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Laboratory methods of experimental phytochemistry

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BRNO 2017

Content

1 BASIC INFORMATION ABOUT PLANT SECONDARY METABOLITES

1.1 Introduction

Interest in plant substances has existed since ancient times. Plants have been used for many purposes, not only as a source of food, but also as colorants, for purposes of shamanism, and for simple medicine. Plants with intoxicating and hallucinogenic properties, or poisonous plants, such as the opium, poppy (*Papaver somniferum*), cannabis (*Cannabis sativa*), belladonna (*Atropa belladonna*), thorn apple (*Datura stramonium*), monk´s hood (*Aconitum* spp.) and hemlock (*Conium maculatum*) were among the most interesting and frequently used plants. In Antiquity knowledge of the use of herbs was widespread, but it stagnated in the Middle Ages and survived only as folk medicine, or in monasteries. The discovery and isolation of the first active plant substances had to await the development of methodology suitable for their isolation and analysis which came only as industry and scientific disciplines grew. Alkaloids were the first to be isolated. They were striking for their strong biological activity, and they were easy to isolate. Given present knowledge we could say their isolation is “trivial“ today, thanks to their chemical properties and abundance in source materials. Some examples: in 1806 Sertürner (Germany) isolated morphine; in 1817-1820 Pelletier and Caventou (France) isolated the crystalline alkaloids strychnine, brucine, and quinine; in 1819 Runge (Germany) isolated caffeine; in 1829 Posselt and Reimann isolated nicotine, and in 1848 Merck (Germany) isolated papaverine. Students of pharmaceutics can be proud that pharmacists actively participated in these first isolations. However, alkaloids were not the only substances which were examined. As early as 1664 Boyle (England) examined plant colorants, such as violet anthocyanins and yellow flavonoids, for use in the textile industry.

As a scientific discipline, phytochemistry was introduced and established at universities around the world at the turn of the 20th century and developed thanks especially to the improving ability of organic chemists to crystallize substances from more complex mixtures and to identify them, using in particular so-called ”chemical degradation“ methods. The first biosynthetic pathways were also examined. By 1957, in addition to alkaloids approximately 2 750 other vegetable substances were known. More than 5 000 vegetable metabolites had been described. This shows the significant importance of the typical properties of alkaloids

(crystalline form, basic character, and significant biological activity) for their isolation and description.

Phytochemistry changed after WWII. Many newly developed identification methods, improved during the war, were introduced in practice. Of these, sophisticated chromatographic techniques were the most important. Improved chromatographic separation enabled the isolation of substances which were not easy to crystallize, and those of amorphous character. Spectral analytical techniques enabled more rapid identification of the isolated substances compared with the lengthy degradation procedure and also worked with milligram quantities, as compared to the prior need of grams. More than 280 000 natural compounds are known and this number is growing.

1.2 Main classes of plant secondary metabolites

1.2.1 Characteristics of primary and secondary metabolism

Substances isolated from plants can be divided into primary and secondary metabolites according to how they are synthesized, whether they are ubiquitous in the plants, and whether or not they play an essential role in the metabolism of the plant. Biochemistry deals with primary metabolites, whereas secondary metabolites are the subject of study of pharmacognosy. We will restrict ourselves here to basic definitions only.

1.2.1.1 Primary metabolites

Primary metabolites include common carbohydrates, proteinogenic amino acids, purines and pyrimidines of nucleic acids, chlorophyll and common vegetable colorants, simple fats, and simple low-molecular weight carboxylic acids. These substances are more or less omnipresent in plants. They are involved in the basic metabolic processes of the so-called primary plant metabolism and are indispensable for the life of plants and other living organisms.

1.2.1.2 Secondary metabolites

Secondary metabolites are compounds formed from precursors originating in the primary metabolism. Their distribution among plant families, and often among species, is limited; they are not ubiquitous in plants (taxonomic restriction). A plant uses specialized enzyme systems for their synthesis and their formation is identified as secondary metabolism. Their role in the life of plants is, to a great extent, unknown and some advantages in the struggle for life are considered. The known functions of secondary metabolites will be mentioned below.

The aforementioned criteria for assigning a substance as primary or secondary metabolite are limited. Natural substances can be of importance in the primary metabolism (*e.g.*, some fatty acids with special structures), and their occurrence may simultaneously be limited to a single family.

Some substances are considered to be secondary metabolites, because the limits of current detection methods prevent demonstrating an ubiquitous nature. An example of such a substance is squalene, which was long considered to be a secondary metabolite that occurred only in the liver of sharks, but is today known as a basic precursor of steroids and higher terpenes.

When a new secondary metabolite is isolated, naming it presents a problem. Although the chemical description of the structure gives the maximum possible information, the chemical name is often too complicated for practical use. In the early days of phytochemistry, trivial names were widely used. A substance was called according to the vegetable species from which it was obtained (strychnine alkaloid – genus *Strychnos*, atropine alkaloid – genus *Atropa*, etc.). Naming after the author who described the substance was also used (peletierine alkaloid – Pelletier), or it might be named for an important property of the isolated substance (morphine alkaloid – according to Morpheus, the god of dreams, diacetylmorphine – heroin – ”heroic“ analgesic). The growing number of known natural substances and their derivatives make it obvious that this system is also limited. It is therefore currently more suitable to use semi-trivial naming, or the indexing of substances by means of numbers or letters of the alphabet (e.g., a mixture of azadirachtines A-G or ginsenosides Rb_1, Rb_2, Rc, etc.).

A biosynthetic system is also used at present for sorting and classifying natural substances. It follows from the division of substances into groups according to the

biosynthetic pathways and precursors used by the plant for their biosynthesis. This system suffers from limited knowledge of the biosynthetic origin of newly isolated or highly complex molecules. Not only single, but also sometimes multiple precursors originating in different parts of the primary metabolism are used in biosynthesis. For the simplified purposes of this text, we will divide secondary metabolites into three main groups: **terpenoids** (1.3), **alkaloids and nitrogen compounds of non-alkaloid nature** (1.4) and **phenolic substances** (1.5).

1.3 Terpenoids

Terpenoids or isoprenoids are usually lipophilic compounds characterized by their biosynthetic origin as substances derived from combinations of 3-methyl-3-butenyldiphosphate and 3-methyl-2-butenyldiphosphate.

Fig. 1: 3-methyl-3-butenyldiphosphate and 3-methyl-2-butenyldiphosphate

They are found especially in the glandular trichomes of plants, in bud exudates, in flowers, and in resins. Chemically, they are usually cyclic hydrocarbons saturated or unsaturated (with varying degrees of unsaturation), and oxidized to varying degrees (alcohols, ketones, etc.). According to the number of biosynthetically connected 5-carbon isoprenoid units, they are simply divided into monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), and triterpenes (C30). Their biosynthetic relationship is depicted briefly in the following figure (Fig. 2).

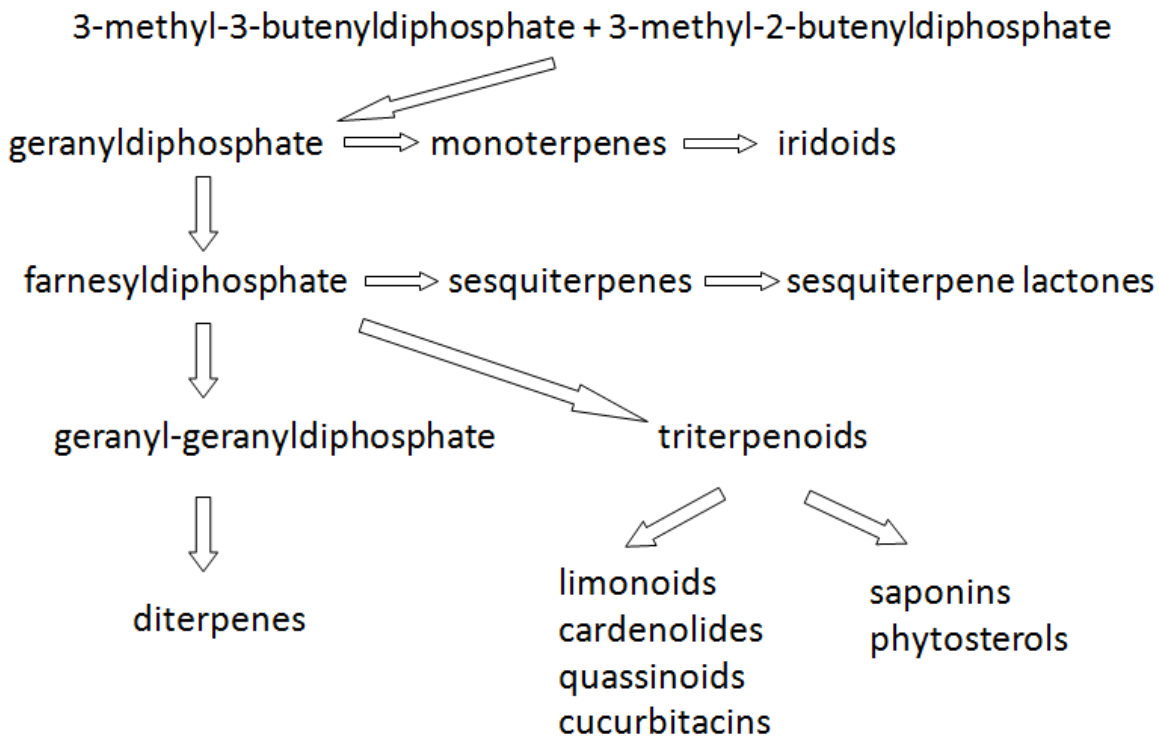

Fig. 2: Simplified overview of the individual classes of terpenoids and their biosynthetic relationships

The volatility of terpenes is related to the number of 5-carbon biosynthetic units; substances with 10-20 carbons have quite low boiling points and are often responsible for a characteristic odor, sometimes pleasant. Sub-groups of substances called iridoids and sesquiterpene lactones (with a lactone ring) can be singled out from the group of monoterpenes and the sesquiterpenes, respectively. Iridoids are substances which often have a bitter flavor (used as amara) or serve as precursors in the biosynthesis of alkaloids. The group of sesquiterpene lactones, represented by more than 3 000 compounds, is especially important because it is typical for plants of the Asteraceae family. Diterpenes include, *e.g.*, giberellins, which are plant growth hormones, or some acids that form the basis of resins. Triterpenoid structures are typical for saponins (substances with foaming and hemolytic abilities), phytosterols, and cardiac glycosides, etc.

1.4 Alkaloids and other nitrogen-containing compounds

Alkaloids are probably the best known nitrogenous secondary metabolites isolated from plants. Genuine alkaloids have an organic base where nitrogen forms part of the structure, usually built-in in the form of a 5- or 6-membered ring. Their precursor is a limited group of amino acids (e.g., ornithine, lysine, phenylalanine, tyrosine, tryptophan). There are other substances, closely related to alkaloids, the so-called protoalkaloids and pseudoalkaloids, but they do not meet all of the particulars described above, *i.e.,* nitrogen does not form part of their heterocycle, or they are formed by other mechanisms and not from the limited number of amino-acid precursors.

