.20; 82.16; 83,50%

Table 6.6.

Technique of calculation for variance analysis of one-factor complex for small samples

Indices	Breed	
	A_1 (large white)	A_2 (large white × landrace)
v value of resultant trait	78,33 76,70 76,37	81,20 82,16 83,50
v^2 – square of values (dates)	6135,59 5882,89 5832,37	6593,44 6750,26 6972,25
n – volume of gradation	3	3
Σv –sum of dates	231,40	246,86
$(\Sigma v)^2$– square of dates sum	53545,96	60939,85
$h=\frac{(\Sigma v)^2}{n}$	17848,65	20313,28
$M=\frac{\Sigma v}{n}$	77,13	82,29

To calculate total, factorial and random variance, it is necessary to determine $H=\frac{(\Sigma v)^2}{n}=\frac{(478,26)^2}{6}=\frac{228732,62}{6}=$ 38122,1

Cy = Συ² - H = 38166,80 - 38122,1 = 44,70;

Cz = Συ² - Σh = 38166,80 - 38161,93 = 4,87;

Cx = Σh – H = 38161,93 - 38122,1 = 39,83;

Cx+ Cz= Cy; Cy = 39,83 + 4,87 = 44,70.

Table 6.7.

Summary of the variance analysis results of monofactor complex for small samples

$H=\frac{(\Sigma v)^2}{n}=\frac{(478,26)^2}{6}=\frac{228732,62}{6}=38122,1$		*Variances*		
		x	*z*	*y*
Cy = Συ² - H = 38166,80 - 38122,1 = 44,70	*C*	*39,83*	*4,87*	*44,70*
Cz = Συ² - Σh = 38166,80 - 38161,93 = 4,87	η^2	$\frac{Cx}{Cy}$	$\frac{Cz}{Cy}$	$\frac{Cy}{Cy}$

Cx = Σh – H=38161,93 - 38122,1=39,83	*η²*	*0,8911*	*0,1089*	*1,00*
Cx+ Cz= Cy; Cy = 39,83 + 4,87= 44,70	*%*	*89,11*	*10,89*	*100*
Number of freedom degrees	*υ*	υ_x *= l-1 = 2-1=1*	υ_z *= n-l = 6-2=4*	υ_y *= n-1 = 6-1=5*
Corrected variance (deviant)	*σ²*	$\sigma_x^2=\frac{Cx}{\upsilon_x}=\frac{39,83}{1}=39,83$ $\sigma_z^2=\frac{Cx}{\upsilon z}=\frac{4,87}{4}=1,22$		
Reliability coefficient Tabulated values	$F=\frac{\sigma_x^2}{\sigma z^2}=\frac{39,83}{1,22}=32,64$ *F* $\begin{cases} 74,1 - P_3 = 0,999 \\ 21,2 - P_z = 0,99 \\ 7,7 - P_z = 0,95 \end{cases}$			

Therefore, in this example, a reliable impact of the factor on the resultant (resulting) trait (at P> 0.99) was established.

Bi-factorial complexc

The structure of bi-factorial complexes is more compound than of mono-factor complexes. To facilitate the calculations in bi-factor complexes, it is necessary that studied factors were independent each of other and frequencies were proportional (to gradations).

When studying the action of more than one factor, it is necessary to consider the impact of factor A and factor B on resultant (resulting) trait, but also their joint impact AB on varying trait.

Therefore, the variance analysis of bi-factorial complex should determine not only *Cy, Cx,* C_A*,* C_B*,* C_Z, but also the variance of C_{AB}. Indicises *Cy, C* and C_Z are calculated in the same way as in the mono-factorial complex.

To calculate factorial variance $C_A$and C_B, the dates processing is somewhat new. To obtain $\Sigma h_A$and Σh_A, the lattices, separated for factors *A* and *B*, are processed, compiling utility tables. In uniform and proportional complexes the variance of factors joint action is calculated $C_{AB} = Cx - C_A - C_B$

General variance Cy = $\Sigma\upsilon^2 - H$

Factorial variance $Cx = \Sigma h - H$

Variance from factor A $C_A = \Sigma h_A - H$

Variance from factor B $C_B = \Sigma h_B - H$

Example. To determine the effect of herbal flour additive *(B)* on sows multiplicity, two gradations of this factor are set (B_1) - first group 0%, (B_2) - second group + 25%, to daily diet according to nutrient value,

Factor *A* – breed of sows, its gradation;

A_1 – large white;

A_2 – myrgorodska.

Two individuals (pregnant sows) were selected for each factor gradation, that showed the following multiplicity over the experimental period:

A_1 – large white	B_1 I group 8 and 11 pigs
	B_2 II group 9 and 13 pigs

A_2 – myrgorodska	B_1 I group 10 and 15 pigs
	B_2 II group 11 and 17 pigs

First factor A	Second factor B	Resulting trait

Let’s transform the numbers of the resultant (resulting) trait by subtracting 5 from each value of the dates; as a result we will obtain the dates more convenient for calculation

B_1 – 3; 6	A_1
B_2 – 3; 8	
B_1 – 5; 10	A_2
B_2 – 6; 12	

Table 6.8.

Calculation technique at variance analysis of bi-factorial complex for small samples

Indices	Breed (A)				*A = 2* *B = 2*	*Factors*	*n*	*Σv*	*(Σv)²*	$h=\frac{(\Sigma v)^2}{n}$	$M=\frac{\Sigma v}{n}$
	Large white		Myrgorodska								
	Herbal flour (B)										
	B_1	B_2	B_1	B_2							
v	6; 6	4; 8	5; 10	6; 12	*Σv = 54*	A_1	4	21	441	110	5 (+5)
v^2	9; 36	16; 64	25; 100	36; 144	*(Σv)² = 430*	A_2	4	33	1089	272	8 (+5)
n	2	2	2	2	$H=\frac{(54)^2}{8}$ *= 365*		8	54		$\Sigma h_A=\frac{(\Sigma v)^2}{n}$ *= 382*	
Σv	9	12	15	18		B_1	4	24	576	144	6 (+5)
(Σv)²	81	144	225	324		A_2	4	30	900	225	7 (+5)
$h=\frac{(\Sigma v)^2}{n}$	40*	72	112	162	*Σh = 386***		8	54		Σh_B = 369	

*81/2 =40,5 – we approximate to 40 for easier calculation

** Results of the five values (underlined) will be used in the calculation formulas of variance analysis.

Table 6.9.

Summary of variance analysis results of bi-factorial complex for small samples

Indices	*Letter code*	*For factor of breed A*	*For factor of herbal flour B*	*Joint action of both factors AB*	*Variance*		
					x factoraial	*z random*	*y general*
Cy = $\Sigma\upsilon^2$ - H = 430-365=65							
$Cz = \Sigma\upsilon^2 - \Sigma h$ = 430 - 386 = 44							
$Cx = \Sigma h - H$ = 386 – 365 = 21	*C*	*17*	*4*	*0*	*21*	*44*	*65*
$C_A = \Sigma h_A - H$ =382-365=17							
$C_B = \Sigma h_B - H$	η^2	*0,26*	*0,06*	*0,00*	*0,32*	*0,68*	*1,00*
$C_{AB} = Cx - C_A - C_B$ = 21-17-4 = 0	η^2	$\frac{Ca}{Cy}$	$\frac{CB}{Cy}$	$\frac{CAB}{Cy}$	$\frac{Cx}{Cy}$	$\frac{Cz}{Cy}$	$\frac{Cy}{Cy}$

Find the impact degree of factor A, B, AB and the ratio of factorial variance to total.

The general conclusion of this analysis can be expressed by the following way: breed impact – 26%; impact of herbal flour additives – 6%; impact of joining breeds with of herbal flour additives – 0%; cumulative effect of organized factors – 32%; effect of unorganized factors – 68%. Impact of all factors – 100%.

Find the impact degree of factor A, B, AB and the ratio of factorial variance to the general, e.i., total (general) impact of factors organized in the experiment on multiplicity.

Chapter 7. Sensory analyses

7. 1. Introduction to sensory analysis

7.1.1 Application of sensory analysis in modern food technology

In modern conditions of increasing competition in the food market, sensory analysis is becoming a very important and indispensable tool not only for quality control, but for marketing as well. Organoleptic attributes of a food product have been considered as important factors since the beginning of the food industrialization process due to their influence on its overall quality.

According to ISO 5492:2008 "Sensory analysis -- Vocabulary" sensory analysis is science involved with the assessment of the organoleptic attributes of a product by the senses (sight, smell, taste, touch and hearing). So, it is used to characterize and measure appearance, aroma, flavour and texture characteristics, as well as sound characteristics (in particular cases), of food products. What's more, four variables affect sensory evaluation: food, people, testing environment and test methods applied.

Sensory analysis can be used for many different purposes. Some sensory analysis questions have the objective of describing characteristics of a product and/ or measuring any differences that are found between products. Other sensory analysis questions have the objective of describing the liking or acceptability of a product. For an instance: «What does this product taste like?», «What are the three most important texture attributes you perceive in this product?», «What are the sensory differences between product A and B?», «Do you like this product?», «How much do you like this product on a scale of 1 to 9?», «Is this product acceptable?», «What do you like most about this product?», «Is product A better than product B?», «Which of the three products A, B, or C do you prefer?».
Given objectives mentioned the aims of sensory analysis in modern food technology can be defined as following:

1. to detect sensory differences among products;
2. to describe qualitatively and quantitatively sensory attributes of food
products in quality control, product development, market research, communication;
3. to evaluate the sensory quality of food: absence of defect and conformity to given standards;
4. to study consumer affective responses to food: food acceptance and preference;
5. to study the relationship between organoleptic properties and consumer affective responses to food (optimization, differentiation, innovation);
6. to study the relationship between chemo-physical composition and sensoryproperties of food (including the effect of operational conditions in food processing).

Sensory analysis is a natural science. The measurements of food sensory attributes should be taken carefully in full accordance with the standardised methodologies. In this case, sensory information can provide great insight into the world. Otherwise, when measures are done poorly they do more to mislead than to inform.
Careful controls must be implemented and followed when conducting sensory analysis, including compliance with the procedure of samples presentation, elimination of subjectivity and bias, application of methods that require panelists to demonstrate their ability rather than relying upon self-reports. Failure to adhere to any of these controls diminishes the value of the sensory data collected. On the other hand, determining appropriate controls and ensuring they are in place will result in reliable data about food which no instrument can measure i.e. their eating quality.

7.1.2. Basic vocabulary and terms

All the vocabulary and terms of sensory analysis are given here in accordance with ISO 5492:2008 "Sensory analysis -- Vocabulary". General terminology includes the following definitions:

- **sensory** (adj) - relating to the use of the senses, i.e. to the experience of a person
- **attribute** (n) - perceptible characteristic
- **organoleptic** (adj) - relating to an attribute perceptible by the senses, i.e. to an attribute of a product
- **sensory assessor** (n) - any person taking part in a sensory test. There are two possibilities: a naive assessor is a person who does not meet any particular criterion and an initiated assessor who has already participated in a sensory test
- **selected assessor** (n) - assessor chosen for his/her ability to perform a sensory test
- **expert** (n) - in the general sense, a person who, through knowledge or experience, has competence to give an opinion in the fields about which he/she is consulted
- **expert sensory assessor** (n) - selected assessor with a demonstrated sensory sensitivity and with considerable training and experience in sensory testing, who is able to make consistent and repeatable sensory assessments of various products
- **sensory panel** (n) - group of assessors participating in a sensory test
- **panel training** (n) - series of sessions in which assessors are oriented to the tasks to be completed by a sensory panel in assessing particular product(s), which may include relevant product characteristics, standard rating scales, techniques of evaluation and terminology

- **panel consensus** (n) - agreement among assessors regarding terminology and intensity of product characteristics
- **consumer** (n) - person who uses a product
- **taster** (n) - assessor, selected assessor or expert who evaluates the organoleptic attributes of a food product, mainly with the mouth
- **tasting** (n) - sensory assessment of a food product in the mouth
- **product** (n) - matter, edible or otherwise, which can be evaluated by sensory analysis
- **sample** (n), **sample of product** (n) - specimen or aliquot of product presented for assessment
- **test sample** (n) - sample of the material under test
- **test portion** (n) - portion of the test sample which is directly tested by the assessor
- **reference point** (n) - selected value (of one or several attributes or of a product) against which samples are assessed
- **control sample** (n) - sample of the material under evaluation, chosen as a reference against which all other samples are compared
- **reference sample** (n) - stimulus/substance, sometimes different from the material under test, carefully selected to define or illustrate an attribute or a specified level of a given attribute to which all others are to be compared
- **hedonic** (adj) - relating to like or dislike
- **acceptability** (n) - degree to which a stimulus is liked or disliked, overall or for particular sensory attributes
- **preference** (n) - selection, by an assessor, of one stimulus or product over others in a given set based on hedonic criteria
- **aversion** (n) - feeling of repulsion provoked by a stimulus
- **discrimination** (n) - act of qualitative and/or quantitative differentiation between two or more stimuli
- **discriminating ability** (n) - sensitivity, acuity, ability to perceive quantitative and/or qualitative differences
- **appetite** (n) - physiological and psychological state expressed by the desire to eat and/or to drink
- **appetizing** (adj) - describes a product capable of exciting the appetite of the individual
- **palatability** (n) - quality of a product which makes it pleasant to eat or drink
- **psychophysics** (n) - study of relationships between measurable stimuli and the corresponding sensory responses
- **olfactometry** (n) - measurement of the response of assessors to olfactory stimuli (refers to the assessors)
- **olfactometer** (n) - apparatus used to present olfactory stimuli to assessors under reproducible conditions
- **odourimetry** (n) - measurement of the odour properties of substances (refers to the products)
- **odourant** (n) - substance whose volatiles can be perceived by the olfactory organ (including nerves)
- **quality** (n) - collection of features and characteristics of a product, process or service that confer its ability to satisfy stated or implied needs
- **quality factor** (n) - one feature or characteristic chosen among others to assess the overall quality of a product
- **attitude** (n) - disposition to respond in a given way toward a class of objects or ideas
- **mastication** (n) - act of chewing, grinding and comminuting with the teeth

For terminology relating to the senses, organoleptic attributes and methods appeal to ISO 5492:2008 "Sensory analysis -- Vocabulary".

There are 39 ISO standards of sensory analysis that are currently relevant. They cover all the necessary points regarding vocabulary, organisation of test rooms, panelists, methodology etc. The full list of them is presented below. However, it is all the time reviewed and supplemented (see the list of ISO under development below).

ISO standarts in sensory analysis:

1. ISO 5492:2008 Sensory analysis -- Vocabulary
2. ISO 20613:2019 Sensory analysis -- General guidance for the application of sensory analysis in quality control
3. ISO 8586:2012 Sensory analysis -- General guidelines for the selection, training and monitoring of selected assessors and expert sensory assessors
4. ISO 11037:2011 Sensory analysis -- Guidelines for sensory assessment of the colour of products
5. ISO 8589:2007 Sensory analysis -- General guidance for the design of test rooms
6. ISO 3591:1977 Sensory analysis -- Apparatus -- Wine-tasting glass
7. ISO 16657:2006 Sensory analysis -- Apparatus -- Olive oil tasting glass
8. ISO 13300-1:2006 Sensory analysis -- General guidance for the staff of a sensory evaluation laboratory -- Part 1: Staff responsibilities
9. ISO 13300-2:2006 Sensory analysis -- General guidance for the staff of a sensory evaluation laboratory -- Part 2: Recruitment and training of panel leaders
10. ISO 6658:2017 Sensory analysis -- Methodology -- General guidance
11. ISO 11132:2012 Sensory analysis -- Methodology -- Guidelines for monitoring the performance of a quantitative sensory panel
12. ISO 8588:2017 Sensory analysis -- Methodology -- "A" - "not A" test
13. ISO 4120:2004 Sensory analysis -- Methodology -- Triangle test
14. ISO 10399:2017 Sensory analysis -- Methodology -- Duo-trio test
15. ISO 5495:2005 Sensory analysis -- Methodology -- Paired comparison test
16. ISO 8587:2006 Sensory analysis -- Methodology -- Ranking
17. ISO 13299:2016 Sensory analysis -- Methodology -- General guidance for establishing a sensory profile
18. ISO 11036:1994 Sensory analysis -- Methodology -- Texture profile
19. ISO 16820:2004 Sensory analysis -- Methodology -- Sequential analysis
20. ISO 29842:2011 Sensory analysis -- Methodology -- Balanced incomplete block designs
21. ISO 13302:2003 Sensory analysis -- Methods for assessing modifications to the flavour of foodstuffs due to packaging
22. ISO 13301:2018 Sensory analysis -- Methodology -- General guidance for measuring odour, flavour and taste detection thresholds by a three-alternative forced-choice (3-AFC) procedure
23. ISO 5496:2006 Sensory analysis -- Methodology -- Initiation and training of assessors in the detection and recognition of odours
24. ISO 11056:1999 Sensory analysis -- Methodology -- Magnitude estimation method
25. ISO 5497:1982 Sensory analysis -- Methodology -- Guidelines for the preparation of samples for which direct sensory analysis is not feasible
26. ISO 11136:2014 Sensory analysis -- Methodology -- General guidance for conducting hedonic tests with consumers in a controlled area
27. ISO 3972:2011 Sensory analysis -- Methodology -- Method of investigating sensitivity of taste
28. ISO 16779:2015 Sensory analysis -- Assessment (determination and verification) of the shelf life of foodstuffs
29. ISO 4121:2003 Sensory analysis -- Guidelines for the use of quantitative response scales
30. ISO 11035:1994 Sensory analysis -- Identification and selection of descriptors for establishing a sensory profile by a multidimensional approach
31. ISO 22308:2005 Cork stoppers -- Sensory analysis
32. ISO 707:2008 Milk and milk products -- Guidance on sampling

33. ISO 22935-1:2009 Milk and milk products -- Sensory analysis -- Part 1: General guidance for the recruitment, selection, training and monitoring of assessors
34. ISO 22935-2:2009 Milk and milk products -- Sensory analysis -- Part 2: Recommended methods for sensory evaluation
35. ISO 22935-3:2009 Milk and milk products -- Sensory analysis -- Part 3: Guidance on a method for evaluation of compliance with product specifications for sensory properties by scoring
36. ISO 18794:2018 Coffee -- Sensory analysis -- Vocabulary
37. ISO 6668:2008 Green coffee -- Preparation of samples for use in sensory analysis
38. ISO 7304-1:2016 Durum wheat semolina and alimentary pasta -- Estimation of cooking quality of alimentary pasta by sensory analysis -- Part 1: Reference method
39. ISO 7304-2:2008 Alimentary pasta produced from durum wheat semolina -- Estimation of cooking quality by sensory analysis -- Part 2: Routine method

Currently under development (as of July, 2019):

1. ISO/FDIS 16820 Sensory analysis -- Methodology -- Sequential analysis (previously ISO 16820:2004)
2. ISO/CD 11132 Sensory analysis -- Methodology -- Guidelines for monitoring the performance of a quantitative sensory panel (previously ISO 11132:2012)
3. ISO/DIS 11036 Sensory analysis -- Methodology -- Texture profile (previously ISO 11036:1994)
4. ISO/DIS 8586 Sensory analysis -- General guidelines for the selection, training and monitoring of selected assessors and expert sensory assessors (previously ISO 8586:2012)
5. ISO/DIS 4120 Sensory analysis -- Methodology -- Triangle test (previously ISO 4120:2004)
6. ISO/DIS 11056 Sensory analysis -- Methodology -- Magnitude estimation method (previously ISO 11056:1999, ISO 11056:1999/Amd 1:2013, ISO 11056:1999/Amd 2:2015)
7. ISO/AWI 22308-1 Cork bark selected as bottling product -- Part 1: Sensory analysis (previously ISO 22308:2005)

7.2 Sensory attributes and their perception

People are the main instrument of sensory analysis, as they use their senses to evaluate sensory properties (attributes) of food products. That is why it is so important to have selected assessors and trained panel. It is crucial for the quality control because sensory analysis can help in answering different questions:

- Identify preferences of consumers and the reasons why they like this particular product
- Quick measurement in a quantifiable manner of product quality attributes
- Other analytical techniques sometimes cannot detect a particular defect in contrast to sensory analysis
- People are the best instrument for detecting odourants at low levels and the only one to measure likings
- Ensures a cost-efficient new or even innovative products
- Monitors processing and formulation changes.

Sensory evaluation is the scientific discipline used to evoke, measure, analyze and interpret reactions to sensory attributes of foods and materials, such as appearance, aroma, flavour, taste, texture and sound, as they are perceived by the human senses of sight, smell, taste, touch and hearing. To understand the power of sensory evaluation, an understanding of the basic psychology and physiology of the senses should be developed. This understanding of the senses will help in further understanding products being tested.

The structure of the human's sensory system is presented on the Figure 1. There are five organs of sense composing our sensory system and helping to interact with the outside world: eyes, ears, nose, skin and tongue. Sometimes vestibular system, through which we aware of our placement in space, maintaining balance, feeling of weight and position, is added to this list as the sixth organ of sense. These organs perceive the information from the outside world, and then transform it into a

nerve impulse that gets into the brain for analyzing and forming response reaction. For each organ of sense corresponds a certain area of cerebral cortex, which processes the impulses.
Sight. Colour is the visual perception resulting from the stimulation of the retina of the eye by light (wavelengths between 380 and 760 nm).

The first attributes often affected the quality of foods are colour and appearance, as well as limpidity in beverages. The importance of colour is also demonstrated in such foods as wine, olive oil and sherbet, in which colour affects perception of other attributes due to the association with their colour. Indeed, people often expects a yellow beverage to have a lemon flavour, as well as red strawberry should be sweet in flavour.

Factors that affect sight perception include: intensity of the light source, distribution of wavelength in the light source, absorption or transmission characteristics, surface characteristics and shape of a product, reflectance, texture or surface of a product. Physiological factors include state of adaptation, angel, sensitivity of the eye to a specific wave lengths and deficiencies in colour vision.

To get reliable test results tasters should be checked for absence of deficiencies in colour vision. This could be done using different tests (see section 3.2). What's more, standards for visual assessment should be determined (memory, physical colour plates or model products). The lightning conditions for evaluation of a particular product must be predetermined - the samples must be evaluated under the same light source in the future. Otherwise metameric matching, when a pair of samples may appear to be a match under one light condition, but mismatched under most other conditions, might occur.

Sound. Sound waves are produced by vibrations squeezing together or spreading apart molecules in the air. Sound can be described in terms of frequency and amplitude. It is measured in frequency (Hz) - the number of cycles that a sound wave can complete in one second. Frequency corresponds to the pitch of sound. Amplitude is the maximum pressure created by sound waves. Amplitude is often measured in decibels and corresponds to the perception of loudness.

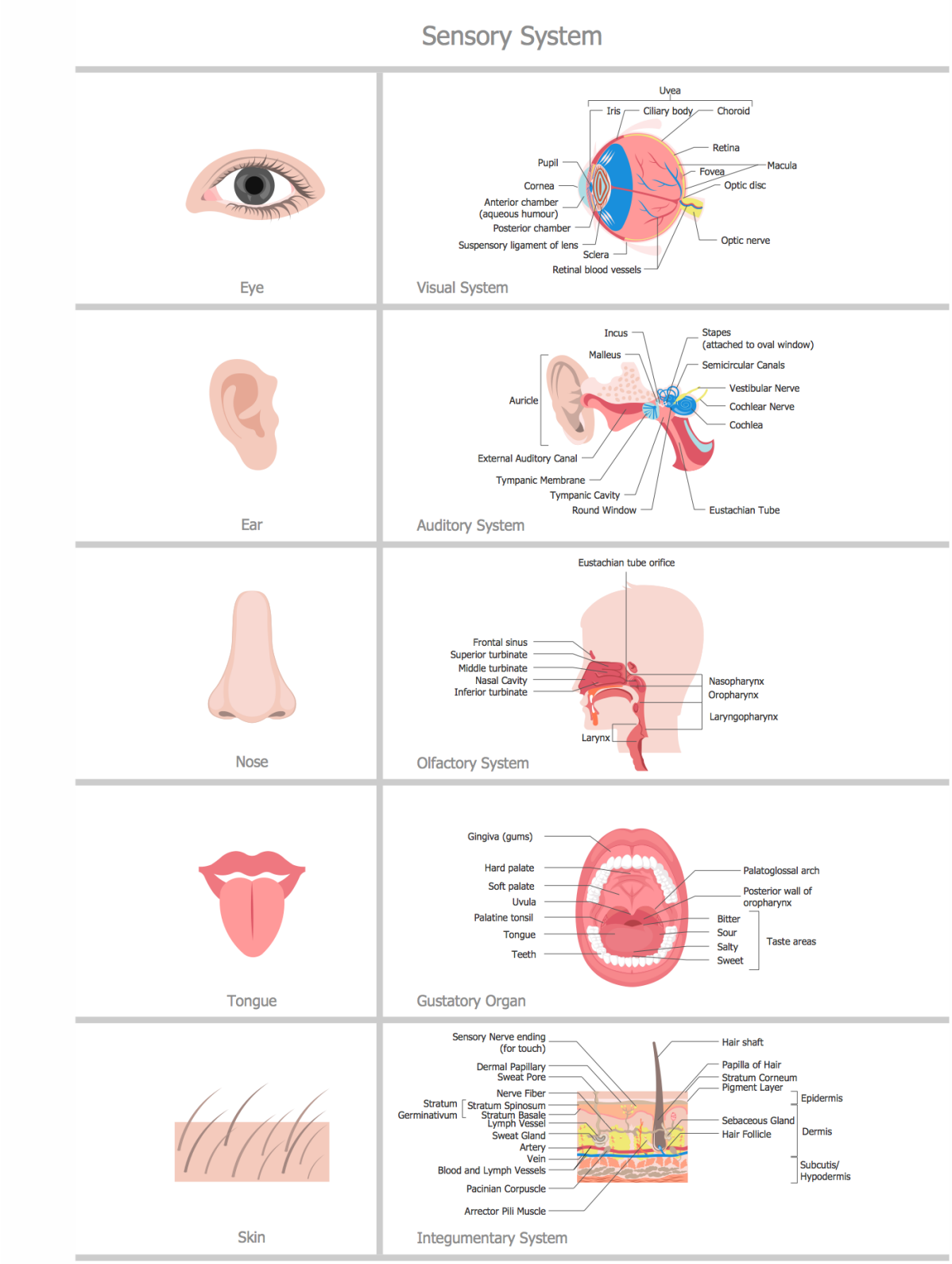


Figure 1 - Structure of the human sensory system
(source: https://www.conceptdraw.com/solution-park/health-human-anatomy)

Sound waves strike the tympanic membrane that causes its vibration. This vibration then is passed through the middle ear to the fluid-filled cochlea in the inner ear. In the inner ear vibration is picked up by sensory cells which then send it to the brain along the auditory nerve.

Factors that affect sound perception include: hearing impairments, conduction deafness, nerve deafness and interaction with other senses.

Sound is the important attribute in assessment of quality characteristics of products such as chocolate, for example.

Aroma and Flavour. The sense of smell is very complex. Aroma and flavour are chemical senses that are stimulated by chemical properties of odour molecules. In order human to feel aroma and flavour these molecules must reach the olfactory bulb to interact with olfactory cells in the olfactory mucosa. Smells are detected by breathing air that carries odour molecules. That is why, in order to smell, molecules must be volatile. The air with volatile odour molecules comes in contact with the tiny smell receptors high in the nasal cavity. These receptors send information firstly to smell nerves, and they then pass it to the brain. The specific reaction with the odour molecule still remains unknown. A term «flavour» is a combination of experiences including sensations of vision, smell, temperature, pain, pressure and texture. By definition, flavour is based on human judgment.

Factors that affect odour sensitivity include interactions with other human senses, such as taste and sight. Colour could sometimes cause the perception of smell when it isn't present in reality, as well as increased perceptions or even distorted perceptions. Odours are subjected to adaption. Other factors that affect dour sensitivity include: gender, age, smoking and olfactory disorders (hyposmia, hypersomnia, anosmia and dysomia).

Taste. The sense of taste is a chemical sense. It is detected by the taste cells, located on the front and back of the tongue and on the sides, back and roof of the mouth. These receptor cells, or taste buds, bind with molecules from the foods or beverages being consumed and send signals to the brain. It is generally accepted that people could distinguish among five to six basic tastes: salty, sweet, bitter, sour, umami and fatty. Factors that affect taste sensitivity include: age, smoking, viscosity of products, taste disorders (ageusia, non-tasters, hypogeusia, hypergeusia, dysgeusia) and temperature.

Tactile. This is the feel of sensations on the skin (including tongue). Human's skin is the largest sensory system that contains numerous receptors. These receptors have free nerve endings with the help of which people sense touch, pressure, hot, cold and pain. Most sensations on the skin are a combination of sensations. Moreover, not all parts of the skin are equally sensitive to pressure, touch, temperature, etc. Sense of touch assists in decision making and helps us to avoid pain. Although this organ of sense is often underestimated in sensory evaluation, tactile attributes play an an important role in evaluating the products we consume. For an instance, hot chocolate, ice lemonade, etc.