Another group of nitrogenous natural substances is called cyanogenic glycosides. These substances differ significantly in their structure; their common feature is that their hydrolysis releases nitrogen in the form of a nitrile. These substances are typical of, *e.g.,* the Rosaceae family and other occurrences are taxonomically relatively limited. Another group is assigned as glucosinolates, structurally characterized by the presence of a double bond between nitrogen and carbon, where the aglycone (containing nitrogen) is further bound *via* sulfur to a carbohydrate residue, usually glucose. Glucosinolates are typical for Brassicaceae plants and after partial hydrolysis and transformation they are responsible for the typical pungent odor and taste of these plants (mustard). For biosynthetic relationships of alkaloids and nitrogen-containing compounds see Fig. 3.

It should be noted that the occurrence of many nitrogenous secondary metabolites is taxonomically quite limited. Moreover, nitrogenous substances are often formed from proteinogenic amino acids, so other biochemical processes, such as protein synthesis, compete for the nitrogen. Also the concentrations of secondary nitrogenous metabolites in plants are usually significantly lower than those of other substances. This is caused by their more complicated metabolism and strong biological activity (the plant must not kill itself) and by the fact that concentrations of these substances differ significantly in different organs and tissues of the plant (*e.g.,* higher concentrations in reserve organs).

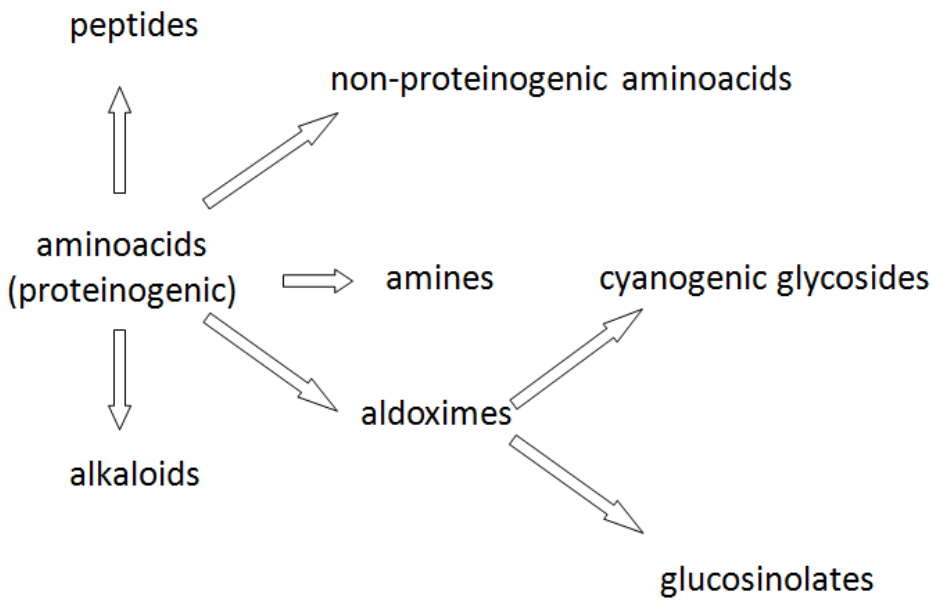

Fig. 3: Simplified overview of nitrogenous plant metabolites and their biosynthetic relationships

1.5 Phenolic compounds

Phenolic compounds are aromatic substances bearing one or more hydroxyl groups (the hydroxyl can be replaced, *e.g.*, by a methyl or carbohydrate residue). Biosynthetically, these substances are usually based on the so-called shikimate or acetate pathways. Simply said, the shikimate pathway represents metabolism based on the formation of sedoheptulose and its transformation into shikimic acid and subsequently into phenylalanine. It is de-aminated to *p*-hydroxycinnamic acid and numerous phenolic compounds are subsequently formed from it. The acetate pathway results in the formation of phenols by connecting acetic units (in the form of malonyl-CoA) with the subsequent formation of a ring. These two biosynthetic pathways can cross each other and result in the formation of combined phenolic compounds, including, *e.g.*, flavonoids. A brief diagram of the formation and transformation of the phenolic group is shown in Fig. 4. The various classes of phenolic substances differ in molecular weight - simple substances, *e.g.*, of the type of benzoic acid in the hundreds, of various pigments of the anthocyanin type in units of thousands, and of polymeric condensed tannins in the tens of thousands of Daltons.

The typical properties of phenols include their ability to ionize. Many phenolic compounds (especially these of shikimic acid origin) are able to form chelates with bivalent or trivalent metals. Some anthocyanins occur in plants as chelates with iron or magnesium. Phenolic substances with *ortho*- or *para*- hydroxyl groups easily oxidize to the corresponding quinones.

The majority of phenols are potentially toxic, therefore plants usually deposit them in vacuoles in the form of glycosides or sulfates (water-soluble compounds). There may be dozens of such glycosides of a single phenolic substance, *e.g.*, more than 130 different glycosides for quercetine, a flavonol aglycone, have been described. In contrast, lipophilic phenols are usually brought to the surface of the plant in the form of various exudates or are localized in surface tissues, probably to protect the plant against feeders, infections, or the influence of the ambient environment.

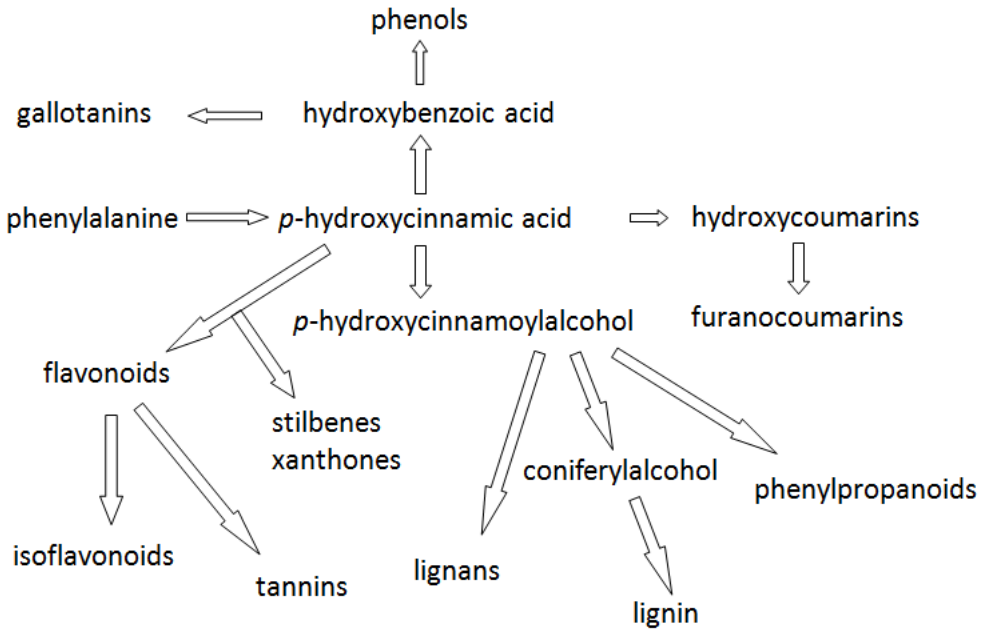

Fig. 4: Simplified overview of plant polyphenols and their biosynthetic relationships

1.6 Function of plant secondary metabolites

It has been quite difficult to determine the real function of secondary metabolism. The first problem is to distinguish between the primary and secondary metabolism. Until quite recently, for example, shikimic acid, was still considered to be a secondary metabolite of the

Japanese tree *Illicium anisatum* (a similar problem occurred with the aforementioned squalene). Certain substances can play roles in both primary and secondary metabolism. An example would be certain non-proteinogenous amino acids that serve to protect against herbivore species and at the same time provide a reserve "metabolic pool" for nitrogen transformation. Such recycling of nitrogen can also occur with alkaloids such as caffeine. Another complication is the multi-functionality of secondary metabolites. Salicylic acid, for example, is considered to be one of signal substances produced by plants in stress situations. At the same time it can operate as a substance stimulating heat production during pollination (in *Calla* species). It is also accumulated in the leaves of various willow trees (*Salix* spp.) as a defense against feeders. Moreover, it is produced and released in *Quercus falcata* as a substance which acts in an allelopathic way to suppress the growth of neighboring plants. The biggest problem in identifying the functions of secondary metabolites is probably the complexity of the materials in which these substances occur. For example, more than 80 alkaloids have been isolated from the Madagascar periwinkle, *Catharanthus roseus*, in varying concentrations. The volatile compounds of the monoterpene and sesquiterpene groups, in mixtures of so-called essential oils, are usually composed of dozens to hundreds of substances. This complexity may be caused by an imperfect system of biosynthesis wherein numerous intermediate products and by-products accompany the main metabolite synthesized. There is also evidence that the accumulation of a mixture of substances is more advantageous for the plant than the synthesis of an individual substance. Typically, this applies to various antifungal, antibacterial, or insecticidal compounds. The following chapters deal with selected known functions of plant secondary metabolites.

1.6.1 Plant growth hormones

One function of secondary metabolites that has been well explained is the control of growth and evolution. The structure and function of such substances have been analyzed only in recent decades because these compounds occur in very small quantities and their effects are detected by means of biological tests at micro or nano concentrations. The importance and function of individual plant hormones have been examined intensively. To date, seven main groups of plant hormones based on ethylene, indolyl-3-acetic acid, gibberellins, cytokinins, abscisic acid, brassinosteroids, and polyamines have been described. Except for ethylene, these substances occur in various structural variants in various plant species. Often they also

occur in conjugated form as a reserve compounds, e.g., derivatives of indolyl-3-acetic acid in conjugation with arabinose, galactose, glucose, or inositol. Gibberellins are the biggest group of plant hormones.

In addition to substances identified as growth hormones, many other secondary metabolites indirectly influence life processes in plants. Some of these act independently, others in synergy with plant hormones. Some secondary metabolites can also intervene in biosynthetic pathways wherein they synthesize, modify, or degrade genuine plant hormones.

1.6.2 Plant pigments and odors

The colors and odors of flowers are important for attracting pollinators (bees and other insects, bats, birds). Various pollinators prefer specific colors and odors. For example, bees prefer yellow and blue flowers (indicating the presence of flavonoids that absorb ultraviolet and visible radiation) and sweet pleasant odors. Bats, which fly at night, are most attracted by white flowers with a fruity and sulfurous odor. These relationships are often species specific. It should be noted that there are numerous pigments and odors and they usually occur in mixtures. For example, anthocyanins as blue and violet pigments accompany flavonoids (yellow), and flavonoids usually occur with carotenoids in yellow flowers.

Plant pigments and odors occur not only in flowers, but often also in leaves and fruits. However, the composition of such pigments differs due to their different function. In fruits, taste and odor should attract feeders and thus spread the plant to the surrounding area, or, alternatively, repel feeders if the seeds could be damaged in their digestive tracts.