Texture. Texture is defined as «the sum total of kinesthetic (muscle sense) and cutaneous sensations derived from manual and oral manipulation». It includes mouthfeel, chewing properties, residual properties and even the visual and auditory properties of foods.

The first bite or initial phase includes the evaluation of the mechanical characteristics of hardness, destructibility and viscosity. In addition, the initial geometric characteristics are also evaluated. The second, the chewing phase, covers the evaluation of the mechanical characteristics of gumminess, chewiness and adhesiveness, as well as the geometric characteristics observed during chewing. The third, residual phase, includes the evaluation of changes caused by mechanical and geometrical characteristics during chewing. The feeling of food is associated with other sensations that occur simultaneously during a «normal» eating.

All the senses together play a key role in product flavour development as they help people to perceive attributes of foods and beverages: colour, aroma and flavour, feel in hand or mouth, sound to the ears and, ultimately, taste.

7.3 Requirements for the laboratories of sensory analysis. National and International recognition

Information given in this section fully corresponds to ISO 8589:2007 Sensory analysis -- General guidance for the design of test rooms. This International Standard provides general guidance for the design of test rooms intended for the sensory analysis of products. It describes the requirements to set up a test room comprising a testing area, a preparation area, and an office, specifying those that are essential or those that are merely desirable.

This International Standard is not specific for any product or test type. Both food and non-food products can be evaluated in such test spaces. However, adaptation of test rooms can be needed for each specialized use.

What's more, although many of the general principles are similar, this International Standard does not address test rooms for specialized evaluation of food products that are inspected or that are under quality control directly at the enterprise.

In different countries this Standard is translated into national languages, and the national introduction is added at the beginning. There is still can be a situation in some countries of Eastern Europe when International Standards are not present in national languages due to the lack of time and resources. However, in all areas the instruction is valid - if there is an International Standard ISO, guide it in the absence of a National Standard.

7.3.1. Test rooms' design

Typical test rooms for sensory analysis of products consist of:

- test sector where work can be performed both individually in test booths and in groups;
- sample preparation sector;
- office;
- dressing room and toilet;
- area for storage of stocks;
- area for storage of samples;
- rest room for tasters.

Minimum requirements are the following:

- test sector where work can be performed both individually in test cabins or in groups;
- sample preparation sector.

The test room should be accessible to tasters and should not be located in areas with heavy traffic flow, unless measures were taken to reduce noise and distracting factors. It is also necessary to take measures to ensure the accessibility of this place for people with disabilities.

It is advisable but not obligatory to have a room for tasters where they could get together or rest before entering the workspace. These rooms should be arranged so that they can be easily cleaned and maintain hygiene.

So, the basic principles that guide the design of test rooms for sensory analysis of products are:

- sensory evaluation should be performed in known and controlled conditions with a minimum of distracting factors;
- the test room should help to reduce the influence of psychological factors and physical conditions inherent to man.

ISO 8589:2007 Sensory analysis - General guidance for the design of test rooms contains 4 examples of test rooms' designs.

Test sector's design

General requirements

Location. The test sector should be located near the sample preparation sectorto facilitate the presentation of samples. However, they must be separate to prevent different types of interference (e.g. noise and odour). Tasters should not enter the test sector or leave it through the sample preparation sector, as this may lead to deviations in the test results.

Temperature and relative humidity. The temperature and relative humidity in the test sector must be controlled. The level of air temperature and relative humidity should be comfortable for the tasters, provided the test of the product does not require unusual conditions.
Noise. The noise level should be minimal during the test, as it can also distract the tasters. It is desirable that the room is soundproof, and the floor minimizes the noise associated with walking or moving objects.
Odours. The test sector should be free of odours. It can be achieved either by installing an air conditioner with activated carbon filters or by creating a light positive pressure in order to reduce air flow from other areas. What's more, the test sector must be equipped with materials that are easy to clean and does not accumulate odours. Furniture, carpets, chairs, etc. must not have odours that may affect the assessment. Detergents used for cleaning should not leave odours in the test sector.
Interior decoration. Walls and furniture colours in the test sector must be neutral so as not to cause changes in the colour of the samples. Recommended colours are matt, not pure white or neutral light grey shades.
Lighting. The source, the type and the lighting level are of great importance in sensory evaluation. Attention should be paid to the general lighting of all areas and, if possible, to the lighting of each booth. The test sector lighting should be uniform, controlled and free from shadows.
When evaluating the colour of products or materials, special lighting may be important. Moreover, special lighting devices to mask the colour or visual difference could be needed in some cases when it is necessary to avoid their influence on the test results. Devices that can be used include: rheostat to regulate the intensity of light; sources of coloured light; colour filters; black colour; monochromatic sources, such as sodium lamps.

Test booths

General requirements. When evaluating with various methods of sensory analysis, tasters are proposed to produce independent individual conclusions about the products that are being analyzed. In order to avoid the possibility of other tasters' influence, as well as in order to minimize other distracting factors' influence, individual booths are used.
Number of booths depends on the availability of space, as well as the type of evaluation performed. It should be such as to enable free movement and supply of samples from the sample preparation sector.
Booths installation. Both permanent and temporary portable booths can be used. Nowadays, all booths are equipped with windows for serving samples. Such windows should ensure the free passage of product samples without any difficulties and should be closed by sliding doors or covers. Windows should be designed in the way that testers cannot see sample preparation or coding.
Sockets should be provided and conveniently located in the booth for the use of electrical equipment. When using computer equipment, it is necessary to locate its components so as not to distract tasters from sensory evaluation. It is also useful to number the booths or label them in some way for easy identification and placement of tasters.
Position and size. The workspace in each test booth should be large enough to accommodate:
- samples;
- instruments;
- spittoon;
- sink;
- rinse aid;
- response forms and pen or device for computer input.

Sufficient space must also be provided in the workspace to fill in response forms or to accommodate computer equipment for transmitting testers' reactions.
The workspace must be at least 0.9 m wide and at least 0.6 m deep. If additional equipment is needed, it may be necessary to increase the size of the booth. What's more, the workspace of the test booths must have an appropriate height in order to test samples in comfort.
The side partitions between the booths should extend beyond the desktop in order to partially hide tasters - 0.3 m is considered sufficient. To ensure complete isolation of tasters, partitions can be placed

from floor to ceiling. However, their design should provide adequate ventilation and cleaning. Suspended partitions are also allowed.
If this is provided by local law, at least one booth should be designed in such a width and height that it can be used for work by a taster with a wheelchair.
Colour of booths. The interior of the general purpose booth should be matt grey with a brightness factor of about 15%. If it is necessary to compare mainly light colours and shades of off-white, the interior of the booth can be painted to provide a brightness factor of 30% or more to reduce the brightness contrast with the test colour.
Lighting guidelines are the same as for the test rooms.

Teamwork sector

General requirements. A teamwork sector should be provided for discussion between tasters and the operator. This sector can be used during initial training sessions and at any time when discussion between tasters is necessary.
This sector should be large enough to accommodate a table with comfortable seats for all tasters who simultaneously perform tests. The table must have sufficient space so that it can be put on it:
- a tray or other device in which there should be response forms and samples for each taster;
- additional materials, such as reference samples, if used, as well as pens, pencils or cups;
- computer (if necessary).

To transfer samples it is useful to provide a movable section in the centre of the table. Also, the table can be equipped with removable panels that divide tasters for individual work. To record moments of discussion, a blackboard/ whiteboard and chalk/ markers or a map must be provided.
Lighting guidelines are the same as for the test rooms.

Sample preparation sector's design

General requirements. The laboratory (or kitchen) for sample preparation should be located in the immediate proximity of the test sector, but, as it has been already mentioned, it should be located so that tasters do not have to pass through it to the test sector.
The sample preparation sector should be well ventilated to remove cooking odours and external odours. Materials selected for the floor, walls, ceiling and furniture should be easy to process, have no smell and be impervious to odours.
equipment
The equipment of the sample preparation sector depends on the range of products to be processed.
The main elements are:
- working surface;
- sink or other equipment necessary for washing the samples;
- equipment, including electrical, for storage, preparation, control, and serving of samples (for example, containers, dishes, various devices, etc.) in satisfactory working condition, calibrated for testing;
- cleaning supplies;
- waste container;
- storage conditions.

Tanks for the preparation and storage of samples, as well as the dishes and knives used in the preparation of samples, should be made of materials that do not give products side smells or tastes and prevent falsification and contamination of samples.

Office

General requirements. The office is a working sector where the paperwork related to the performance of sensory evaluation is carried out. It is necessary that the office is separated from the teat sector, but adjoined to it.
Size. Enough space should be provided for planning tests, developing response forms, sorting and decoding response forms, statistical data analysis, written reports and, if necessary, for meetings with clients to discuss tests and results.

Equipment. Depending on the specifics of the task to be performed in the office, it can be equipped with the following equipment: a desk, a filing cabinet, a bookcase, chairs, a telephone, a calculator and a computer for statistical data analysis.
The office can also accommodate a means of photocopying and storage of documentation.
ISO 8589:2007 Sensory analysis - General guidance for the design of test rooms contains examples of a test sector design, booths designs, booths windows design and examples of different booths placement.

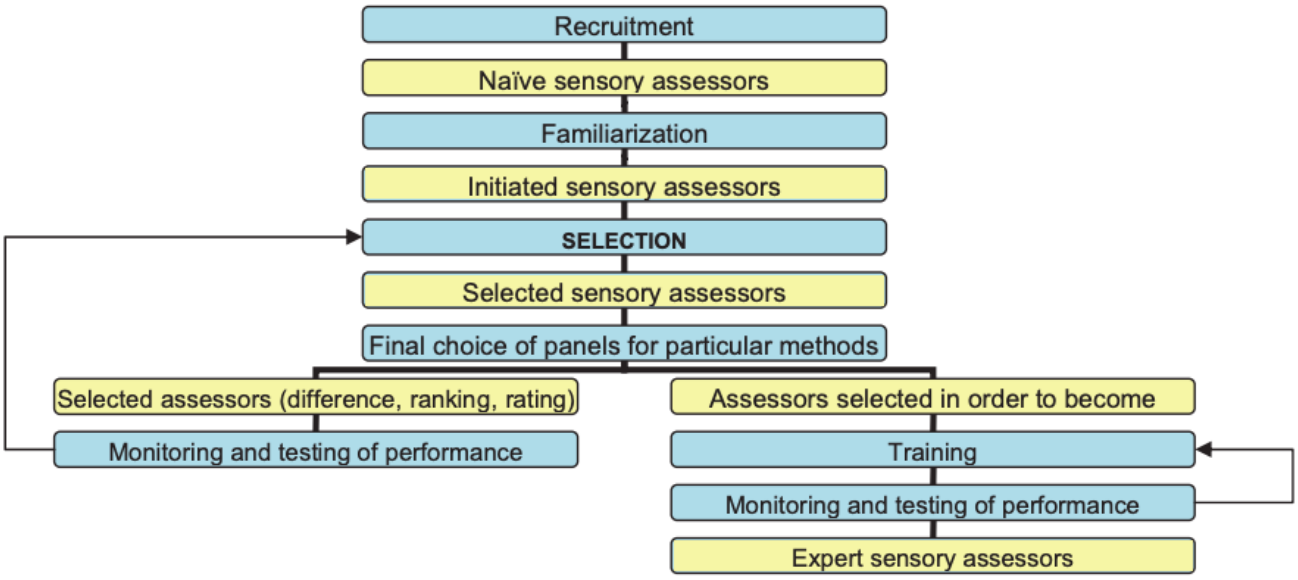
7.3.2. Panel selection and training

Information given in this section fully corresponds to ISO 8586:2012 Sensory analysis -- General guidelines for the selection, training and monitoring of selected assessors and expert sensory assessors. This International Standard specifies criteria for the selection and procedures for the training and monitoring of selected assessors and expert sensory assessors.

Given that a sensory analysis panel constitutes a true «measuring instrument», the results of the analysis depends on its each member. That is why the recruitment of persons willing to participate in a panel needs to be carried out with care and to be considered as a real instrument, both in time and money. Not every single person has an ability to carry out sensory evaluation. What's more, even if the person has demonstrated the ability to participate in sensory evaluation, the further trainings should be carried out for different types of products.
Sensory assessment can be performed by three types of assessors:

1. *Sensory assessors* - any people taking part in a sensory test. They can be «naive assessors» who don't have to meet any precise criterion, or «initiated assessors» who have already participated in sensory tests.
2. *Selected assessors* are chosen for their ability to perform a sensory test.
3. *Expert sensory assessors* - selected assessors with a demonstrated sensory sensitivity and with considerable training and experience in sensory testing, who are able to make consistent and repeatable sensory assessments of various products.Any person before participation in sensory tests should be assessed by the special procedure. First of all, it is always necessary to undertake a preliminary selection of the candidates at the recruitment stage, in order to eliminate those who would be unsuited for sensory analysis. However, the final selection can only be made after selection and training. The selection and training methods to be employed depend on the tasks to intend for the "selected assessors" and "expert sensory assessors". According to ISO 8586:2012

Sensory analysis -- General guidelines for the selection, training and monitoring of selected



assessors and expert sensory assessors, the entire process can be algorithmized as it is described on the Figure 2.

Figure 2 - Process of selection, training and monitoring of selected assessors and expert sensory assessors (given from ISO 8586:2012 Sensory analysis -- General guidelines for the selection, training and monitoring of selected assessors and expert sensory assessors)

So, the recommended procedure involves:

- recruitment and preliminary screening of naive assessors;
- familiarization of naive assessors who are to become initiated assessors;
- selection of initiated assessors in order to determine their ability to perform particular tests, who then become selected assessors;
- possible training of selected assessors to become expert sensory assessors.

The exact procedures of recruitment and familiarization and the nature of the tests in further steps depend on the tasks intended for the panel.

Sensory assessors work as a panel which is managed by a panel leader. In certain cases (especially for descriptive sensory analysis), the panel may be divided into specialized subgroups. In case of a panel of the expert sensory assessors, the panel leader is responsible for the general monitoring of the group and for their training. The expert sensory assessors are not responsible for the choice of tests used, the presentation of the samples or for the interpretation of results. These matters are the responsibility of the panel leader who also decides how much information is given to the panel.

In case of a panel of the selected assessors, their performance should be monitored regularly to ensure that the criteria by which they were initially selected continue to be met.

Selection of assessors

General. The following general characteristics are desirable for willing participants for training:

- then shall be motivated and interested in further developing their sensory skills;
- they shall be willing to participate.

Recruitment. During this stage it is necessary to answer the following questions:

1. From where should people be sought to constitute the group?
2. How many people shall be selected?
3. How shall the people be selected?

There are 2 types of recruitment:

- Internal - when organizations recruit staff of their departments;
- External - when organizations recruit people from outside.

What's more, a mixed panel constituted from both internal and external assessors can be organized.

There are advantages and disadvantages of both types of recruitment. For the internal recruitment the main advantage is that people are available all the time, and it is not necessary to pay them extra money for the sensory tests. However, they are influenced in their judgements by knowledge of the product. Another advantage is that in the case of internal recruitment a better confidentiality is ensured, which is important for research work. On the other hand, replacement of candidates is more difficult and the choice of people is less. Moreover, organizations can face lack of availability of staff members during sensory tests.

Among the advantages of the external recruitment are wider range of choice, a subsequent supply of new persons, much easier selection (without the risk of offending people if they are unsuitable), easy availability. On the other hand, the disadvantages are:

- the big costs,
- high efficiency only in urban communities,
- since it is necessary that the individuals be available, a disproportionate number of retired people, unemployed women or students are sometimes encountered;
- after having paid for the selection and training, there is always a risk that people leave.

Number of persons to be selected. Experience has shown that approximately half the people is eliminated during the selection procedures due to different reasons. So, the numbers of persons to be recruited varies depending on the following:

- the financial means and the recruitments of the organization;
- the types and frequency of tests to be conducted;
- Whether or not it is necessary to interpret the results statistically.

It is highly desirable that a panel has at least 10 selected assessors. So, it is generally accepted that in order to obtain a panel of this number persons, 40 to 60 persons should be recruited and a minimum of 20 persons should be selected.

Background information and preselection

Background information is usually obtained from questionnaires filled by candidates, as well as by interviewing candidates by persons experienced in sensory analysis.

General criteria. Among the general criteria for the selection of candidates there are the following:

1. Availability. Candidates shall have time to attend both training and assessments.
2. Attitudes to foods. Candidates shall not have strong dislikes for certain foods and beverages, (especially for those under sensory evaluation), as well as cultural and any other prohibitions for consuming products.
3. Knowledge and aptitude. The initial sensory perceptions of the candidates have to be interpreted and expressed, requiring certain physical and intellectual abilities, in particular the capacity to concentrate and to remain unaffected by external influences.
4. Ability to communicate and describe. This ability is extremely important for candidates selected for descriptive analysis as it involves the necessity to describe their sensations perceived during the assessment. It is desirable that candidates have good sensory memory and are able to describe in words characteristics of a product.

Health. Candidates must have good health. They should not have chronic diseases which can affect the senses, as well as allergies. Candidates should not take medicines that can weaken sensory

perception and, therefore, make their judgments unreliable. It is also useful to find out whether or not candidates have dentures, as this may affect some types of assessment regarding the texture or taste of products. Colds or temporary conditions (e.g. pregnancy) should not be a reason for rejecting a candidate.

Psychological criteria that are important:

- Interest and Motivation;
- Awareness of responsibility and ability to concentrate;
- Ability to make judgments;
- Desire to cooperate.

Other factors. In addition to the above mentioned information, during the recruitment of candidates can also be obtained the following information: name, surname, age group, gender, nationality, education, current job and experience in conducting sensory analysis. It is also can be noted whether candidate smokes or not. However, smoking, as a rule, should not be the reason for rejection of a candidate.

Screening

Tests that can be used for screening candidates are separated into 3 groups:

a) tests aimed at identifying non-compliance with basic requirements;
b) tests aimed at determining the development of the senses;
c) tests aimed at identifying a candidate's ability to describe and transfer feelings.

The aim of these tests is familiarization of candidates with methods and materials that are used during sensory analysis.

Colour vision. Candidates with colour vision anomalies are not suitable for conducting tests that include colour assessment. A colour vision test can be performed using an effective test, such as the Ishihara test or Farnsworth-Munsell 100 HueColor vision test.

Loss of taste and smell. It is advisable to check the sensitivity of candidates to substances that may be present in small quantities in products, in order to reveal a loss of taste, smell or a possible lack of sensitivity. Samples of substances are prepared for tasting by taste and smell with concentrations that significantly exceed the threshold values. Examples of substances that can be used:

- tastes: sweet, sour, bitter, salty, umami, astringent, metallic etc.;
- smells: lemon, vanilla, thyme, floral etc.

The methodology of the test, as well as substances and their concentration for the preparation of solutions are described in detail inISO 8586:2012 Sensory analysis -- General guidelines for the selection, training and monitoring of selected assessors and expert sensory assessors.

Training

Training is necessary to provide assessors with basic knowledge of the procedures performed during sensory analysis, and to develop their ability to identify, recognize and describe stimuli, as well as to enable assessors to use experience in this way to be fluent in these methods for specific products.

The number of assessors undergoing training should be 1.5-2 times more than it is required for the final formation of the panel. To ensure the right approach to sensory analysis, all training should be carried out in an appropriate setting in accordance with the recommendations outlined in ISO 6658:2017 Sensory analysis -- Methodology -- General guidance.

Assessors shall be instructed that they should always be objective and neglect their addictions or dislikes. What's more, they should not use flavoured cosmetics before or during tests, and refrain from smoking or contact with smokers or strong tastes and smells at least 60 minutes before the test. Otherwise they could bring extrinsic odours to the test room that can influence test results.

Training to perceive colour, taste, smell and texture includes 6 types of tests.

1. *Tests for determining the stimulus.* These tests are based on the triangle method (see section 4). During one session only one substance is examined. Each candidate is provided with two samples of the test substance and one sample of water or another neutral substance/ or one sample of the

test substance and two samples of water or another neutral substance. The concentration of the test substance must be above the threshold level. Inability to identify differences after several repeated tests indicates the unsuitability of the candidate to carry out tests of this type.

2. *Tests for determining various levels of stimulus intensity.* These tests are based on methods using the scales and categories (see section 4). Tests are carried out using stimuli for taste, smell (only in very small concentrations), touch (in mouth and by hand), and colour vision. In each test candidates are randomly provided with four samples of different concentrations of the test substance, which candidates must place in order of the stimulus increasing intensity. Random order must be the same for all candidates so that when comparing the results, it was obvious that they did not depend on the order of familiarization with the samples. A satisfactory result in this test can only be determined on the basis of particular concentrations.
3. *Ability to describe feelings.* These tests are aimed at checking the ability of candidates to describe their feelings. It is recommended to carry out two tests: one based on the perception of odours, the other - on touch.
4. Odour description. Candidates are given 5 to 10 samples of various olfactory stimuli, preferably related to products that will be analyzed further. The kit should consist of several easily recognizable samples, and the rest should be less recognizable. The intensity should greatly exceed the threshold of perception, but not too much exceed the concentration in those products that will be further analyzed. A satisfactory result in this test can only be determined on the basis of particular substances.
5. *Texture description test.* Candidates receive sample kits in no particular order and must describe their texture. Samples of solid products should be presented in the form of blocks of the same size, and liquid substances should be presented in opaque containers. A satisfactory result in this test can only be determined based on the substances used.
6. *Recognition of the differences in texture.* Method used: ranking test (see section 4). A satisfactory result in this test - at least 80% of the samples are located correctly.

Preparation to identify and recognize tastes and odours. The test for the detection of identical substances, paired comparison test, triangle and duo-trio methods in accordance with ISO 6658:2017 Sensory analysis -- Methodology -- General guidance are used at this stage. Moreover, identification tests are used to develop the sensitivity of the assessors to odours. At first, stimulus are provided as aqueous solutions of the relevant substances, but as assessors gain experience, they can proceed to testing real foods and beverages. Distortion of the sample appearance (for example, using colour lighting) is particularly useful in order to emphasize the need to be objective when trying to identify differences in other characteristics.

Preparation to use scales. At this stage assessors shall be introduced to the estimated, classification, interval and ratio scales, depending on which scale will be used. Various evaluation procedures are used to assign reasonable values when testing samples. The most common first to test aqueous solutions, then real foods and beverages with mixed stimuli, which can vary independently of each other.

Training in the compilation and use of descriptions (profiles). Training participants should know how descriptions (profiles) are compiled. To achieve this, they need to be offered samples of simple products and asked to compile vocabularies for describing the characteristics of these products in such terms that will allow differentiation of samples. The terms are developed individually, then after discussion a general list of terms is developed from at least 10 definitions. This list is used further to compile product descriptions, first choosing terms appropriate for each sample, and then evaluating their intensity using different scales mentioned above.

All the theoretical training described above alternates with practical tasks so that assessors can expand their experience. After basic training, assessors can be trained to evaluate a real product. Formation of panels is carried out for certain types of tests from candidates who demonstrate success in the relevant tests. Candidates who are eligible for one type of test need not be suitable for another, and candidates excluded from the number of assessors in one test should not automatically be excluded from carrying out others.

Expert training. The experts are trained from selected фыыуыыщкы who already have knowledge of the physiology of taste and smell. Their training is aimed at improving sensory knowledge and, especially, memorizing descriptors for the sensory profile and their intensity values, as well as acquiring skills to create high-quality sensory product profiles (repeatability, reliability, distinctive abilities). Training usually includes:

- development of sensory memory;
- semantic and motivic study of sensory descriptors;
- development of descriptor vocabularies;
- training in the conditions of certification.

All the methodologies of the tests mentioned are described in detail in ISO 8586:2012 Sensory analysis -- General guidelines for the selection, training and monitoring of selected assessors and expert sensory assessors.

7.3.3. Serving procedures

General information about material to be tested, apparatus for it and sampling is presented in ISO 6658:2017 Sensory analysis -- Methodology -- General guidance. What's more, each ISO on methodology of sensory analysis contains short instruction regarding this topic.

According to accepted rules, sampling for testing should be carried out according to the standards for sensory analysis of the product or products to be tested. In the absence of such standards, one should be guided by the sampling rules for this product or reach an agreement between interested parties.

Methods for preparing and presenting samples should be appropriate to the specific type of product and the specific purpose of its testing. Products must be tasted in the same conditions in which they are usually consumed, except when tasters are asked to focus on some special cases. For example, a product that is usually consumed hot should be cooked in the usual way and tested hot, and a product that is usually consumed in chunks should not be made homogeneous (homogenized), since it is necessary to preserve its inherent characteristics of the texture. However, it is necessary to ensure that parts of the samples evaluated by each taster are as uniform as possible.

Tasters should not make conclusions about the nature of the samples based on the way they are provided with these samples. Samples must be prepared in the same way (identical containers, identical quantities of product, identical appearance, identical temperature conditions etc.). Temperature of the samples should be controlled and kept constant.

When assessing the appearance of samples, it is necessary to create certain lighting conditions. When differences in smell and taste are only determined, the appearance of colour differences can be partially smoothed out by creating lighting conditions whereby colour differences are minimized.

The product container should not affect its characteristics or performance of the subsequent test. They can be ceramic or glass containers that are easy to clean and rinse, as well as disposable plastic or paper containers. They should not contain chemicals that cause the formation of coloured spots. In particular, the containers should be washed only with detergents, odourless and colourless, and rinsed with water. Polymer and paper containers, including those with thermal insulation (for hot or chilled samples) must be odourless and must be colourless. Apparatus should be selected by the panel leader according to the nature of the product to be analyzed, the number of samples, etc., and should not in any way affect the test results.

When conducting a blind tasting (anonymous sample serving), production packaging, labels, i.e. all information about a producer, should be removed from the samples. Containers containing tested samples must be coded using three-digit numbers chosen at random. The coding must be different for each test. Code values should be known only to the test organizer who does not participate in the work as a taster.

Samples of one type of product are collected in a series. The order of testing products in one series is set according to the degree of increase in the intensity of smell or the amount of seasoning, or by increasing the mass fraction of constituent elements, such as fat, salt, sugar, ethyl alcohol, etc.

First of all, products with a faint odour, then - moderate, further - strongly expressed. The same procedure is followed when evaluating taste: less salty and spicy samples are tested first.

During sensory analysis, an important factor is the number of samples evaluated by a panel in one session. It is better to analyze a small number of samples which depends on the type of products.

The problem of taste dullness, benefits of mouth rinsing and standard intervals between testing different samples should also be discussed. Testing of various samples should ensure the restoration of perception acuteness, but should not be too long so that testers do not lose the ability to compare and distinguish samples.

It is allowed for tasters to use means to neutralize the odour and taste in the mouth between the testing of samples or test periods. However, it is necessary to ensure that these products do not affect the taste and smell of the product being evaluated. Between samples testing or test periods carbonated or non-carbonated water, as well as fresh-flavoured products (for example, unsalted biscuits), are allowed.

Depending on the product properties, after evaluating five to eight samples, a break is taken for at least 15 minutes to restore sensory abilities.

7.4 The main sensory tests

In the sensory evaluation of the organoleptic characteristics of foods, different methodologies are used depending on the purpose of the tests and the information that they want to obtain. It is possible, first of all, to distinguish laboratory tests (or analytical tests) from those for consumers. The first group can be further subdivided into three categories as shown in Figure 1. With qualitative discriminant tests it is possible to evaluate the existence or not of differences between two products, with quali-quantitative discriminate tests to obtain information related to the entity of existing differences between multiple samples or the category to which a sample belongs. With descriptive tests, it is possible to identify and quantify the sensorial attributes (descriptors) that best characterize a food. The distinction between the three groups finds its justification, in addition of course in the purpose of the test and therefore in the type of information obtainable, also in the different difficulty of each test and, consequently, in the different degree of training and numerosity of the panel.

Laboratory test		
Qualitative discriminant tests	Quali-quantitative discriminant tests	Descriptive tests
1. Paired difference test 2. Triangular test (triangle test) 3. Duo-trio test (duo-trio test).	1.Sorting test 2.Range classification test 3.Score test (report scales)	1. QDA 2. Flavour Profile 3. Texture Profile
Consumers tests		
1. Acceptability test 2. Preference test 3. Hedonistic test		

Figure 1 Classification of the main sensory tests

7.4.1 Quality discriminating tests

A number of surveys conducted in the United States and England have shown that qualitative discriminatory tests represent the most widely used sensory method in the food sector. The widespread use of these methods is attributable to the simplicity of the experimental procedure, to the rapidity of performing the tests and to the ease of interpretation of the results. Qualitative discriminant tests are used for a specific purpose, namely to assess whether two samples can be judged to be different with predetermined statistical probability levels. The question that is actually asked of

the tasters is of the type "which is the different sample?" "Which is the best sample ..." or "which is the favourite sample?"