1.6.3 Plant repellents of herbivores

Repelling herbivores is currently considered to be one of the main functions of secondary metabolites. Herbivores are among the biggest natural enemies of plants and as a result of co-evolution and in the course of time, plants have developed means of fighting these herbivores. Plants have developed various techniques to prevent consumption, including the accumulation of secondary metabolites. Herbivores have then tried to adjust by developing certain detoxification mechanisms which enable limited grazing.

The aim of such toxic effects may differ; secondary metabolites can be toxic specifically for a single species or, alternatively, for all forms of life, including the parent plant itself. To prevent plant auto-toxicity, poisonous compounds are either secreted to the surface of plant organs or deposited in vacuoles in a bound form. Sometimes a certain metabolic activation of the secondary metabolite, either by the enzymatic apparatus of the plant or in the digestive tract of the consumer is required to achieve the toxic effect.

The production of secondary metabolites is often sharply increased at the moment a herbivore attacks. The substances are maintained at a certain level in the resting state, an attack activates biosynthesis and the concentrations of the substances increase as much as several fold. This phenomenon is typical for an attack by insects where the devouring is not very fast and the plant has a relatively long period of time to respond. The idea that damage to the plant tissue might immediately stimulate the release of volatile secondary metabolites, which initiate defense mechanisms in the surrounding plants has also been considered. A certain kind of chemical communication might thus occur.

Some substances do not have the potential to kill a feeder directly, but they rather operate using the principle of repelling it with a disgusting taste (usually bitter) or odor. The toxic effects of repellent taste and odor are combined in many substances.

1.6.4 Plant antifungal substances

The ability of plants to resist infections is caused by the formation and presence of many physical defensive barriers formed by proteins, lignification, waxy cuticle, etc. Higher plants also often synthesize secondary metabolites for protection against microbial infections and fungi. These substances are often secreted to the surface of tissues, where they inhibit the ability of pathogens to propagate, or inside tissues where they can prevent the spread of an infection.

It is not uncommon that not just a single substance, but a whole complex of substances participate in such a defensive barrier. This applies to substances for which synthesis can increase after the plant is attacked by a pathogen; such substances can be formed *de novo* at the moment of attack or metabolic activation may be necessary for their effect. Such substances also accumulate at the place of infection. When the infection has been suppressed, these substances usually metabolize, i.e., shortly after the suppression of the infection their

concentrations become quite low. The detection of such substances is sometimes complicated by the fact that the pathogen produces certain metabolites during infection which can spread *via* plant tissues. These substances, so-called ”mycotoxins“, can then be toxic not only for the plant, but also for any consumer of the plant if they are present in the parts consumed (fruits, seeds).

1.6.5 Plant substances with the activity of animal hormones

The discovery of insect hormones in plant tissues has been one of the most interesting issues in phytochemical research. Quite large quantities of ”phytoecdysons“, steroid hormones of insects, have been found, e.g., in the yew tree (*Taxus baccata*) and polypody (*Polypodium vulgare*), which shows that these substances are spread in relatively remote plant families. The high activity of these substances - which show up to 20 times greater efficacy than similar zoo-ecdysons - is also striking.

Another group is the so-called insect juvenile hormones. Terpenoid compounds which are able to intervene especially in the final part of the transformation by means of the juvenile hormone mechanisms have been isolated from plants.

Another interesting connection between plants and animals can be found in substances with estrogenic activity. This activity is relatively high for substances, especially of the stilbenoid and isoflavonoid type, and can cause abortions in mammals.

2 METHODS FOR ISOLATING PLANT SECONDARY METABOLITES

This chapter deals with various phytochemical procedures for isolating secondary metabolites. Understanding the methods and selecting the right extraction and separation methods are important considerations for the phytochemist and should not be underestimated. An incorrect application costs both time and money and in a worst case can completely destroy the processed material.

2.1 Basic approaches and phytochemical literature

Before carrying out an extraction and separation, phytochemists must determine the purpose for which they want to isolate a secondary metabolite from the plant material. It is sometimes necessary to obtain large quantities of material for parallel tests of biological and pharmacological activity or for a partial synthesis. It may be necessary to examine an extract for its initially promising biological activity, to obtain very pure substances for the determination of the structure, or to perform a chemical-taxonomic study. There are always relatively big differences between phytochemical approaches to given topics and different procedures are suitable for each purpose.

The material should first be evaluated according to the available information. We can usually acquire the most knowledge by studying the specific phytochemical literature. There is usually a relatively wide spectrum of resources which can be examined. At present, when we have the internet, the simplest way is to search for literature by means of electronic databases. However, this depends on the approach of the institutions which usually pay for such databases. The Web of Knowledge and SciFinder cover a relatively wide range of magazines, but they do not always provide full access to the text. For full texts, it is possible to use ScienceDirect, the websites of journals of the American Chemical Society (acs.pubs.org), or the websites of the publishers Elsevier or Springer-Verlag. Again there are some restrictions because all of the resources are not freely accessible and the information must be paid for. Individual scientific periodicals are usually topic specific. In the field of phytochemistry, several important periodicals are issued, usually monthly. Periodicals where in you can find information about the isolation and/or biological activity of natural substances

are as follows: the Journal of Natural Products, Phytochemistry, Planta Medica, Phytochemistry Letters, and Fitoterapia. These magazines provide the most interesting information about the methodology of isolation, identification, and biological activity in the experimental sections of articles. Basic chromatographic separations of natural substances are usually published in the Journal of Chromatography A/B, the Journal of Liquid Chromatography, and Chromatographia. Further information can also be found, e.g., in the Journal of Agricultural and Food Chemistry, Food Chemistry and Toxicology, and Chemical and Pharmaceutical Bulletin. Ethnopharmacological research is published in the specialized Journal of Ethnopharmacology.

Of course there are numerous specialized monographs concerning, e.g., various biosynthetic groups of secondary metabolites. Examples are Modern Methods of Plant Analysis, Studies in Natural Products Chemistry, and Natural Products Reports.

Studying the literature requires a healthy criticism as well. Although papers published in the literature are subject to relatively strict review procedures, it can happen that published experiments have not been described perfectly. Therefore, data and methods adopted from the literature should not be applied without first verifying them on a small quantity of the sample.

The objective of an isolation from the plant material must be defined and a procedure protocol created accordingly. If possible, information about the physical and chemical properties of the potentially isolated substances and about the matrix which contains them should be obtained. The polarity of the substances to be isolated needs to be known, as it is a key factor in the extractions and for various chromatographic separations. It is good to know about the presence of basic or acidic functional groups, which give us an idea about the ionization of the substance. For example, substances are basic due to the presence of an amino group, and they are able to form salts in an acidic environment (typical for alkaloids). Further information, e.g., the ability to absorb ultraviolet or visible radiation, can be useful for detecting substances. The presence of labile functional groups, which can cause instability, and the decomposition of substances under conditions of extraction and separation should be estimated.

The plant matrix from which the substances are isolated may contain undesirable or interfering components. This basic and highly useful information can be obtained by carefully studying the literature, applying knowledge of organic chemistry, and experience. The following chart (Fig. 5) represents a hierarchy of problems and questions which should be

addressed before the experiment. Priorities need to be set according to the needs of the analysis. It is necessary to select either preparatory or only analytical processing of the sample. Methods suitable for the qualitative analysis of a complicated mixture of secondary metabolites need not be sufficiently efficient and suitable for isolating larger quantities of the substances; a typical example would be thin layer chromatography. Some methods may require equipment which is not available during the ”field“ analysis. Decisions on the selection of a method and processing of a sample are influenced by the level of knowledge of the sample composition, the number of target products, the required quantity, purity, laboratory equipment, etc. A different approach is required for sample processing with regard to quantitative and qualitative analysis, and the need for fast and routine processing of a large number of samples also requires a different approach than working with a unique, hard-to-obtain material intended for the isolation of new compounds.

The required degree of purity of the isolated substance is one of the most important factors. There is no linear relationship between the degree of purity achieved and the effort expended. It is usually quite easy to start with a raw material and eliminate ½ - ¾ of the ”impurities“ from it. The real problem arises with the elimination of minor substances to achieve a purity of the resulting product of 99.5% or 99.9%. The relationship between the purity and the resulting quantity of the isolated substance is the same. Isolation, as a chemical reaction, does not have 100% yield. Losses occur at all levels of isolation and it is sometimes necessary to use up the majority of the processed material to obtain a highly pure product. It is also important to try the procedure with a small, representative quantity of the sample. During an experiment a mistake can be made, not all parts of the experiment are as successful as desired, and an ill-considered use all of the material can cause significant problems and damage.

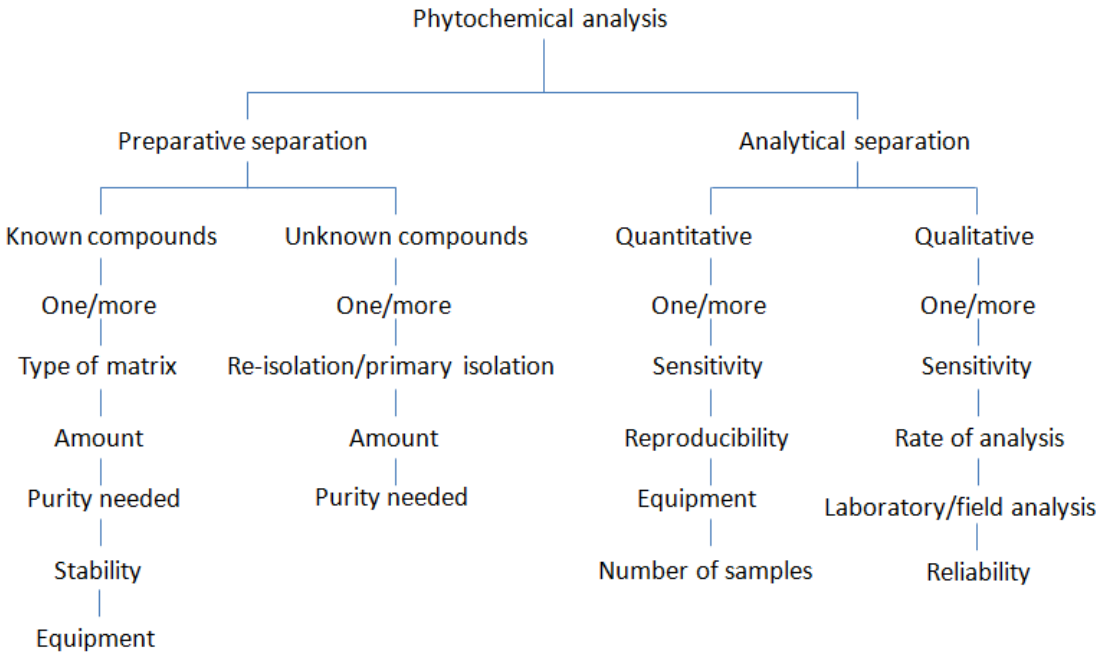

Fig. 5: Important elements of phytochemical analysis

2.2 Selection and preparation of plant material

Every type of a phytochemical survey requires a specific strategy for collecting the plant material to be determined. It was assumed in 1999 that 20-30% of the 250 000-500 000 species of plants had been subjected to phytochemical and pharmacological research in some way. Precise information cannot be found and the data on tests of the various biological activities of plant extracts are even more imprecise. This is because pharmaceutical companies often do not publish their research results in order to protect the data, and only a part of the academic research performed achieves the stage of publishing. Scientific journals only rarely publish data on re-isolations of known substances from new sources or negative results (finding low or no activity in bioactivity tests). Therefore, the number mentioned above could have doubled in the intervening years. Furthermore, phytochemical and pharmacological databases depend on published papers and a large part of scientific results is not published. It is therefore possible that a lot of scientific institutions waste time working on previously performed (probably even repeatedly) experiments.