The products to be evaluated with these methods must differ only in the first feature being considered. If two samples, for example, have to be evaluated on the basis of consistency, they must have the same appearance, the same shape, the same color, etc. , and must be presented in the same quantity. Qualitative discriminating tests are used both in the selection and training phase of the panel, and in the evaluation of the products. The three main qualitative discriminating methods are:

1. Paired difference test

2. Triangular test (triangle test)

3. Duo-trio test (duo-trio test).

7.4.1.1. Paired difference test

Test preparation and execution

The purpose of the comparison test in pairs is to assess the existence or not of differences between two samples and / or to establish a preference, based on a global assessment or in relation to a specific sensory attribute. This test is also used to select and train tasters.

In practice an equal number of samples of the two products is prepared (product A and product B) to be compared. Each sample is identified by a code. The coding can be different for the two products (for example product A: 376 and product B: 914) and the same for all tasters or, better, different for the two products and for the tasters. In this case it is indispensable, for the preparation of the samples, to use a card of the type shown in Figure 2 on which the answers of the tasters will then be recorded, In the card it is indicated, in addition to the progressive identification number of the taster , the order in which the two samples will be served. In fact, it is important to ensure that half of the panel evaluates sample A first and the other half first evaluates sample B. In the event of a repetition of the test, the order must be reversed so that each taster will have evaluated both sample A and sample B first.

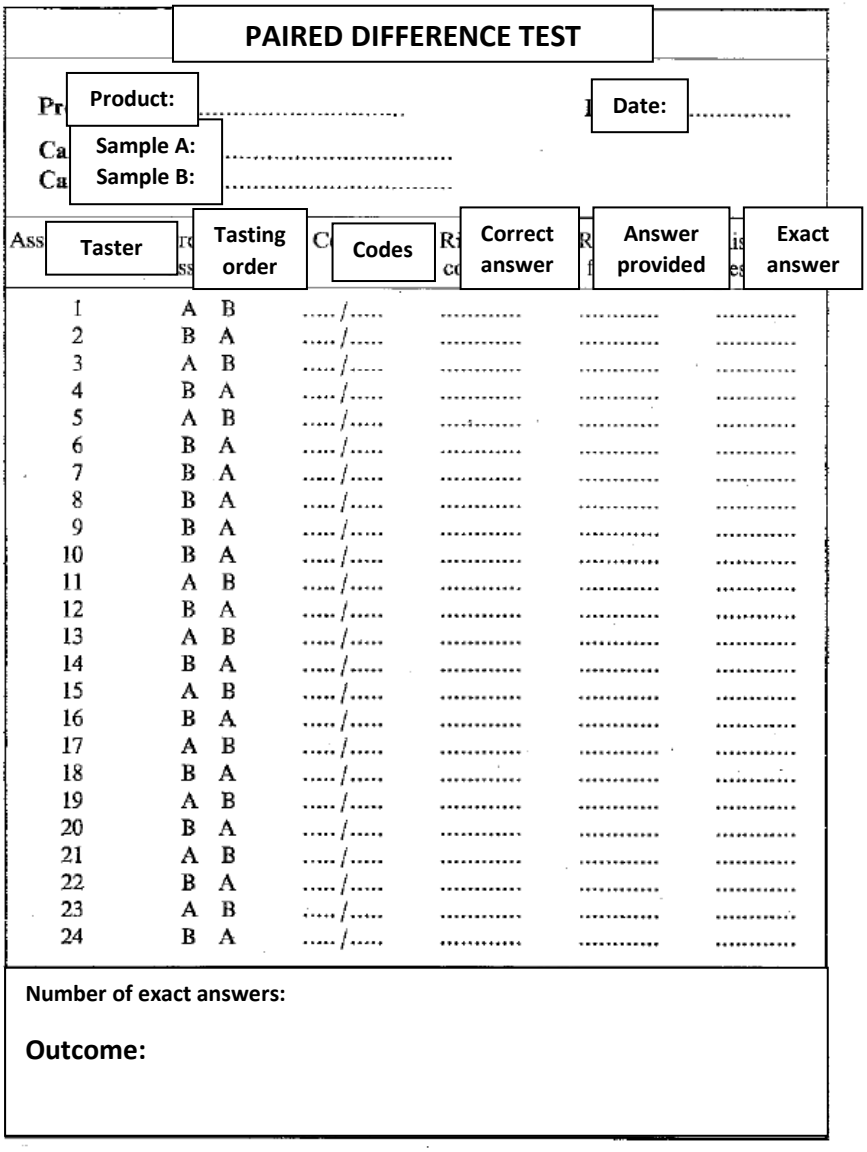


Figure 2 General card of a paired difference test

To simplify the preparation of the samples and avoid possible errors it is advisable, first of all, to code the containers in which the samples will be presented. The choice of the sequence of the three digits that make up the code can take place through the table of random numbers starting from a random position and then proceeding in a certain direction (left, right, up or down) and composing 3-digit numbers (table 5). The encoded containers are then divided into two equal groups: the first group will receive sample A, the second sample B. The coded samples are distributed to the tasters in the following way: samples A are initially distributed and then samples B are given in the tasting order indicated, for each taster, in the general tab of the test (figure 2). At the same time, the codes assigned to each sample for the different tasters are recorded in the third column. In the same tab, three other columns are provided, first for indicating the correct answer, the second for indicating the answer provided and the third for identifying the tasters who provided a correct answer.

TABLE OF RANDOM NUMBERS

2217686584	6895239235	8702225751	6109439606	5824820347
1936275946	1379933755	3977327709	8552053062	4783516274
1677230277	0961872521	2806242593	1671135978	2305474725
7843767161	2044903264	9767639961	4638039322	6981219021
0328282608	7337320405	6930160905	8869582899	3507447547
9322536439	0710637635	8703047988	0813138551	5534577269
7876585474	9238709692	5206797945	8263182744	6966921909
2368352600	9953936128	5270054834	5665056186	9092107080
1539257099	9386527765	1533590528	2287260747	8696982906
5871963024	1846233427	8513992444	4918097949	7416322302
5735273372	2453639409	4110764791	4404954966	3960045981
4850865448	2206347252	8221156520	3329947111	1591291203
6196489503	0716393366	9856105679	7721302712	9049222362
3693894126	2970836351	9974205236	8709411509	9860160303
1887004231	5790120207	2347371731	5408018863	3941889210
8856532759	3335726747	7734554570	0818273890	1695867075
0972958429	4941310670	4238064518	6484733165	5253379715
1296881731	6519690283	6075869068	2464193551	5661873912
8594572416	9209843876	2200276985	2981947870	2194479012
3864435998	9877876807	9151676244	4098059378	2332654118
5344094272	0041867979	6847220020	3555315151	0083632255
4076662684	5799999037	3663320858	3740136897	8764810783
0217791805	1259525702	2207904703	2814113079	2069224098
9517820653	3151109646	9206880777	5611508169	4023725139
3576224292	9611834480	3468354877	3342409060	7396539786
2629135641	8547046608	3472575913	8243804615	3826617004
7780207582	7282329990	6395737663	8973449905	4867264318
4640664452	9136744353	3082135400	7845639835	5503366768
3756081809	7753844647	3191189558	2416741153	4410138557
6165616866	3727473919	8483700748	5321400671	9506798854
9343696407	3418045235	5627092486	6185538345	1990709900
2196601299	1120994518	4813935534	1837794990	6597382046
9520479797	2737832871	0006414174	4589093984	5157115249
9786217873	1065819259	5876171497	0476621617	1795704580
6992063413	5971741732	2755102419	2371821374	6352520141
0431172156	3373991987	2672392767	5377576893	6061972261
6106980391	8714774396	4300659850	4560330107	9899465047
8593858688	7287086240	1606108920	2321347497	7638032963
2174324745	7396079452	0965907747	2576161933	5305705330
1569538280	7996235310	6539071629	4533024370	0287404145
0289080449	2021146886	8763939517	1129019580	3514973533
8718158979	8543017273	0861745169	8974398215	9451334167
9883719422	5997509952	6852850840	8780616531	9151803244
1008582166	7268492931	8985844606	5973198523	6509297563
4790561008	8802842783	4229722319	6656456579	2071532025
2285616890	4964928544	1640128988	5014498106	0182774512
6780437933	1283114116	2558196870	7702540052	5343371526
2762509672	7944614015	1453406539	2731585028	1139033425
3378808715	3830063821	1447470726	5496875332	4036409676
1313926699	4724495774	3225436117	1097116984	9963223298

Table 1 Table of random numbers

Each taster is presented, together with the two samples to be evaluated, a card with instructions for carrying out the test on which the answers will be reported and any cards are shown in the application examples of the test (figure 3).

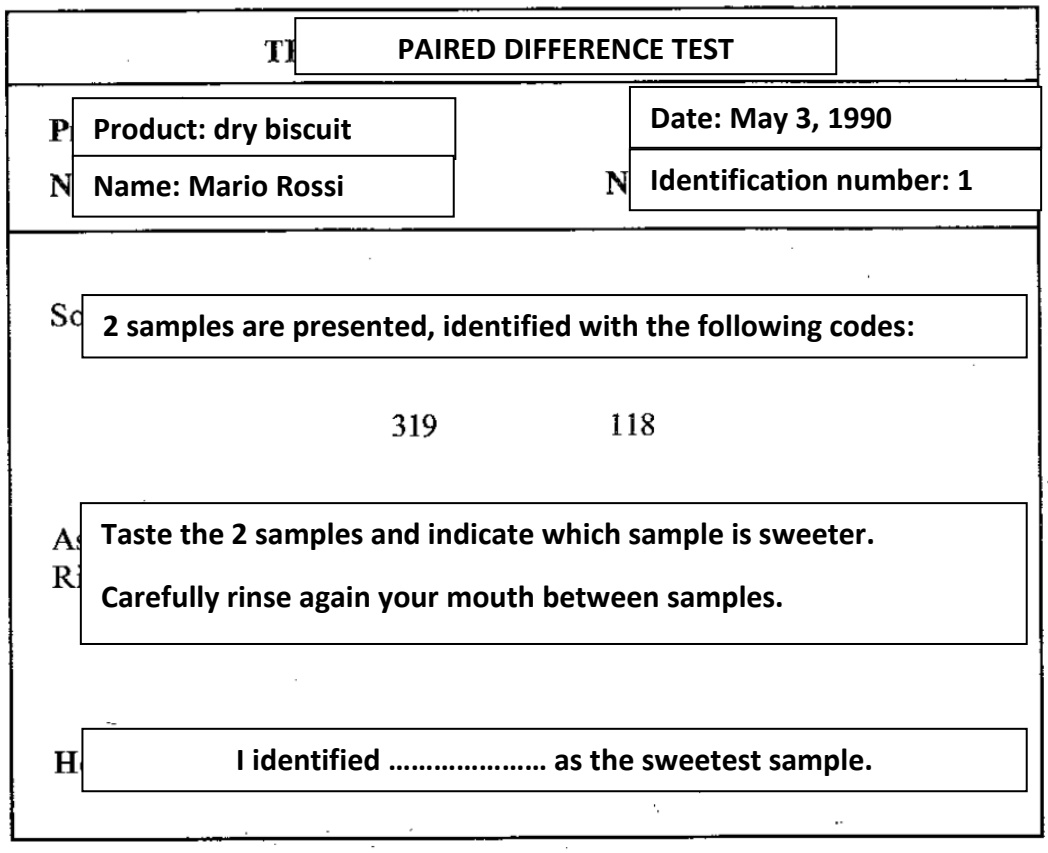


Figure 3 Card for performing the test

It is useful for the panel leader to verbally specify the test execution modalities, for example:

-evaluate the appearance of the sample first, then smell it and finally taste it;

-eliminate or swallow the sample, after tasting and rinse again the mouth thoroughly. If necessary, chew a small piece of cracker or apple or other to eliminate the sensations left by the first sample, rinse the mouth again, and, only after eliminating the taste of the previous sample, taste the second sample;

-taste both samples once or a limited number of times, indicate the answer on the card avoiding to taste the samples repeatedly: this procedure causes, especially with some samples, a rapid fatigue of the senses and therefore a lower capacity for discrimination;

- equally provide an answer even if you are unable to identify a difference or express a preference;

-do not hesitate to write comments on the card.

It is also important not to inform panel members of the problem that made the tasting necessary or to inform them in a way that does not affect their response.

At the end of the session, it is a good rule to inform the tasters on the reason for which the test was performed, on the nature of the differences between the samples and also on the validity or not of their answers; this in order to motivate their work. If the knowledge of this information can, in some way, influence the answers of the tests that will be carried out later on, it is good not to present the results, but to assure the tasters who will be informed as soon as possible.

Processing of results

After collecting the cards, the answers of each taster are shown in the general tab of the test (figure 2). The number of people who have provided the correct answer is then calculated in the case of the assessment of differences between the samples, or the number of tasters who have indicated the sample A or the sample B, in the case of the evaluation of preferences or in the identification of the sample that has a certain attribute with greater or lesser intensity.

Table 2 allows you to evaluate if the differences between the two samples have been highlighted randomly or if they can, on the contrary, be considered significant. The table, which refers only to unilateral tests, shows three distinct columns for three different levels of significance.

Number of answers	Minimum number of correct answers to establish a significant difference			Number of answers	Minimum number of correct answers to establish a significant difference		
N ri	= 0,05 (*)	$\alpha = 0,01$ (**)	$\alpha = 0,001$ (***)		= 0,05 (*)	$\alpha = 0,01$ (**)	$\alpha = 0,001$ (***)
7	7	7	—	53	33	36	39
8	7	8	—	54	34	36	39
9	8	9	—	55	35	37	40
10	9	10	10	56	35	38	40
11	9	10	11	57	36	38	41
12	10	11	12	58	36	39	42
13	10	12	13	59	37	39	42
14	11	12	13	60	37	40	43
15	12	13	14	61	38	41	43
16	12	14	15	62	38	41	44
17	13	14	16	63	39	42	45
18	13	15	16	64	40	42	45
19	14	15	17	65	40	43	46
20	15	16	18	66	41	43	46
21	15	17	18	67	41	44	47
22	16	17	19	68	42	45	48
23	16	18	20	69	42	45	48
24	17	19	20	70	43	46	49
25	18	19	21	71	43	46	49
26	18	20	22	72	44	47	50
27	19	20	22	73	45	47	51
28	19	21	23	74	45	48	51
29	20	22	24	75	46	49	52
30	20	22	24	76	46	49	52
31	21	23	25	77	47	50	53
32	22	24	26	78	47	50	54
33	22	24	26	79	48	51	54
34	23	25	27	80	48	51	55
35	23	25	27	81	49	52	55
36	24	26	28	82	49	52	56
37	24	27	29	83	50	53	56
38	25	27	29	84	51	54	57
39	26	28	30	85	51	54	58
40	26	28	31	86	52	55	58
41	27	29	31	87	52	55	59
42	27	29	32	88	53	56	59
43	28	30	32	89	53	56	60
44	28	31	33	90	54	57	61
45	29	31	34	91	54	58	61
46	30	32	34	92	55	58	62
47	30	32	35	93	55	59	62
48	31	33	36	94	56	59	63
49	31	34	36	95	57	60	63
50	32	34	37	96	57	60	64
51	32	35	37	97	58	61	65
52	33	35	38	98	58	61	65

Table 2 Significance of thepaired difference test(p=1/2) (Unilateral test)

An error level of 5% (a = 0.05) means that the two samples compared have been correctly identified as a result of the case once out of twenty (there is a 5% probability of refusing without any statistical justification the null hypothesis which states an equality between the two samples). This concept is expressed by saying that one is confident, with 95% probability (P = 95%), that the two

samples are different. In the case of an error level of 1% there is the probability of 1% obtaining a correct answer solely due to the case.

The use of tables is very simple. Based on the number of responses or the number of judgments (number of tasters multiplied by the number of replies) the minimum number of exact (or concordant) responses is reported to establish a statistically significant difference (or preference) between the two samples. If the number of correct or concordant answers obtained in the test is equal to or greater than the tabulated value, it is fair to state or that there are significant differences between the two products or that one of the two products has a characteristic with a statistically greater (or lower) intensity for a certain level of significance (thus rejecting the null hypothesis).

7.4.1.2. Triangular Test

Test preparation and execution

The triangular test is the most widely used discriminating qualitative method both during the selection and training of Panel tasters and for the evaluation of food products. To perform this test, three differently coded samples are presented to each panel member, two identical and one different. The taster will have to identify the different sample. For the preparation of the samples to be subjected to the test, similarly to what previously reported for the comparison test in pairs, it will be possible to use a card of the type shown in figure 4.

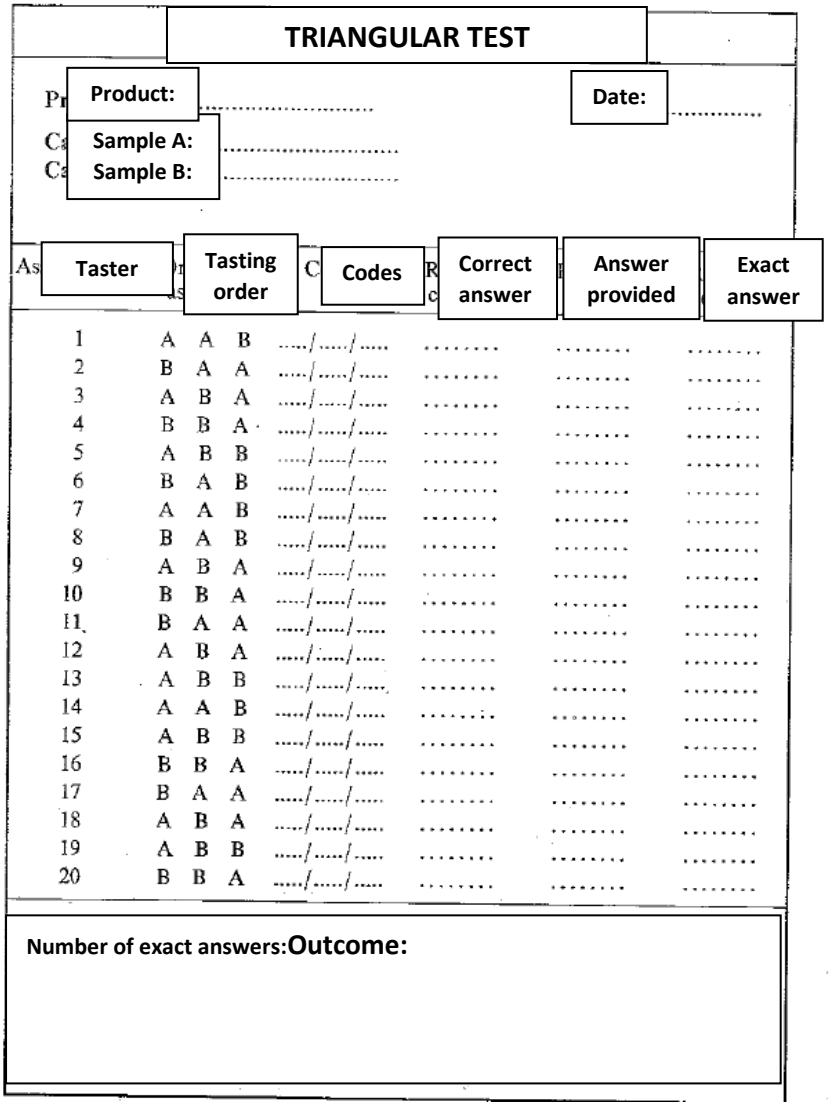


Figure 4 General card of a triangular test

In addition to the taster's identification number, the card shows the order in which the three samples will be served to each panel member. It is in fact important that the tasters assess the three samples with a different order and that half the panel receives sample A as a single sample, while the other half receives sample B as a single. If the test is to be repeated with the same tasters, it will be it is advisable to invert the order in which the samples are presented in the second test. This order derives, as indicated in the sheet, from a sequence of random combinations. The choice of codes for each sample can be made on the basis of the table of random numbers, as previously reported.

To avoid errors in the distribution of the two products (sample A and sample B) in the previously coded containers, it is necessary to fill them separately, form the tasting triads and report in the third column of the general test sheet the sample codes provided to each taster. The three samples will be submitted to the panel's judgment accompanied by a form of the type shown in Figure 3 but with 3 codes and this type of sentence: “2 of these samples are the same and one is different; indicate which one is different:”.

Also in this case it is useful to verbally specify the test execution methods, similarly to what was reported for the comparison test in pairs to which reference is made for more details.

Processing of results

After collecting the cards, the answers of each taster are shown in the general tab of the test (fifth column) and, from the comparison between the correct answers and those provided, the number of correct answers provided globally by the tasters is evaluated. This number is compared with the value reported in Table 3 in relation to the number of tasters who carried out the test or the number of judgments (number of tasters for the number of replies) for the different levels of significance. If the global number of correct answers is equal to or greater than the value shown in the table, it can be stated that the two products examined are statistically different. Table 3 differs from that relating to the comparison test in pairs since, in the triangular test, the probability of obtaining a correct response at random is 33.3% (p = 1/3).

Number of answers	Minimum number of correct answers to establish a significant difference			Number of answers	Minimum number of correct answers to establish a significant difference		
	$\alpha = 0{,}05$ (*)	$\alpha = 0{,}01$ (**)	$\alpha = 0{,}001$ (***)		$\alpha = 0{,}05$ (*)	$\alpha = 0{,}01$ (**)	$\alpha = 0{,}001$ (***)
5	4	5	—	53	24	27	29
6	5	6	—	54	25	27	30
7	5	6	7	55	25	27	30
8	6	7	8	56	25	28	31
9	6	7	8	57	26	28	31
10	7	8	9	58	26	29	31
11	7	8	9	59	27	29	32
12	8	9	10	60	27	29	32
13	8	9	11	61	27	30	33
14	9	10	11	62	28	30	33
15	9	10	12	63	28	31	34
16	9	11	12	64	29	31	34
17	10	11	13	65	29	32	34
18	10	12	13	66	29	32	35
19	11	12	14	67	30	32	35
20	11	13	14	68	30	33	36
21	12	13	15	69	30	33	36
22	12	13	15	70	31	34	37
23	12	14	16	71	31	34	37
24	13	14	16	72	32	34	37
25	13	15	17	73	32	35	38
26	14	15	17	74	32	35	38
27	14	16	18	75	33	35	39
28	14	16	18	76	33	36	39
29	15	17	19	77	33	36	39
30	15	17	19	78	34	37	40
31	16	17	19	79	34	37	40
32	16	18	20	80	35	37	40
33	16	18	20	81	35	38	41
34	17	19	21	82	35	38	42
35	17	19	21	83	36	39	42
36	18	20	22	84	36	39	42
37	18	20	22	85	36	39	43
38	18	20	23	86	37	40	43
39	19	21	23	87	37	40	44
40	19	21	24	88	38	41	44
41	20	22	24	89	38	41	44
42	20	22	24	90	38	41	45
43	20	23	25	91	39	42	45
44	21	23	25	92	39	42	46
45	21	23	26	93	39	43	46
46	22	24	26	94	40	43	46
47	22	24	27	95	40	43	47
48	22	25	27	96	41	44	47
49	23	25	28	97	41	44	47
50	23	25	28	98	41	45	48
51	24	26	28	99	42	45	48
52	24	26	29	100	42	45	49

Table 3Significance of the triangular test (p = 1/3)

Extension of the triangular test

Often it is not sufficient to establish the existence or not of a difference between two samples, but it is necessary to obtain, with the same test, information relating to the entity of the difference, or of opinions of preference. In this case the question related to the identification of the sample other than a series of other questions is followed. For the statistical elaboration of the second part of the test only the answers of the tasters who have correctly identified the different sample are considered. Also in this case we compare the number of responses that indicate a sample as the favourite, or as the one that presents a certain characteristic with a greater (or lesser) intensity, with the minimum value of answers necessary to obtain a significant difference between the two samples.

The same tables (tables 2 and 4) previously reported for the comparison test in pairs are used, calculated on the probability 1/2, since in the second part of the test the choice or comparison is made between two samples. Also in this case the distinction must be made between unilateral tests and bilateral tests. In assessing a preference, the test is typically bilateral: both samples can in fact be preferred by tasters. In choosing a product based on the intensity of a certain characteristic, the test appears as one-sided when there is a known difference between the two samples compared, such as, for example, in the case of solutions with different concentrations used for the selection of tasters. On the contrary, the test will be bilateral if the difference between the two products is not known; it will be the test itself that will provide us with information about the differences.

Number of answers	Minimum number of correct answers to establish a significant difference			Number of answers	Minimum number of correct answers to establish a significant difference		
	$\alpha = 0,05$ (*)	$\alpha = 0,01$ (**)	$\alpha = 0,001$ (***)		$\alpha = 0,05$ (*)	$\alpha = 0,01$ (**)	$\alpha = 0,001$ (***)
7	7	—	—	53	35	37	39
8	8	8	—	54	35	37	40
9	8	9	—	55	36	38	41
10	9	10	—	56	36	39	41
11	10	11	11	57	37	39	42
12	10	11	12	58	37	40	42
13	11	12	13	59	38	40	43
14	12	13	14	60	39	41	44
15	12	13	14	61	39	41	44
16	13	14	15	62	40	42	45
17	13	15	16	63	40	43	45
18	14	15	17	64	41	43	46
19	15	16	17	65	41	44	47
20	15	17	18	66	42	44	47
21	16	17	19	67	42	45	48
22	17	18	19	68	43	46	48
23	17	19	20	69	44	46	49
24	18	19	21	70	44	47	50
25	18	20	21	71	45	47	50
26	19	20	22	72	45	48	51
27	20	21	23	73	46	48	51
28	20	22	23	74	46	49	52
29	21	22	24	75	47	50	53
30	21	23	25	76	48	50	53
31	22	24	25	77	48	51	54
32	23	24	26	78	49	51	54
33	23	25	27	79	49	52	55
34	24	25	27	80	50	52	56
35	24	26	28	81	50	53	56
36	25	27	29	82	51	54	57
37	25	27	29	83	51	54	57
38	26	28	30	84	52	55	58
39	27	28	31	85	53	55	59
40	27	29	31	86	53	56	59
41	28	30	32	87	54	56	60
42	28	30	32	88	54	57	60
43	29	31	33	89	55	58	61
44	29	31	34	90	55	58	61
45	30	32	34	91	56	59	62
46	31	33	35	92	56	59	63
47	31	33	36	93	57	60	63
48	32	34	36	94	57	60	64
49	32	34	37	95	58	61	64
50	33	35	37	96	59	62	65
51	33	36	38	97	59	62	66
52	34	36	39	98	60	63	66

Table 4 Significance of the paired difference test (p=1/2) (Bilateral test)

7.4.1.3 Duo-trio test

The duo-trio test is used both in the selection and training phase of the panel and in the analysis of food products. It is particularly indicated in the evaluation of foods that have a pungent taste or unpleasant aftertaste, as this test requires a more limited number of tastings than the triangular test.

In the duo-trio test each taster must evaluate three samples, two equal and one different: one of the two identical samples is identified with the letter R and represents the reference sample. The taster must identify which of the two coded samples is the same as the reference sample. For the preparation and coding of the samples to be submitted to a duo-trio test a general form can be used, as has been reported for the previous tests.

For the statistical processing of the results, reference is made to table 2 since, similarly to the pair comparison test, the probability of identifying the sample at random as the reference one is of the 50%.

Also in this case, in addition to identifying the sample as the reference one, it is possible to ask the taster other questions concerning both the preference and the existence in a sample of some features present with greater or lesser intensity. Tables 2 or 4 are used for the statistical processing of this second part of the test depending on whether the test is unilateral or bilateral.

7.4.2. Discriminating qualitative tests and quantities

Some sensory analysis methods allow the estimation of one or more characteristics of a food product with reference to a measurement scale. The main scales are: ordinal scales, the scales at intervals and the scales of relationships. If the products to be evaluated are ordered based on the increasing or decreasing intensity of a specific attribute (sorting test) the ordinal scales are used. When the judgment of the taster is formulated on a scale characterized by one or more reference points, the other two types of scales are used. In the interval classification test, the scale is made up of a series of categories, each of which identifies a specific intensity of the feature considered or the level of appreciation a particular product elicits. The punching test, which uses a scale of relationships, instead provides for the measurement of the characteristics of a product in relation to one or more reference products.

7.4.2.1. Sorting test

With this test, panel members must order a certain number of samples based on the increasing or decreasing intensity of a specific attribute (colour, consistency, aroma intensity, etc.). The different samples can also be ordered according to their degree of acceptability (preference). Among the samples to be tested it is possible to include one of control or a standard, in order to obtain an ordering of the other samples with reference to the one with known characteristics. Unlike the tests described above, more than two samples are compared at a time in the sorting test.

This rapid sensory procedure allows the evaluation of several samples at the same time and the results obtained are easy to elaborate, but provide information on the order of intensity of a specific attribute, without allowing any quantification of the entity of the differences existing between the samples. The sorting test is used for the selection of the panel (tests carried out with the four basic flavors), in the preliminary phase of selection, by a larger group, of some samples to be subjected to more in-depth tests and finally in the preference tests carried out with a large group of tasters or consumers. For the execution of the test, the panel is required to order a certain number of samples based on the increasing or decreasing intensity of a given characteristic (figure 5). The test can be organized with the aid of a card that shows the order in which the products are served different tasters and the codes assigned to each sample (figure 6). The following examples show some evaluation sheets, used for sorting the samples.