The selection of the plant is therefore a key question. Numerous criteria can be used for selection: random selection, selection based on ethnobotanical or chemotaxonomic information, or selection based on geographic parameters. The majority of plant selections require the cooperation of botanists or ethnobotanists and approaches which are unusual in synthetic chemistry.

2.2.1 Random selection

A random selection of plants for phytochemical analysis is very difficult to perform. It can be made, e.g., according country of origin or location. One of the methods used in the phytochemical research of large companies is the random selection and collection of plants from a single environment, the preparation of their extracts, and the serial testing of their biological activities. More attention has been paid recently to the areas of rain forests endangered by civilization. These locations are the focus of numerous bioprospective studies because they contain many disappearing plant species which have not yet been examined phytochemically. As ethnobotanical information is lacking, plants from these locations are selected randomly.

This kind of random selection also requires a very wide spectrum of tests for biological activity. A pilot program of this type was the screening for cytotoxic activity performed by NCI (The National Cancer Institute) in the U.S.A. This program discovered, e.g., taxol and camptothecin. The statistics of this program showed the probability of discovering an anti-tumor medical preparation which would be marketable to be approximately 1:8000, compared to 1:10000 for synthetic substances. It should be noted, however, that other programs performed on the basis of random selection of plant sources have been less successful.

2.2.2 Use of ethnobotanical information

Ethnobotanical research brings abundant useful information to phytochemical research – and a lot of success. Statistical estimates show that about three quarters of the known biologically active natural substances have been discovered by means of ethnobotanical research. Interestingly, the biological effect identified does not always correspond to the original ethnobotanical use of the plant. For example, *Catharanthus roseus* was originally studied for its antidiabetic activity, but vincristine and vinblastine, two compounds isolated from it, are currently used as anti-tumor medical preparations.

Ethnobotanical research includes three main steps: 1. First hand information must be obtained from original users of the plants, 2. This information must be critically assessed to identify and determine the intensity of potential activities, 3. In the last stage the plants must be collected, and the substances of interest isolated and tested for their biological activity.

The collection and assessment of ethnobotanical information is a complex process. It is often too expensive for small organizations or university teams. Botanists, ethnobotanists, and field research must be financed for the relatively long periods of time necessary for gathering the information. Only then can phytochemists and pharmacologists come into the picture.

Gathering the information is complicated and the quality of information depends on many factors. It depends, e.g., on the age of the provider, personal experience, education, etc. It is also necessary to select a criterion which identifies a plant as biologically active. Interesting ”markers“ of biological activity include, e.g., antifungal or antiviral activity. Another problem is the ethnobotanical form of use, e.g., aqueous extracts of plants often show

certain antiviral activity (the presence of polyphenols) but this activity is not selective and extracts are not suitable for the development of drugs. Many companies therefore currently perform various pre-separation steps intended to eliminate these non-specifically active polyphenols.

2.2.3 Use of chemotaxonomic information

Chemotaxonomy uses secondary metabolites to research and confirm the phylogenetic relationship of plants. Diversity in the production of secondary metabolites is related to the position of the species in the plant kingdom. Knowledge of the plant classification in a genus or family can help in foreseeing the spectrum of substances which could be isolated from the plant. However, phytochemical studies have shown that some substances are present in several genera or families that are not connected taxonomically.

Taxonomic information is then useful in cases where an interesting bioactive substance has been isolated from one plant and other, related plants can provide more active analogs. Typically, pharmaceutical companies immediately provide an active substance to organic chemists who seek to synthesize more active variants. However, it may be more effective and cheaper to perform intensive chromatographic separation of the extracts before such synthesis in an effort to isolate derivatives of the substance.

2.3 Collection and drying

There is a big difference between the collection of individual plants for research purposes and collecting them in large quantities for use in phytotherapy. Significantly less material is needed for basic phytochemical research where free collection in the country is common, especially in the early stages of the research.

Herbs are currently cultivated culturally for phytotherapeutic uses. This is advantageous because of the production yield and the standard level of content of secondary metabolites. To grow the plants, it is necessary to consider: correctly selecting the land and choosing the variety to be grown, and the time of collection. Varieties that are highly resistant to molds,

insects, frost, drought, etc. are grown especially. Economic aspects in such growing play the main role.

The time of collection plays an important role in the content of secondary metabolites in a source material. The concentrations of secondary metabolites change during the seasons of the year, during the life time of the plant, and during the diurnal cycle. For example, the content of menthol in mint is higher in older plants, whereas younger plants contain rather more precursors. For example, camphor is obtained from 40 year-old trees. The content of substances also fluctuates during the year. We usually collect underground parts in the autumn, whereas seeds and fruits are collected at the time of maturity and other above-ground parts of plants are collected at the time of blooming. The variability of the content of substances in plants depends on the alternation of light and darkness and on temperature and humidity.

Certain principles of collection and material processing must be adhered to both therapeutic use and research. In extraordinary cases fresh plants are used for phytotherapy for practical reasons, usually in homoeopathy. Fresh plants are sometimes used directly to prepare extracts because of the high energy demands of the drying process. However, the fresh material is more frequently used for research. Drying is the fastest and most economical means of conservation. Eliminating water from the material avoids metabolic processes which could degrade the contained substances. Correct drying preserves the contained substances without loss. The dried material is also less susceptible to the invasion of micro-organisms and molds. During conservation, it is necessary to proceed forcefully and quickly. The primary task is to stop metabolic processes in the material caused by undesirable changes in the contained substances. This is achieved by the action of ethanol vapor on the material, or by freezing. In some cases, the metabolism is not stopped because desirable so-called post-mortal synthesis occurs, and the required substances are formed only after the collection of the material, e.g., by fermentation. Examples are foxglove and black tea. If the plant material needs to be preserved in any manner, this is usually carried out by drying, or especially by lyophilization.

3 DECOMPOSITION OF SUBSTANCES AND FORMATION OF ARTEFACTS

One of the problems which accompany phytochemical analysis is the decomposition of secondary metabolites and the formation of so-called artefacts. An artefact is defined as a compound which was isolated and identified but which was not present in the original material or was present only at trace level. Every process which occurs during the acquisition of the material (internal - plant metabolism, and external - extraction, separation, and analysis) can contribute to the formation of artefacts. Accordingly, three different types of artefacts can be distinguished:

1. Well-defined artefacts formed spontaneously by chemical reactions, such as hydrolysis, oxidation, dehydration, isomerization, or cyclization of the substances originally present in the plant material without any added chemical agents. In this case, it is quite difficult to rank substances among artefacts as they are in fact formed immediately after collection or during the simplest processing.

2. Well-defined artefacts formed by the reaction of genuine (original) substances with materials used in processing the plant (e.g., reactions with solvents or impurities present in the chromatographic medium). If such substances are described, it is relatively easy to rank them among the artefacts. An example is the reaction of tertiary amines with ethylene chloride. Another example is the formation of chamazulene from sesquiterpene lactones during the steam distillation of chamomile flowers.

3. It is often possible to identify foreign substances ”imported“ into the material obtained from a plant during extraction or separation (impurities from the solvents and chromatographic materials used), contamination with fungi, molds, or other micro-organisms.

4. Difficult-to-characterize artefacts of the polymer type formed by photodegradation, oxidation, or thermal degradation. These are usually of a polar nature, dark and insoluble in organic solvents or water. They often reduce the yield of a chromatographic separation.

The material can of course contain all of the aforementioned types of artefact in parallel, which makes their identification more difficult. Therefore, during any processing of plant material, separation of substances, and analysis it is necessary to be careful not to use an

aggressive procedure and if possible, to use re-distilled solvents and high-quality chromatographic sorbents. The material should be stored in a dark and cold place (a refrigerator, if possible). If a “suspicious“ substance is isolated (a chemotaxonomically unusual compound, a complex substance in a small quantity, etc.), the process of isolation should be repeated in another manner to confirm the result and to verify all procedures including the identification of the material.

4 EXTRACTION

4.1 Introduction

Only rarely can plants be analyzed for the substances they contain directly, without any separation or extraction to isolate such substances. However, some advanced mass spectrometric techniques can do this). It may happen that a plant substance of interest crystallizes at high concentration, e.g., directly from a vegetable resin or other exudate, but this is a rather rare case. At least one extraction step is usually needed, followed by purification (purifying the sample before analysis). The extraction serves both to detach the secondary substances we want to analyze from the high-molecular weight ballast (which forms the major part of the plant material matrix), and to separate the target compound from low-molecular weight primary and secondary metabolites, as they would interfere with subsequent steps in the analysis. Ballast substances comprise a mixture of cellulose, hemicellulose, lignin, etc. and usually constitute to the major portion of the dry matter obtained by processing plant material. They are usually insoluble in extraction media. Soluble ballast includes various primary metabolites, chlorophyll, pectin, starch, glycoproteins, and salts. The proportion of secondary metabolites in the plant material does not usually exceed 10%, but this certainly depends on the plant species and the part of the plant that is used.

Secondary substances in plants are usually bound in various ways or their presence is masked, which makes the extraction procedure more difficult. We can call the interaction between secondary metabolites and the plant material ”the matrix effect” and it can be caused by both chemical and physical barriers. If we want to successfully extract secondary metabolites from a plant, we have to nullify the binding in the matrix of the plant and overcome the barriers. An example of the destruction of the matrix effect can be the extraction of strychnine, an alkaloid, from the seeds of *Strychnos nux-vomica*. The seeds of this plant are very hard and covered with trichomes with a waxy cuticle. Alkaloids, such as strychnine, can be easily extracted using dilute sulfuric acid, but this medium cannot penetrate the highly rigid and very hard seeds. Moreover, the waxy cuticle and a high content of fat prevent penetration into the material. Therefore, the physical barrier must be first destructed by crushing the seeds and then degreasing them, using, e.g., petroleum ether or hexane. After these procedures, the dilute sulfuric acid can be applied to achieve a relatively selective extraction of the alkaloids. The large majority of lipophilic compounds pass into the non-polar

hexane; strongly hydrophilic sulfuric acid extracts the alkaloids and the part of the substances with medium-polarity remains in the crushed seeds. If subsequently needed, these substances can be extracted using methanol or ethyl acetate.

In the first stage of the extraction process, free molecules of the secondary metabolites are relatively quickly dissolved. These molecules are on or near the surface of the material where the solvent easily penetrates. In the next stage, the solvent needs to penetrate deeply into the material and the physical-chemical barrier blocking the release of substances must be destroyed (breaking the matrix effect). A solvent can make extraction easier in various ways. The material can swell; enlarged pores make the passage of substances easier. In the third stage only the substances dissolve in the extraction medium and diffusion is the only limiting factor. The matrix effect and the process of diffusion significantly affect the yield of an extraction. The diffusion can be described by means of Fick`s First Law:

$$\Delta n/\Delta t = -(D \times A/h) \times (c_0 - c) \quad (1)$$

$\Delta n/\Delta t$	change in the amount of substance per unit time (the velocity of diffusion)
D	diffusion coefficient
A	diffusion area
H	thickness of the diffusion layer
(c_0-c)	concentration gradient

All of the parameters of the diffusion process can be influenced in one way or another. The goal is the greatest transfer of mass possible in the shortest time.

Of course the rate of diffusion is also influenced by the solubility of the extracted substances in the solvent used.

4.1.1 Diffusion coefficient

The diffusion coefficient depends on the character of the extracted substances and that of the extraction medium (solvent). The yield of the extraction can be improved significantly by correctly selecting the extraction medium. Another parameter significantly influencing the

diffusion coefficient is the temperature. The diffusion coefficient grows with increasing temperature and diffusion thus accelerates.

4.1.2 Diffusion area

The area over which diffusion acts should be as large as possible. It can be most influenced by pulverizing the material into the smallest pieces possible. A greater degree of pulverization makes more surface of the material accessible for the extraction process. The degree of pulverization can be measured using a set of screens for which the dimensions of the mesh are defined by the pharmacopoeia.

The rule that the smallest particles are the most suitable for extraction does not always apply. If the material is powdered to a fine dust, sedimentation occurs in the extraction medium during dispersion and paradoxically forms a kind of mud which the solvent penetrates with difficulty. Very fine particles also quickly clog filters when the extract is discharged. It is therefore necessary to compromise when selecting the degree of pulverization and the size of particles, depending on the other extraction parameters.

4.1.3 Thickness of the diffusion layer and concentration gradient

The thinner the diffusion layer, the faster the diffusion. The size of the diffusion layer can be reduced by powdering the material. On the other hand, the concentration gradient must be as high as possible for fast diffusion and thus extraction. In practice, it can be influenced by simply mixing. The movement of the extraction medium towards the material breaks up the diffusion layer, the concentration gradient increases along with diffusion, and extraction thus accelerates. As the extraction proceeds, the concentration of substances in the material being extracted decreases and that in the extract increases, which results in a diminishing concentration gradient. This phenomenon can be observed especially in periodic extraction methods (such as maceration) and it can be addressed by using semi-continuous or continuous methods, as described below.

4.2 Types of extraction

In principle, four types of media can be used for extraction: 1. liquids, 2. gases, 3. steam and 4. supercritical liquids. Other possibilities, e.g., solid extraction or eutectic solvents, are currently coming to the forefront. The first two types and, in a way, also solid extraction, in fact use the physical principle of dissolution, steam and other gases are rather used as carrier gases for the extraction and transport of volatile components out of the material. Each of these extraction media has its advantages and disadvantages and is used under different conditions which need to be selected correctly. Technically, we distinguish several types of extraction. According to the relationship between the extracted material and the solvent, we distinguish periodic or continuous extraction.

The following parts of the text deal with individual types of extraction, including descriptions of typical examples of use.

4.2.1 Liquid extraction

Liquid extraction is probably the most commonly used technique. Liquid extractions can be divided into two methods according to their purpose: 1. total extraction, 2. selective extraction. Total extraction is used to obtain, as far as possible, all of the extractive substances from the material regardless of their chemical character or biological activity. We usually try to achieve the maximum total yield.

Unlike total extraction, selective extraction is unable to extract the maximum number of substances, and we obtain a narrower group of substances. In this case, an extraction medium is able to dissolve only a narrow group of substances and we either obtain only the target analytes or only the impurities we want to eliminate from the material. A typical example of the selective approach is the aforementioned strychnine extraction, wherein we first selectively extract lipophilic fats and waxes (we remove the barriers) and only then do we selectively extract the alkaloids using dilute sulfuric acid. Another example is the elimination of large quantities of chlorophyll from extracts of the above-ground parts of plants.

There is a simple chemical rule for the selection of the total or selective extraction medium: *similia similibus solvuntur* - like dissolves like.

For the extraction of organic substances, such as secondary metabolites, we usually select from a relatively limited number of solvents which are ranked in the eluotropic series according to their increasing polarity. Non-polar solvents are mainly represented by simple alkanes (heptane, hexane), the series continues through chlorinated hydrocarbons (chloroform, ethylene chloride), and further to more polar solvents, such as acetone and ethyl acetate. At the polar end of the eluotropic series are alcohols (ethanol, methanol) and water. Polar (hydrophilic) substances dissolve well in polar solvents and non-polar (lipophilic) substances in non-polar solvents. The main guide for the selection is the selectivity of the solvent. For a total extraction, we select solvents from the polar end of the eluotropic series, usually aqueous solutions of methanol or ethanol (70-80%). These solvents are very ”strong“ and quickly and easily extract both polar and non-polar substances, but this results in low selectivity. For a selective extraction, we proceed from the non-polar end of the eluotropic series, mainly various saturated hydrocarbons. These solvents extract only non-polar substances selectively and well. They have high selectivity, but the efficiency of the extraction is significantly lower. The ability of a solvent to extract efficiently increases from the non-polar to polar end of the series, whereas the selectivity decreases. A relatively large jump to greater selectivity is seen between water and methanol. Selecting the most suitable solvent usually requires compromise: the solvent selected should be sufficiently strong, but also sufficiently selective not to dissolve too many impurities in addition to the target substance.

4.2.2 Periodic extraction

Periodic extraction is the easiest extraction as far as technical equipment and performance are concerned. It is carried out in tightly closed vessels that minimize the loss of extraction medium by evaporation and are protected from light, if possible. A single dose of material is extracted with a fixed quantity of extraction agent for a fixed period of time. This system is called maceration. As necessary, the extraction time can differ from a few minutes to hours or days. Other parameters, such as the ratio of extracted material to extraction agent (usually 1:10), and/or temperature can be changed and mixing is possible. The yield of extraction is also influenced by the water content of the extracted material. A typical example of maceration and the possibilities of influencing the process is the preparation of tea.

Maceration can be repeated. After some time (usually 24 hours), we remove the extract and add a new batch of solvent, significantly increasing the concentration gradient and accelerating the extraction. This procedure is usually repeated three times or as many times as required until the selected detection method shows the absence of the target substance in the last portion of extract.

Maceration carried out at a higher temperature is called digestion. Increasing the temperature usually shortens the extraction to tens of minutes but this approach also reduces the selectivity and working at laboratory temperature removes concerns about losing thermally labile compounds.

The main advantages of maceration are as follows. It is a very simple and cheap method, undemanding for laboratory equipment and if the process is set at a high quality and the solvent is well selected it is relatively efficient and selective. Its disadvantages are that it requires more time and is less efficient compared with other methods (unless we use permanent mixing or heating).

4.2.3 Continuous extraction

In continuous extraction, we insert a single dose of the material to be extracted into the extractor and subsequently continuously add fresh solvent. A typical example can be percolation or a Soxhlet extractor.

A percolator is usually a glass or metal tube with a drain controlled by a tap at the lower end. The tube is filled with the material to be extracted and the material is then left to saturate with the extraction agent for several days (air bubbles are released and the material "homogenizes"). A solvent is then added from the top and, by gravity, passes through the material and extracts substances. The extraction is carried out for as long as the selected analytical method of detection shows the presence of the target substances in the discharged extract. With this method, it is possible to dry the material with one solvent following extraction and then continue using a different solvent. An example of a simple percolator is a coffee machine.

An advantage of this method is the possibility of complete extraction and the delicacy of this technique for the extracted substances. However, the extractor must be supervised by an operator, the extraction is quite slow, and large amounts of the extraction agent are needed.

Another alternative for continuous extraction is a Soxhlet extractor. This is a simple laboratory instrument that uses repeated re-distillation of the extraction medium fed continuously to a single batch of the extracted material in a closed system. A Soxhlet extractor is depicted in Fig. 6. The extracted material is inserted into the extraction cartridge (paper or glass with sintered glass in the lower part). The extraction medium is heated up and enters the cooler in the form of a vapor. There it condenses and pours over the material to be extracted. After filling the extraction chamber, the extract flows back to the boiling flask where it concentrates by evaporation. This process is continuously repeated many times. This method has its pros and cons. Its advantages are the possibility of automating the system, the use of relatively small quantities of the extraction medium, and the resultant concentrated extracts. The extraction can be profound and of high quality. Its disadvantages are the need for heating and cooling, the possible thermal decomposition of the extracted substances, and the limited quantity of material that can be processed (the extractor has a limited volume).

At present, automatic extractors of the percolator type that contain several steel cartridges in which it is possible to carry out extraction under pressure and at elevated

temperature thus accelerating the extraction and increasing the yield have been introduced into practice. They also significantly reduce the consumption of solvents.