ORDERING TEST
Product: …….. Date: …….. Name: …….. Code of taster: ……..
Five samples are presented identified by the following codes: ……. …… …… …… ……. Order the five samples based on your preference [or the increasing or decreasing intensity of a particular attribute]. To do this, write the code of the sample you prefer more in the box marked “1” and the abbreviation of the sample less appreciated or not in the box marked with “5”. Place the other samples in the intermediate boxes.

Preference order 1 2 3 4 5 [Intensity order]
Code

Figure 5Card for carrying out a sorting test

ORDERING TEST		
Product: Date: Sample A: Sample B: Sample C: Sample D: Sample E:		
Sample Code for different replicas 1 2 3		
A 937 B 130 C 725 D 418 E 512		
Taster,	Tasting order,	Sample code
1	BCDAE	
2	CDAEB	
3	BACDE	

Figure 6General sheet for preparation of the test of the order.

A quick processing of the results, even if incomplete, involves the calculation, for each sample, of the total scores (sum of the scores provided by all the tasters) and their comparison with the values obtained from tables developed by Kramer (tables 5-6 99% significantly level and 7-8 95% significantly level).

Tabella 9. Totale dei punteggi richiesti

Number of answers	Number of samples or treatments								
ris	2	3	4	5	6	7	8	9	10
2	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	3-19
3	—	—	—	—	—	—	—	—	4-29
	—	—	—	4-14	4-17	4-20	5-22	5-25	6-27
4	—	—	—	5-19	5-23	5-27	6-30	6-34	6-38
	—	—	5-15	6-18	6-22	7-25	8-28	8-32	9-35
5	—	—	6-19	7-23	7-28	8-32	8-37	9-41	9-46
	—	6-14	7-18	8-22	9-26	10-30	11-34	12-38	13-42
6	—	7-17	8-22	9-27	9-33	10-38	11-43	12-48	13-53
	—	8-16	9-21	10-26	12-30	13-35	14-40	16-44	17-49
7	—	8-20	10-25	11-31	12-37	13-43	14-49	15-55	16-61
	8-13	9-19	11-24	12-30	14-35	16-40	18-45	19-51	21-56
8	9-15	10-22	11-29	13-35	14-42	16-48	17-55	19-61	20-68
	9-15	11-21	13-27	15-33	17-39	19-45	21-51	23-57	25-63
9	10-17	12-24	13-32	15-39	17-46	19-53	21-60	22-68	24-75
	10-17	12-24	15-30	17-37	20-43	22-50	25-56	27-63	30-69
10	11-19	13-27	15-35	18-42	20-50	22-58	24-66	26-74	28-82
	11-19	14-26	17-33	20-40	23-47	25-55	28-62	31-69	34-76
11	12-21	12-29	17-38	20-46	22-55	25-63	27-72	30-80	32-89
	13-20	16-28	19-36	22-44	25-52	29-59	32-67	35-75	39-82
12	14-22	17-31	19-41	22-50	25-59	28-68	31-77	33-87	36-96
	14-22	18-30	21-39	25-47	28-56	32-64	36-72	39-81	43-89
13	15-24	18-34	21-44	25-53	28-63	31-73	34-83	37-93	40-103
	15-24	19-33	23-42	27-51	31-60	35-69	39-78	44-86	48-95
14	16-26	20-36	24-46	27-57	31-67	34-78	38-88	41-99	45-109
	17-25	21-35	25-45	30-54	34-64	39-73	43-83	48-92	52-102
15	18-27	22-38	26-49	30-60	34-71	37-83	41-94	45-105	49-116
	18-27	23-37	28-47	32-58	37-68	42-78	47-88	52-98	57-108
16	19-29	23-41	28-52	32-64	36-76	41-87	45-99	49-111	53-123
	19-29	25-39	30-50	35-61	40-72	46-82	51-93	56-104	61-115
17	20-31	25-43	30-55	35-67	39-80	44-92	49-104	53-117	58-129
	21-30	26-42	32-53	38-64	43-76	49-87	55-98	60-110	66-121
18	22-32	27-45	32-58	37-71	42-84	47-97	52-110	57-123	62-136
	22-32	28-44	34-56	40-68	46-80	52-92	59-103	65-115	71-127
19	23-34	29-47	34-61	40-74	45-88	50-102	56-115	61-129	67-142
	24-33	30-46	36-59	43-71	49-84	56-96	62-109	69-121	76-133
20	24-36	30-50	36-64	42-78	48-92	54-106	60-120	65-135	71-149
	25-35	32-48	38-62	45-75	52-88	59-101	66-114	73-127	80-140

Table 5 Total scores required for 99% significance (page 1)

per una significatività del 99%

Number of answers	Number of samples or treatments								
ris	2	3	4	5	6	7	8	9	10
21	26-37	32-52	38-67	45-81	51-96	57-111	63-126	69-141	75-156
	26-37	33-51	41-64	48-78	55-92	63-105	70-119	73-127	80-140
22	27-39	34-54	40-70	47-85	54-100	60-116	67-131	74-146	80-162
	28-38	35-53	43-67	51-81	58-96	66-110	74-124	82-138	90-152
23	28-41	36-56	43-72	50-88	57-104	64-120	71-136	78-152	85-168
	29-40	37-55	45-70	53-85	62-99	70-114	78-129	86-144	95-158
24	30-42	37-59	45-75	52-92	60-108	67-125	75-141	82-158	89-175
	30-42	39-57	47-73	56-88	65-103	73-119	82-134	91-149	99-165
25	31-44	39-61	47-78	55-95	63-112	71-129	78-147	86-164	94-181
	32-43	41-59	50-75	59-91	68-107	77-123	86-139	95-155	104-171
26	33-45	41-63	49-81	57-99	66-116	74-134	82-152	90-170	98-188
	33-45	42-62	52-78	61-95	71-111	80-128	90-144	100-160	109-177
27	34-47	43-65	51-84	60-102	69-120	77-139	86-157	94-176	103-194
	35-46	44-64	54-81	64-98	74-115	84-132	94-149	104-166	114-183
28	35-49	44-68	54-86	63-105	72-124	81-143	90-162	99-181	108-200
	36-48	46-66	56-84	67-101	77-119	88-136	98-154	108-172	119-189
29	37-50	46-70	56-89	65-109	75-128	84-148	94-167	103-187	112-207
	37-50	48-68	59-86	69-105	80-123	91-141	102-159	113-177	124-195
30	38-52	48-72	58-92	68-112	78-132	88-152	97-173	107-193	117-213
	39-51	50-70	61-89	72-108	83-127	95-145	106-164	117-183	129-201
31	39-54	50-74	60-95	71-115	81-136	91-157	101-178	112-198	122-219
	40-53	51-73	63-92	75-111	86-131	98-150	110-169	122-188	133-208
32	41-55	52-76	62-98	73-119	84-140	95-161	105-183	116-204	126-226
	41-55	53-75	65-95	77-115	90-134	102-154	114-174	126-194	138-214
33	42-57	53-79	65-100	76-122	87-144	98-166	109-188	120-210	131-232
	43-56	55-77	68-97	80-118	93-138	105-159	118-179	131-199	143-220
34	44-58	55-81	67-103	78-126	90-148	102-170	113-193	124-216	136-238
	44-58	57-79	70-100	83-121	96-142	109-163	122-184	135-205	148-226
35	45-60	57-83	69-106	81-129	93-152	105-175	117-198	129-221	141-244
	46-59	59-81	72-103	86-124	99-146	113-167	126-189	140-210	153-232
36	46-62	59-85	71-109	84-132	96-156	109-179	121-203	133-227	145-251
	47-61	61-83	74-106	88-128	102-150	116-172	130-194	144-216	158-238
37	48-63	61-87	74-111	86-136	99-160	112-184	125-208	137-233	150-257
	48-63	63-85	77-108	91-131	105-154	120-176	134-199	149-221	163-244
38	49-65	62-90	76-114	89-139	102-164	116-188	129-213	142-238	155-263
	50-64	64-88	79-111	94-134	109-157	123-181	138-204	153-227	168-250
39	51-66	64-92	78-117	92-142	105-168	119-193	133-218	146-244	160-269
	51-66	66-90	81-114	97-137	112-161	127-185	142-209	158-232	173-256
40	52-68	66-94	80-120	94-146	109-171	123-197	137-223	150-250	164-276
	53-67	68-92	84-116	99-141	115-165	131-189	146-214	162-238	178-262

Table 6 Total scores required for 99% significance (page 2)

Tabella 10. Totale dei punteggi richiesti

Number of answers	Number of samples or treatments								
	2	3	4	5	6	7	8	9	10
2	—	—	—	—	—	—	—	—	—
	—	—	—	3-9	3-11	3-13	4-14	4-16	4-18
3	—	—	—	4-14	4-17	4-20	4-23	5-25	5-28
	—	4-8	4-11	5-13	6-15	6-18	7-20	8-22	8-25
4	—	5-11	5-15	6-18	6-22	7-25	7-29	8-32	8-36
	—	5-11	6-14	7-17	8-20	9-23	10-26	11-29	13-31
5	—	6-14	7-18	8-22	9-26	9-31	10-35	11-39	12-43
	6-9	7-13	8-17	10-20	11-24	13-27	14-31	15-35	17-38
6	7-11	8-16	9-21	10-26	11-31	12-36	13-41	14-46	15-51
	7-11	9-15	11-19	12-24	14-28	16-32	18-36	20-40	21-45
7	8-13	10-18	11-24	12-30	14-35	15-41	17-46	18-52	19-58
	8-13	10-18	13-22	15-27	17-32	19-37	22-41	24-46	26-51
8	9-15	11-21	13-27	15-33	17-39	18-46	20-52	22-58	24-64
	10-14	12-20	15-25	17-31	20-36	23-41	25-47	28-52	31-57
9	11-16	13-23	15-30	17-37	19-44	22-50	24-57	26-64	28-71
	11-16	14-22	17-28	20-34	23-40	26-46	29-52	32-58	35-64
10	12-18	15-25	17-33	20-40	22-48	25-55	27-63	30-70	32-78
	12-18	16-24	19-31	23-37	26-44	30-50	33-57	37-63	40-70
11	13-20	16-28	19-36	22-44	25-52	28-60	31-68	34-76	36-85
	14-19	18-26	21-34	25-41	29-48	33-55	37-62	41-69	45-76
12	15-21	18-30	21-39	25-47	28-56	31-65	34-74	38-82	41-91
	15-21	19-29	24-36	28-44	32-52	37-59	41-67	45-75	50-82
13	16-23	20-32	24-41	27-51	31-60	35-69	38-79	42-88	45-98
	17-22	21-31	26-39	31-47	35-56	40-64	45-72	50-80	54-89
14	17-25	22-34	26-44	30-54	34-64	38-74	42-84	46-94	50-104
	18-24	23-33	28-42	33-51	38-60	44-68	49-77	54-86	59-95
15	19-26	23-37	28-47	32-58	37-68	41-79	46-89	50-100	54-111
	19-26	25-35	30-45	36-54	42-63	47-73	53-82	59-91	64-101
16	20-28	25-39	30-50	35-61	40-72	45-83	49-95	54-106	59-117
	21-27	27-37	33-47	39-57	45-67	51-77	57-87	63-97	69-107
17	22-29	27-41	32-53	38-64	43-76	48-88	53-100	58-112	63-124
	22-29	28-40	35-50	41-61	48-71	54-82	61-92	67-103	74-113
18	23-31	29-43	34-56	40-68	46-80	51-93	57-105	62-118	68-130
	24-30	30-42	37-53	44-64	51-75	58-86	65-97	72-108	79-119
19	24-33	30-46	37-58	43-71	49-84	55-97	61-110	67-123	73-136
	25-32	32-44	39-56	47-67	54-79	62-90	69-102	76-114	84-125
20	26-34	32-48	39-61	45-75	52-88	58-102	65-115	71-129	77-143
	26-34	34-46	42-58	50-70	57-83	65-95	73-107	81-119	89-131

Table 7 Total scores required for 95% significance (page 1)

per una significatività del 95%

Number of answers	Number of samples or treatments								
	2	3	4	5	6	7	8	9	10
21	27-36 28-35	34-50 36-48	41-64 44-61	48-78 52-74	55-92 61-86	62-106 69-99	68-121 77-112	75-135 86-124	82-149 94-137
22	28-38 29-37	36-52 38-50	43-67 46-64	51-81 55-77	58-96 64-90	65-111 73-103	72-126 81-117	80-140 90-130	87-155 99-143
23	30-39 31-38	38-54 40-52	46-69 49-66	53-85 58-80	61-100 67-94	69-115 76-108	76-131 85-122	84-146 95-135	91-162 104-149
24	31-41 32-40	40-56 41-55	48-72 51-69	56-88 61-83	64-104 70-98	72-120 80-112	80-136 90-126	88-152 99-141	96-168 109-155
25	33-42 33-42	41-59 43-57	50-75 53-72	59-91 63-87	67-108 73-102	76-124 84-116	84-141 94-131	92-158 104-146	101-174 114-161
26	34-44 35-43	43-61 45-59	52-78 56-74	61-95 66-90	70-112 77-105	79-129 87-121	88-146 98-136	97-163 108-152	106-180 119-167
27	35-46 36-45	45-63 47-61	55-80 58-77	64-98 69-93	73-116 80-109	83-133 91-125	92-151 102-141	101-169 113-157	110-187 124-173
28	37-47 38-46	47-65 49-63	57-83 60-80	67-101 72-96	76-120 83-113	86-138 95-129	96-156 106-146	106-174 118-162	115-193 129-179
29	38-49 39-48	49-67 51-65	59-86 63-82	69-105 74-100	80-123 86-117	90-142 98-134	100-161 110-151	110-180 122-168	120-199 134-185
30	40-50 41-49	51-69 53-67	61-89 65-85	72-108 77-103	83-127 90-120	93-147 102-138	104-166 114-156	114-186 127-173	125-205 139-191
31	41-52 42-51	52-72 55-69	64-91 67-88	75-111 80-106	86-131 93-124	97-151 106-142	108-171 119-160	119-191 131-179	130-211 144-197
32	42-54 43-53	54-74 56-72	66-94 70-90	77-115 83-109	89-135 96-128	100-156 109-147	112-176 123-165	123-197 136-184	134-218 149-203
33	44-55 45-54	56-76 58-74	68-97 72-93	80-118 86-112	92-139 99-132	104-160 113-151	116-181 127-170	128-202 141-189	139-224 154-209
34	45-57 46-56	58-78 60-76	70-100 74-96	83-121 88-116	95-143 103-135	108-164 117-155	120-186 131-175	132-208 145-195	144-230 159-215
35	47-58 48-57	60-80 62-78	73-102 77-98	86-124 91-119	98-147 106-139	111-169 121-159	124-191 135-180	136-214 150-200	149-236 165-220
36	48-60 49-59	62-82 64-80	75-105 79-101	88-128 94-122	102-150 109-143	115-173 124-164	128-196 139-185	141-219 155-205	154-242 170-226
37	50-61 51-60	63-85 66-82	77-108 81-104	91-131 97-125	105-154 112-147	118-178 128-168	132-201 144-189	145-225 159-211	159-248 175-232
38	51-63 52-62	65-87 68-84	80-110 84-106	94-134 100-128	108-158 116-150	122-182 132-172	136-206 148-194	150-230 164-216	164-254 180-238
39	52-65 53-64	67-89 70-86	82-113 86-109	97-137 102-132	111-162 119-154	126-186 135-177	140-211 152-199	154-236 168-222	169-260 185-244
40	54-66 55-65	69-91 72-82	84-116 88-112	99-141 105-135	114-166 122-158	129-191 139-181	144-216 156-204	159-241 173-227	173-267 190-250