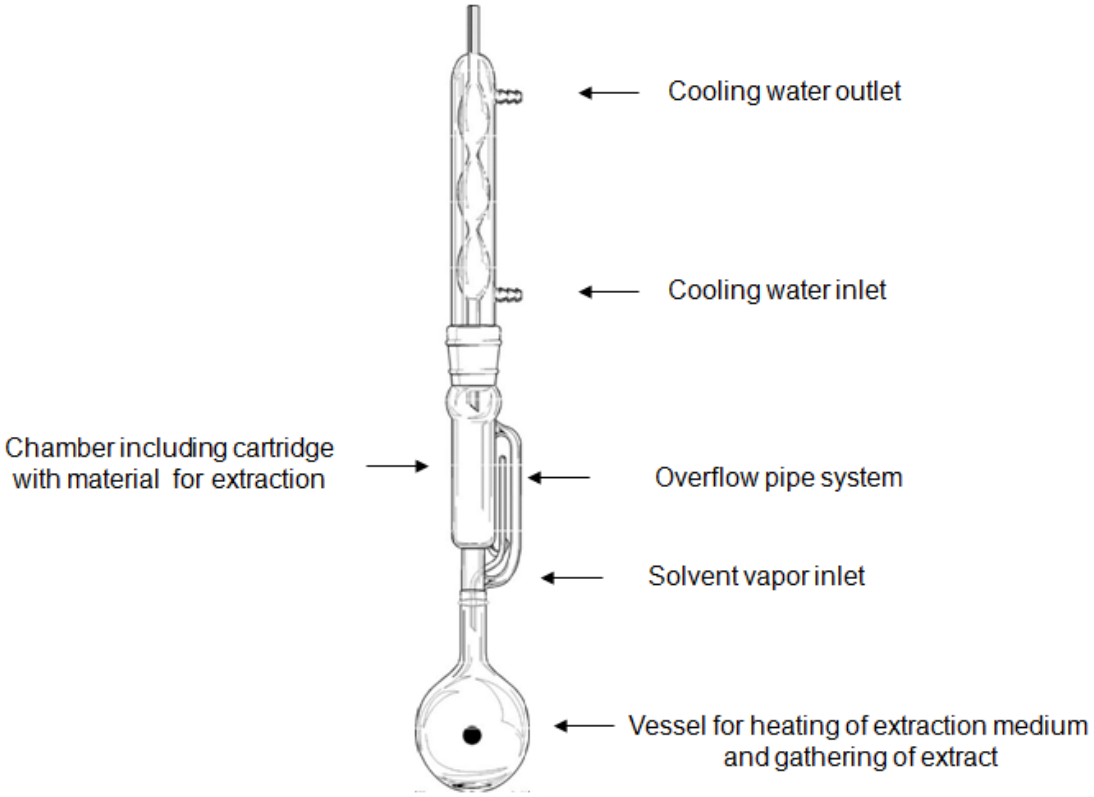

Fig. 6: Soxhlet extractor

4.2.4 Supercritical fluid extraction (SFE)

Supercritical fluid extraction is a fast-developing modern method for the preparation of vegetable extracts using the properties of supercritical liquids. Supercritical liquids are media having the properties of both liquids and gases. They are formed at pressures and temperatures which exceed the "critical values". For CO_2, which is probably the most frequently used supercritical medium, the critical temperature t_c = 31 °C and the critical pressure p_c = 73 atm apply. Supercritical liquids have some properties close to gases and some to liquids. Like liquids, they have a similar slightly lower density and the ability to solvate, i.e., the ability to dissolve. However, their diffusion coefficient is close to gases, which enables a fast transfer of mass. Their viscosity and surface tension are significantly lower than for liquids, which results in good penetration through the extracted material. The aforementioned facts show that supercritical media combine the best properties of gases and liquids for fast and efficient extraction. In the past, this method was rather more likely to be used in industry because of the large volumes. Now it is expanding at a smaller scale into phytochemical laboratories as well.

The equipment for SFE can be described schematically as follows (Fig. 7). Extraction medium is fed from a tank (a pressure vessel) to the extraction space, where it is heated and compressed to above-critical values. It is then transferred to the extraction vessel where the material to be extracted is inserted in a cartridge. The material to be extracted can be inserted separately or mixed with an inert material to achieve better permeability and increase the extraction surface. Methanol may be added at this point to increase the polarity of the extraction medium. High temperature and pressure are maintained in the extraction vessel as required (usually 35-150°C and 120-680 atm). The extraction occurs there and can be static or dynamic. A static extraction is similar to maceration (single batch of the material and one of the extraction medium). A dynamic extraction is similar to percolation (a single batch of the material and continuous addition of fresh extraction medium along with continuous discharge of the extract). The extraction medium in the supercritical state and enriched with the extracted substances, leaks under pressure from the cell through a restrictor. The restrictor is a thin capillary of variable length that serves as a simple pressure valve, enabling the expansion of the supercritical medium to atmospheric pressure. The extract flows through the restrictor to a collecting vessel under the gradually diminishing pressure when the supercritical medium expands as the pressure declines, it cools sharply and loses its extraction capabilities. At the

end of the restrictor, the cooled liquid or gaseous CO_2 is discharged (depending on the length and diameter of the restrictor) separately from the mixture of extracted substances. Usually the CO_2 gas containing extracted substances is bubbled through a medium in the collecting vessel. The extracted substances are captured and the CO_2 escapes. A solid medium can be used to capture the extracted substances, and thus SFE is combined with SPE (solid phase extraction, as described below). In industrial plants or more sophisticated equipment, supercritical CO_2 is allowed to expand only to a certain level, and is then recycled because retrieving and pressurizing it to super-critical condition represent the biggest operating costs.

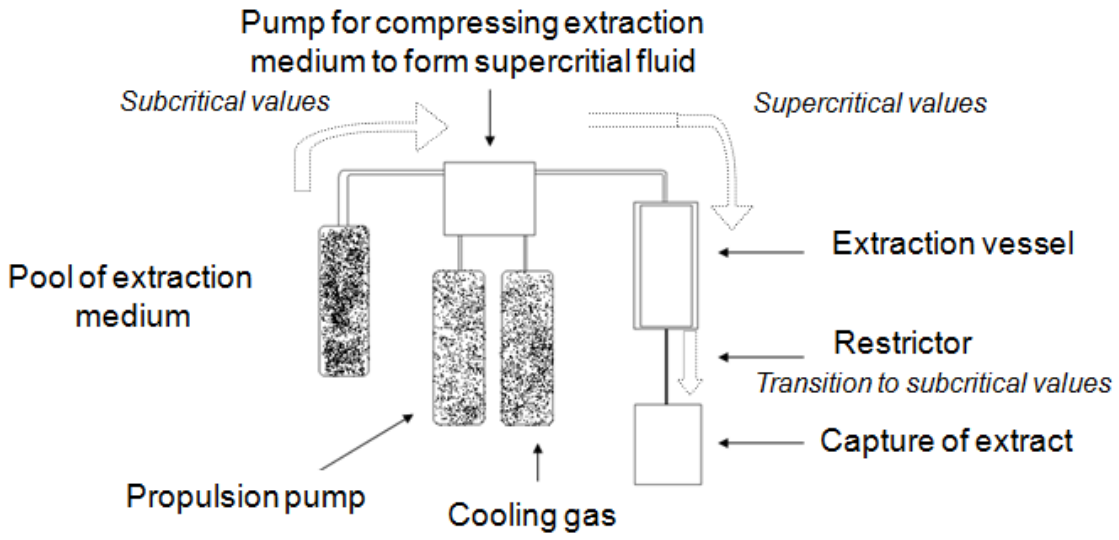

Fig. 7: Schematic chart of equipment for SFE

The aforementioned characteristics enable understanding of the specific advantages of SFE compared to classical extraction methods (as CO_2 is most frequently used for supercritical extraction in phytochemistry, the following description will relate to it). SFE is fast if it uses CO_2 and is highly environmentally friendly. It is relatively selective if defined conditions are used. Changing the pressure and temperature above the super-critical values, changes the density and can thus affect the solvation ability according to the polarity of the analytes. Different mixtures of substances can be selectively extracted by changing the pressure and the temperature. CO_2 is non-corrosive and cheap. There are of course certain disadvantages. The ability of CO_2 as a solvent is relatively low. As a non-polar substance, CO_2 can dissolve only non-polar secondary metabolites, such as waxes, fats, essential oils,

and triterpenes. The polarity of supercritical CO_2 can be increased by adding a polar solvent, such as anhydrous methanol, to the extraction mixture. However, doing so loses one of the advantages of the method, the absence of the need to use classical solvents. Problems in extraction can arise if there is a large quantity of undesirable non-polar substances in the material, such as the chlorophyll in leaves. Such substances are extracted together with the analytes and the method becomes non-selective. Another disadvantage of the method is the relatively high price of the equipment, which is significantly more complicated than that used for classical extractions. It is usually possible to process only small quantities of material in the laboratory equipment made for SFE.

Like other extractions, the SFE process can be monitored using various analytical methods, such as TLC or HPLC with UV/Vis or other detection.

There are many examples of the use of SFE. Its main advantages are that it is non-toxic, and ecologically wholesome and it treats the material gently. No residues harmful for humans or the environment are left when CO_2 is used. Therefore, this method is used extensively in the food, pharmacy, and cosmetics industries, and generally in the making of products which in some way come into direct contact with humans. The properties of CO_2 as a non-polar solvent predetermine that the method is suitable for the extraction of non-polar substances. Using SFE, the bitter substances in hops (*Humulus lupulus*) are extracted for both brewing and pharmaceutical purposes, coffee is decaffeinated (and the caffeine is also used), odor- and taste-significant essential oils are extracted for aromatherapy, spices extracts are prepared, etc. As far as other natural substances are concerned, SFE is used, e.g., to obtain gingkolides from *Gingko biloba*, lignans from *Schisandra chinensis*, flavonoids, some coumarins, and catechins.

4.2.5 Steam distillation

Steam distillation is one of the oldest extraction techniques. The method is highly selective and easily obtains water-insoluble non-polar substances. It can be used for various plant materials. The selectivity of the method is caused by the fact that only volatile substances are extracted and simple compounds are thus obtained. The extract is free of all non-volatile non-polar substances, such as fats, waxes, or fatty acids. Volatile substances (most frequently components of an essential oil) are usually present in very small quantities in plant material. Concentrations are usually below 5%, often less than 1%. Such small quantities are not suited to the use of other extraction methods such as liquid maceration. Furthermore, steam distillation is a very simple method because simple apparatus is used and no special steps for the preliminary preparation of the sample are needed.

The principle of steam distillation is based on application of Dalton`s Law: the total pressure of steam a mixture of two immiscible substances (in our case volatile secondary metabolites and water) equals the sum of their partial pressures. A mixture, wherein the main part is water, will have a boiling point of 100°C under normal atmospheric pressure. The evaporation of substances with boiling points higher than water is thus facilitated and, e.g., sesquiterpenes, with boiling points of about 300°C, are isolated by this method at a temperature of 100°C. There are two procedures for steam distillation, which do not differ in principle: 1. In so-called genuine steam distillation, steam is prepared in a generating unit and subsequently forced into the plant material, 2. In hydro-distillation, the steam is generated *in situ* by boiling the suspended plant material in water.

The second type is especially popular in laboratory practice and is specified by numerous pharmacopoeias for the quantitative determination of essential oils in plant materials. One apparatus for hydro-distillation is the Clevenger apparatus (Fig. 8). Genuine steam distillation is rather used in organic chemistry or for the industrial processing of large quantities of material.

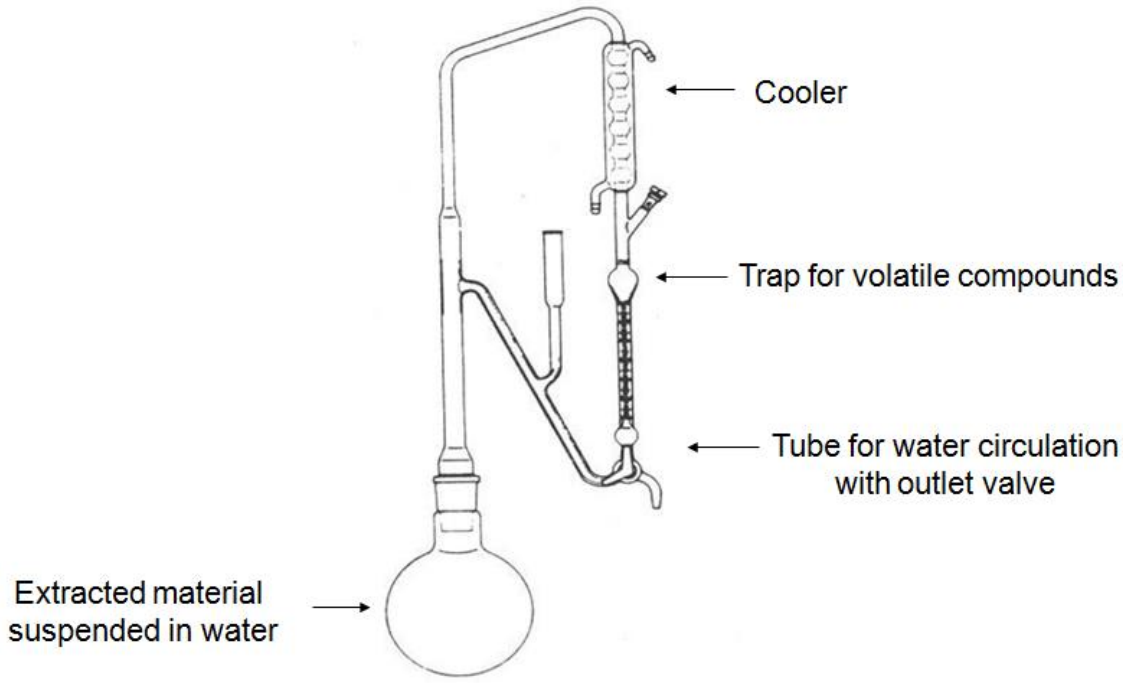

Fig. 8: Clevenger apparatus

The ”matrix effect“ and diffusion processes need to be taken into account during distillation. If volatile substances are located on the surface of the plant material (e.g., in trichomes, small glands, etc.) diffusion plays a primary role and the substances are distilled quickly. If the substances to be distilled are located deeper within the material (as in seeds or bark, etc.), the speed of distillation is influenced more by the matrix effect than by diffusion. This is caused by the fact that volatile non-polar substances are insoluble or nearly insoluble in water and easily remain associated with other substances, such as fats, etc., making diffusion and movement towards the surface more complicated.

Steam distillation is a classical technique used in phytochemistry for isolating essential oils. Essential oils are substances typically contained in, e.g., plants of the Asteraceae, Apiaceae, Rutaceae, and Lamiaceae families. Special rules for steam distillation can apply to selected plant species according to the physico-chemical character of the essential oil. The distillation of the essential oil from the chamomile flower *Matricariae flos* (*Chamomilla recutita*, Asteraceae) can serve as an example. This essential oil contains a relatively large quantity of sesquiterpenes and sesquiterpene lactones. It is heavier than water and therefore does not remain trapped on the surface of water in the Clevenger apparatus but rather falls and recirculates. Another complication is the relatively high solubility of some types of essential

oils in water (e.g., the essential oil of *Cinnamomi cortex* – *Cinnamomum zeylanicum* – the cinnamon tree). This essential oil contains water-soluble phenylpropanoids. In such cases, hexane or another lipophilic compound lighter than water is added to the water as a distillation medium to capture the essential oil and enable its isolation.

The steam distillation method is not suitable for isolating thermally labile substances which can decompose in a water environment at a temperature of 100°C, or less.

4.2.6 Steam-distillation extraction (SDE)

SDE is a special adaptation of steam distillation. It is used in cases where the plant material contains only really very small quantities of the volatile substances we want to isolate, or when the desired volatile substances are partially water-soluble. This technique uses a Godefroot or Likens-Nickerson apparatus or one of their modifications (Fig. 9).

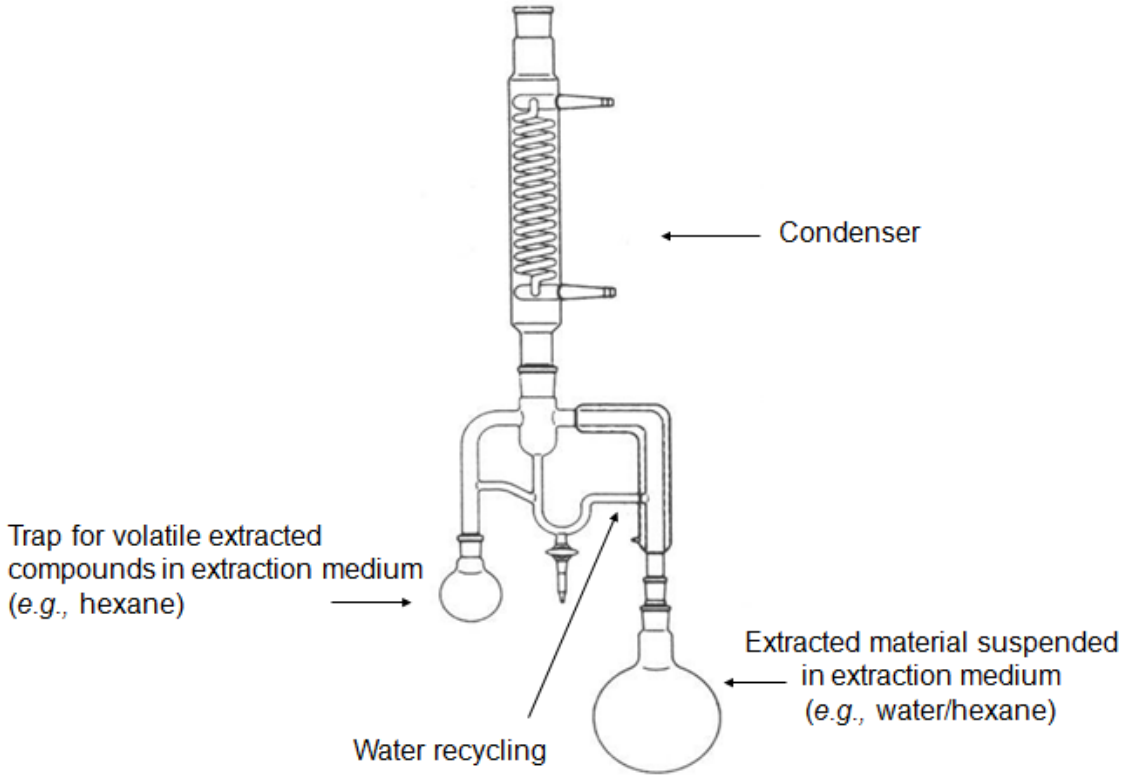

Fig. 9: Schematic chart of equipment for SDE

A mixture of water and a highly volatile organic solvent (pentane or diethyl ether) is used for the extraction. The material is suspended in this mixture and heated. Lipophilic secondary metabolites are extracted into the organic phase and at the same time water vaporizes with the organic solvent. During condensation, the immiscible water phase is separated from the organic phase and the organic phase containing the extracted volatile substances flows to a special vessel. The organic solvent can then be eliminated and the extracted and concentrated substances can be further processed or analyzed. An example of the use of this technique is the extraction of the substances contained in *Nicotiana tabacum*. Freshly processed tobacco has a pungent odor and makes irritating smoke; it is therefore

fermented and aged. The ageing process is necessary for the production of cigarettes and the analysis of volatile substances is necessary in order to obtain marketable quality tobacco. The volatile substances in tobacco are highly complex mixtures of different compounds. In some cases these are soluble in water and SDE is an advantageous method for their extraction. The extraction of volatile components from honey is another example.

4.2.7 Gas-solid extraction

Gas-solid extraction is a technique used to obtain volatile compounds. The term ”headspace“ is applied to a limited space surrounding an object that contains volatile substances (such as a flower). Volatile substances are released into this space and can be collected and isolated there. Headspace techniques can be divided into two categories: equilibrium (static), and dynamic.

The static headspace technique is used for analytical purposes. A part of the plant is put into a closed container which can be heated. After some time, equilibrium between the concentrations of the substance in the plant and in the surrounding space is reached. The concentration depends on the boiling points of the substances, the temperature and, of course, the matrix effect. Substances can then be analyzed, usually by means of gas chromatography.

The dynamic headspace technique is based on a different principle. A flow of nitrogen is forced into the headspace where the sample has been inserted and subsequently forced, along with any compounds released, through an absorbent which captures and concentrates the substances. The absorbent trap is washed out with an organic solvent after a certain time and the substances are further analyzed. If the quantity is sufficient, the mixture can be divided (using preparative gas chromatography) into the individual components so that the structures of the substances can be determined, or tests of biological activity performed.

One of the most modern headspace techniques is headspace-SPME (solid phase micro extraction). For the last two decades, this technique has been used to analyze substances at very low concentrations (ppb-ppt). The technique uses a silicon fiber which captures and concentrates the volatile substances in the static or dynamic headspace. The trapped substances can be desorbed from the fiber at the injection port of a gas chromatograph. A schematic chart is given in Fig. 10.

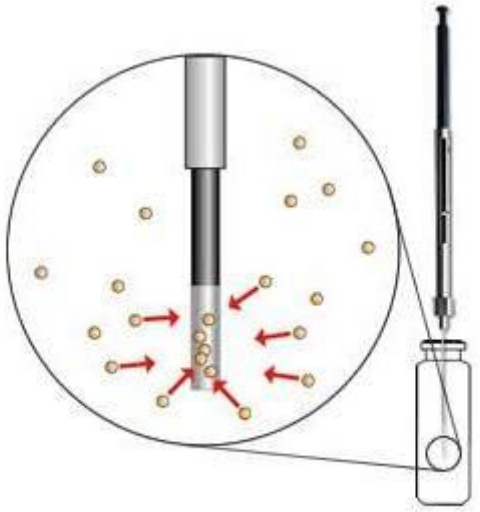

Fig. 10: Schematic chart of headspace - SPME (adopted from http://www.labhut.com/products/autosamplers/autosampler_ht280t.php)

All of the different forms of headspace separation are highly conservative and selective for volatile substances. Comparing the analysis of the volatile substances obtained using the headspace technique to an extract obtained by steam distillation can provide completely different results for the same plant. The results can be influenced by both the matrix effect and the conditions of extraction. The headspace technique is advantageous because samples can be analyzed *"in vivo"* and it is very fast, sensitive, and selective. On the other hand, its usage for preparative purposes is complicated and a relatively complex apparatus is necessary.

The headspace technique is used, for example, to analyze the aromas of coffee (*Coffea arabica*), or beer (*Humulus lupulus*) and for the analysis of the simple sesquiterpenes (farnesene) found when examining the defensive responses of plants.

5 PURIFICATION

5.1 Introduction

Using the correct type of extraction obtains the highest and lowest possible portions of desirable substances and undesirable impurities, respectively. After the extraction, the remaining impurities must also be separated, usually in a single separation or purification step. This step is necessary for almost all of the other experiments carried out subsequently (identification of substances, measurement of biological activity). The following chapters deal with particular purification techniques and their useful applications. The survey highlights chromatographic techniques, described in Chapter 5.6, as deserving significant attention.