Table 8Total scores required for 95% significance (page 2)

In the tables, for each combination number of samples and number of answers, there are two pairs of numbers. The first pair indicates the interval outside which the global score of at least one sample must be found to conclude that there is a significant difference between the samples. The second pair

is used to identify which sample (or which samples) differs from the others. Once established, comparing the scores of the different samples with the first pair of numbers, which there are significant differences, we compare each score with the second pair of numbers. If the score of a sample is lower than the first number of the couple, it means that that sample is significantly different from the others (for example because it is less sweet or because it is less appreciated), if it is higher than the second number of the pair, there will be a similar result, but in the opposite direction (for example, because it is sweeter or more pleasing). With this method it is possible to classify the samples in no more than 3 categories: those that have a score lower than the first number of the pair, those whose score is located within the pair and, finally, those samples that have a score higher than the second number of the couple. Within each group it is not possible to have information on the existence or otherwise of differences between the different samples. With the method proposed by Nemenyi and Dunn-Runkin (method of multiple comparison between the sums of scores) it is possible to make a direct comparison between the total scores of each sample, calculating the difference. between each pair of scores and comparing these differences with tabulated values (table 9-10), depending on the number of samples compared and the number of tasters. Another method of processing the results involves the use of the analysis of variance after transforming the positions of each sample into the score by means of a special table.

Tabella 11. Differenze critiche per il

Number of answers		Number of samples or treatments												
		3	4	5	6	7	8	9	10	11	12	13	14	15
5	0,01	9	12	16	19	23	26	29	33	37	40	44	47	51
	0,05	8	11	14	17	20	23	26	30	34	37	40	43	47
6	0,01	10	14	17	21	25	29	33	37	41	45	49	53	57
	0,05	9	12	15	19	22	26	29	33	37	41	43	48	52
7	0,01	11	15	19	23	27	31	36	40	44	49	53	58	62
	0,05	9	13	16	20	24	28	32	36	40	44	48	52	56
8	0,01	12	16	20	25	29	34	38	43	47	52	57	62	67
	0,05	10	14	17	21	25	30	34	38	42	47	51	56	60
9	0,01	12	17	22	26	31	36	41	46	51	56	61	66	71
	0,05	10	14	18	23	27	31	36	40	45	50	54	59	64
10	0,01	13	18	23	28	33	38	43	49	54	59	65	70	75
	0,05	11	15	19	24	28	33	38	43	47	52	57	62	67
11	0,01	14	19	24	29	35	40	46	51	57	62	68	74	78
	0,05	11	15	20	25	30	35	40	45	50	55	60	65	71
12	0,01	14	20	25	31	36	42	48	54	59	65	71	77	83
	0,05	12	16	21	26	31	36	41	47	52	58	63	68	74
13	0,01	15	21	26	32	38	44	50	56	62	68	74	80	87
	0,05	12	17	22	27	32	38	43	49	54	60	65	71	77
14	0,01	16	21	27	33	39	45	52	58	64	71	77	84	90
	0,05	13	17	23	28	34	39	45	50	56	62	68	74	80
15	0,01	16	22	28	34	41	47	54	60	67	73	80	87	94
	0,05	13	18	24	29	35	40	46	52	58	64	70	76	83
16	0,01	16	23	29	36	42	49	56	62	69	76	83	90	97
	0,05	13	18	24	30	36	42	48	54	60	67	73	79	86
17	0,01	17	23	30	37	43	50	57	64	71	79	86	93	100
	0,05	14	19	25	31	37	43	50	56	62	69	75	82	88
18	0,01	17	24	31	38	45	52	59	66	74	81	85	96	103
	0,05	14	20	26	32	38	45	51	57	64	71	77	84	91
19	0,01	18	25	32	39	46	53	61	68	76	83	91	98	106
	0,05	14	20	27	33	39	46	52	59	66	73	80	86	93
20	0,01	18	25	33	40	47	55	62	70	78	85	93	101	109
	0,05	15	21	27	34	40	47	54	61	68	75	82	89	96
21	0,01	19	26	33	41	48	56	64	72	79	87	95	104	112
	0,05	15	21	28	35	41	48	55	62	69	76	84	91	98
22	0,01	19	27	34	42	49	57	65	73	81	89	98	106	114
	0,05	16	22	29	35	42	49	56	64	71	78	86	93	101

Table 9 Critical differences when comparing multiple pairs of samples (page 1)

confronto tra più coppie di campioni

Number of answers		Number of samples or treatments												
		3	4	5	6	7	8	9	10	11	12	13	14	15
23	0,01	20	27	35	43	51	59	67	75	83	91	100	108	117
	0,05	16	22	29	36	43	50	58	65	72	80	88	95	103
24	0,01	20	28	36	44	52	60	68	76	85	93	102	111	119
	0,05	16	23	30	37	44	51	59	66	74	82	89	97	105
25	0,01	21	28	36	44	53	61	70	78	87	95	104	113	122
	0,05	17	23	31	38	45	52	60	68	75	83	91	99	107
26	0,01	21	29	37	45	54	62	71	80	88	97	106	115	124
	0,05	17	24	31	38	46	54	61	69	77	85	93	101	109
27	0,01	21	30	38	46	55	63	72	81	90	99	108	117	127
	0,05	17	24	32	39	47	55	62	70	78	87	95	103	113
28	0,01	22	30	39	47	56	65	74	83	92	101	110	120	129
	0,05	18	25	32	40	48	56	64	72	80	88	97	105	113
29	0,01	22	31	39	48	57	66	75	84	93	103	112	122	131
	0,05	18	25	33	41	49	57	65	73	81	90	98	107	116
30	0,01	23	31	40	49	58	67	76	86	95	104	114	124	133
	0,05	18	26	33	41	49	58	66	74	83	91	100	109	117
31	0,01	23	32	41	50	59	68	77	87	97	106	116	126	136
	0,05	18	26	34	42	50	58	67	75	84	93	102	110	119
32	0,01	23	32	41	50	60	69	79	88	98	108	118	128	138
	0,05	19	27	35	43	51	59	68	77	85	94	103	112	121
33	0,01	24	33	42	51	61	70	80	90	100	110	120	130	140
	0,05	19	27	35	43	52	60	69	78	87	96	105	114	123
34	0,01	24	33	42	52	61	71	81	91	101	111	121	132	142
	0,05	19	27	36	44	53	61	70	79	88	97	106	116	125
35	0,01	24	34	43	53	62	72	82	92	103	113	123	134	144
	0,05	20	28	36	45	53	62	71	80	89	99	108	117	127
36	0,01	25	34	44	53	63	73	83	94	104	114	125	136	146
	0,05	20	28	37	45	54	63	72	81	91	100	109	119	129
37	0,01	25	35	44	54	64	74	85	95	105	116	127	137	148
	0,05	20	29	37	46	55	64	73	82	92	101	111	121	130
38	0,01	25	35	45	55	65	75	86	96	107	118	128	139	150
	0,05	20	29	38	46	56	65	74	84	93	103	112	122	132
39	0,01	26	35	45	56	66	76	87	98	108	119	130	141	152
	0,05	21	29	38	47	56	66	75	85	94	104	114	124	134
40	0,01	26	36	46	56	67	77	88	99	110	121	132	143	154
	0,05	21	30	39	48	57	66	76	86	95	105	115	125	136

Table 10 Critical differences when comparing multiple pairs of samples (page 2)

7.4.2.2. Range classification test

It is one of the most frequently used methods to highlight and quantify the differences between two or more food products or to establish their level of satisfaction. It is a test that can be carried out both with selected tasters and with normal consumers. This method allows the evaluation of the intensity of a certain characteristic, or the acceptability of a food product, using intervals scales. Each point on the scale identifies the intensity of a particular characteristic or the level of satisfaction expressed with respect to the different products evaluated (hedonistic-verbal scales). Numbers can be associated to each interval of the scale in order to favor the tasters' choices and the processing of the results. Some examples of interval scales are shown in Figure 7. Generally the scales are formed by a number of variable intervals from 5 to 11 (more frequently 7-9). It is not advisable to use ladders with too small a number of intervals in order to limit the so-called end effect or distortion of scores at the extremes of the scale attributable to the reluctance of the tasters to assign too high or too low scores. In evaluating the results, standard statistical methods are used, such as the t-Student test or the analysis of variance, in order to highlight whether there are significant differences between the attributes or the scores assigned to the different samples. These processing procedures assume that the points of the scale divide it into intervals of equal amplitude, that is to say that, for example, the difference in acceptability. between two listed products I and 2 is the same as that existing between two listed products 8 and 9. It has been shown that this is not always true both for the different interpretation of the words by the tasters and for the aforementioned reluctance of the tasters to assign extreme scores. Despite these approximations, the interval classification method is considered one of the most reliable methods to measure both the preference and the acceptability of the products, and to quantify the intensity of a particular characteristic of a product.

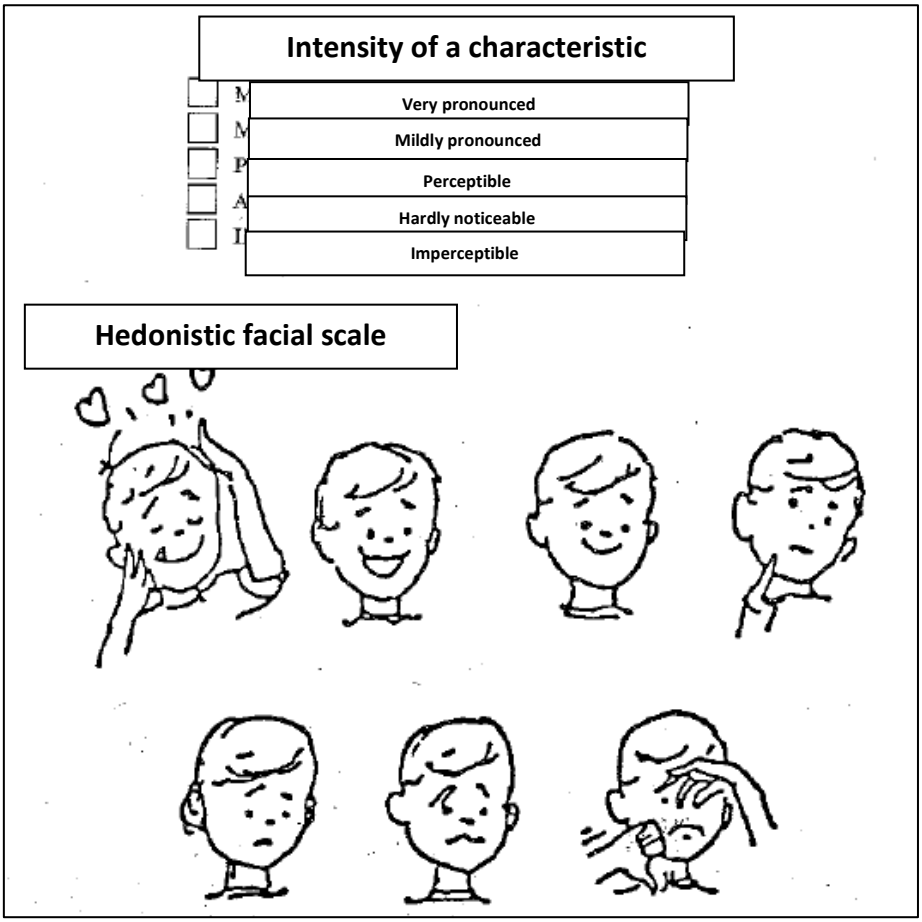


Figure 7 Examples of verbal and graphic interval scales

7.4.2.3. Score test (report scales)

Interval scales only provide information about the differences in intensity between the attributes of two or more food products, but do not provide any information regarding the relationships between the aforementioned attributes. For example, a hardness evaluated 8 with an interval scale is four intervals from a hardness evaluated 4, but this does not mean that a product rated 8 has a hardness twice that estimated.

With the relationship scales, on the other hand, we evaluate the intensity of a certain characteristic compared to a reference. Suppose you want to evaluate the intensity of the sweet taste of an orange juice (sample A) in comparison with that of a reference juice (sample R). The question that will be formulated to the tasters will be of the type: "how many times more (or less) dessert is the sample A compared to the sample R?" To respond to a question of this type, the taster must be appropriately trained on the use of relationship scales.

The previous example uses an open scale; alternatively it is possible to delimit the scale with two references having minimum and maximum intensity of the characteristic to be evaluated. From the comparison with the two reference samples, the relative intensity of the organoleptic characteristic of the unknown sample is evaluated. The answer is given on a straight line segment (measurement scale) by drawing a line perpendicular to the scale at the point which, in terms of distance from the ends, represents the estimate provided by the adjuster of the intensity of the unknown characteristic with respect to that of the regions of reference. In this case the taster receives,

in addition to the sample to be evaluated, two references: one of these, which is assigned the score I, has a minimum intensity of the characteristic to be evaluated, the other, to which the score is assigned 9 , presents the characteristic with maximum intensity. The taster must establish at which point on the ladder the vertical section is to be used using a simultaneous comparison procedure with the two references. The following question will then be asked: "is the intensity of the characteristic of the sample to be evaluated halfway between that of the other two?" If so, it will put the vertical stroke at the value 5 and so on. The scales used can be divided into a series of equal intervals (structured scales), or they can be unstructured when only the extremes are defined.
The results are processed with the t-Student test or, when the unknown samples are. more than two, by analyzing the variance and the Duncan test.