5.2 Distribution between immiscible solvents (liquid-liquid extraction)

Purification can be based on the distribution of substances between immiscible solvents. In this extraction, the equilibrium concentrations of the dissolved substance between the two liquid phases are established; the equilibrium is defined by the distribution coefficient k as $k = [c1]/[c2]$, where $c1$ and $c2$ are the concentrations of the substance in the two immiscible phases of the mixture. In practice, the distribution ratio q is of greater significance; it is the ratio of the total concentration of the substance in both phases (the substance may be present in one or both phases in the form of several different constituents). The total amount of the substance that has been extracted is determined by the degree of extraction E (%), which is influenced by the solubility of the extracted substance in the solvents used. It therefore depends on the distribution ratio (q).

$$E = \frac{100q}{q+V/Vo} \qquad (2)$$

where V_0 and V are the volumes of the organic and aqueous phases. It follows from Equation (2) that the degree of extraction increases with a decreasing V/V_0 ratio. However, it is more productive to perform multiple extractions with several small volumes of the organic phase rather than a single extraction with a large volume. For example, if we have a substance with $q = 2$ and we use the same volumes of the organic and aqueous phases for the extraction ($V_0 = V$), then the yield of a single extraction with the entire volume of organic phase is 66.6 % of

the total substance (see Equation 2). If we divide the same volume of organic phase into halves and perform two sequential extractions, the extraction yield after the first step is 50% and with 50% extracted from the remnant in the second step; the combined extracts contain 75% of the total substance. From a practical point of view, there is no sense in performing more than five sequential extractions. If the degree of extraction is not sufficient even then, it is necessary to increase the value of *q* by changing the extraction system.

One of the devices most frequently used for extraction in the phytochemical laboratory is the separating funnel. Liquid-liquid extraction in a separatory funnel is common mainly for preparative separation. For analytical purposes, it has been replaced by so-called solid-phase extraction (SPE). Rough extracts are not suited to preparing a sample for chromatography because they usually contain too wide a spectrum of substances of different polarities. Moreover, an economic aspect plays a role, too, as a chromatographic analysis of smaller amount is more effective and provides greater recoveries and the use of costly chromatographic sorbents and solvents is also significantly reduced.

Liquid-liquid extraction exploits so-called immiscible solvents. Immiscible solvents can be selected from an eluotropic series according to their polarities. Usually, solvents from opposite ends of the eluotropic series cannot be mixed with each other. Place a sample dissolved in one solvent into a separating funnel and add another, immiscible, solvent. Shaking the funnel forms a temporary emulsion as the substances to be extracted diffuse and equilibrium concentrations are established according to the solubility of each substance in each solvent. After the phases have separated, the liquids (extracts) can be removed, separately, and the entire procedure repeated with a new solvent. The ratio of solvents used is usually 1:1 by volume. If water is one of the constituents, the ratio is increased in favor of a greater content of water. In a more practical step, the organic constituent is also divided into several parts and the entire extraction is repeated individually with smaller volumes (this is typical for a mixture of water and $CHCl_3$). After being separated, the extracts obtained from the organic solvent can be concentrated and dried out with anhydrous sodium sulfate or another appropriate drying agent.

The benefits of distribution by immiscible solvents are obvious. The separation is not accompanied by any irreversible adsorption and a volatile solvent can be removed by evaporation, if necessary. The apparatus for extraction is very simple. The amount of

substances separated is limited only by the volume of the separating funnel. The types of solvents can be selected according to the character of the substances to be extracted.

The disadvantages are the limited number of possible combinations of solvents, the impossibility of automation, and potential problems with the formation of emulsions or so-called interphases. Interphases are formed mainly when the aqueous extract contains surface-active agents, usually of a polar nature (e.g., saponins) and a water/$CHCl_3$ system is used. In such cases, the mixture $CHCl_3$:ethanol 2:1 can be used as the organic constituent; the organic fraction is then dissolved in water after evaporation of the solvent and extracted again with chloroform.

Examples of the most often used separation systems are shown below. To remove interfering lipophilic substances such as fats, waxes, chlorophyll, etc., the system methanol:hexane is used. To improve the separation, 10% of water can be added to the methanol:hexane system. The extraction of polar substances, e.g., glycosides, can be performed using the system water:butanol. The general procedure for this isolation is as follows: The solvent is removed from the total methanol extract, the extract is dissolved or suspended in water, filtered, and extracted with butanol. The glycosides pass into the butanol; the strongly polar impurities remain dissolved in the water. However, disadvantages of butanol are its high boiling point, which makes it difficult to evaporate, and its limited miscibility with water. The aqueous phase must be saturated with butanol in advance. Compounds with middling polarity, e.g., aglycones, are often extracted in the systems water:chloroform or water:ethyl acetate. If we must process an unknown sample without having perfect knowledge of the substances contained in the plant and want to analyze for all possible substances, we can use a procedure like the one shown in Fig. 11.

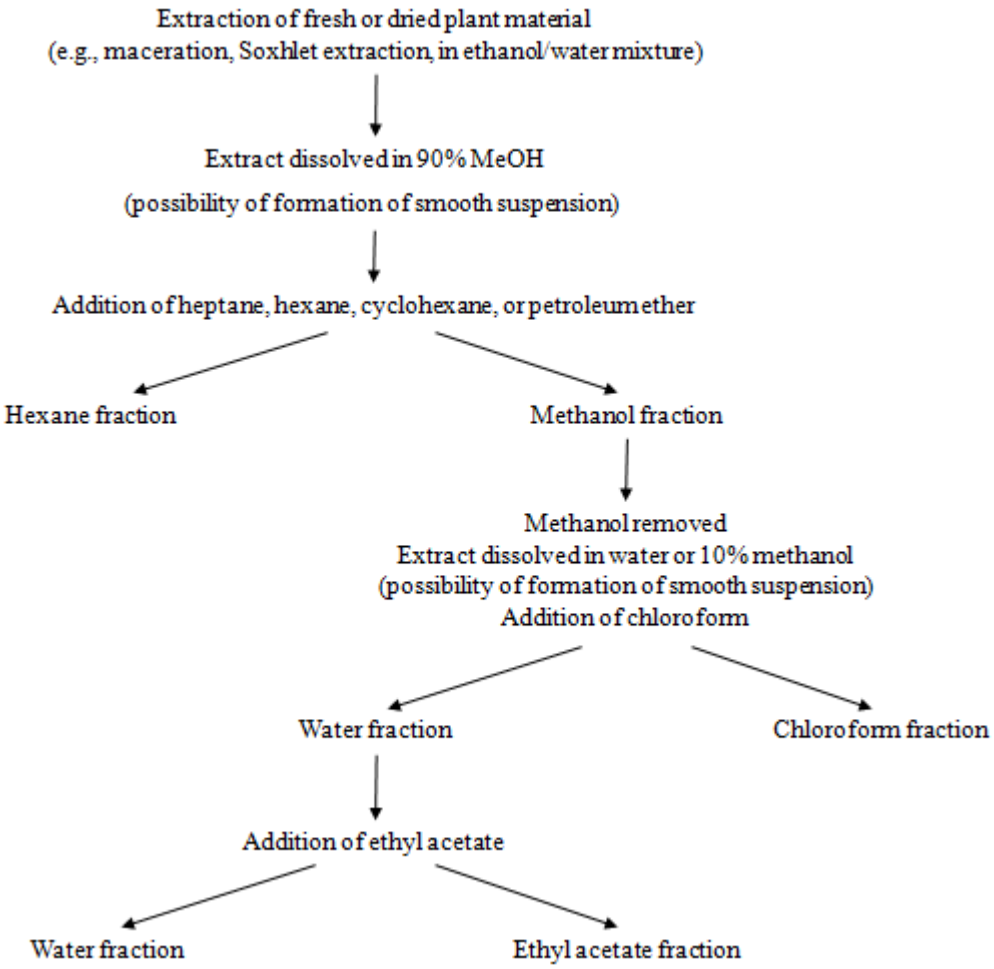

Fig. 11: A typical procedure for separation by extraction between immiscible solvents

This type of extraction is also used to purify alkaloids. Alkaloids are substances with alkaline character caused by the presence of a secondary or tertiary nitrogen. When extracted in an acid environment (dilute sulfuric, hydrochloric, or citric acid), they easily form salts and are soluble in water. When such a solution is extracted with a non-polar organic solvent, non-polar impurities are extracted. The organic layer is separated, fresh solvent is added and the entire system is alkalized (usually with dilute ammonia). An alkaline substance displaces an alkaloid from a salt and the alkaloid becomes soluble in the lipophilic organic layer. After the separation, the organic layer can be repeatedly extracted into an acidified aqueous phase. The salts formed from the alkaloids are transferred into the aqueous phase again. In this way, alkaloids can be easily separated from ballast or, with a controlled pH change, fractionated into groups with similar structures according to their dissociation constants.

5.3 Lyophilization (freeze-drying)

Lyophilization is the most gentle method of removing water from a sample after extraction or separation. Reducing the pressure and temperature to approx. 1 mbar and -50°C enable the easy removal of water by sublimation. The water vapor is caught on a spiral at a very low temperature and then removed from this condenser in the form of ice. Simple commercially supplied lyophilizers in variable sets are currently used in laboratory conditions. With various adjustments, this method is also suitable for removing other sublimable solvents (e.g., tetrahydrofuran or DMSO).

The method is used mainly when the substances in the extract are thermolabile and there is no other way to remove water without heating. The resulting lyophilizate is a material with a large surface area and is easily dissolved for subsequent processing.

5.4 Precipitation

Precipitation is used to remove either target substances or undesirable impurities from a solution or extract. Precipitation is followed by decantation, centrifugation, or filtration. Unlike chromatographic separation, for example, this method can be very simple, cost-effective, and fast and can be used when other techniques are not appropriate. However, precipitation can be used only when a sufficiently selective precipitating reagent is available and there is no danger of co-precipitation.

The precipitation of alkaloids using Mayer´s reagent (potassiomercuric iodide) is an example. In an acid environment, it forms complexes with tertiary and quaternary alkaloids. A heavy white sediment precipitates and can be easily filtered off from the primary solution. The precipitate is then dissolved in a mixture of acetone, methanol, and water. This solution is subsequently pressed through a column with an ion exchanger in Cl^- cycle, which exchanges $[HgI_4]^{2-}$ complex for chloride ions and the alkaloids are therefore isolated in the form of chlorides.

An example of removing impurities by curdling is the precipitation of phenolic compounds with plumbous ions or polyvinylpyrrolidone. Phenolic substances, such as catechins or gallates, often interfere in chromatographic or spectrophotometric analyses for other substances. Precipitation with lead can be used to selectively remove phenol derivatives with an *ortho* dihydroxy group.

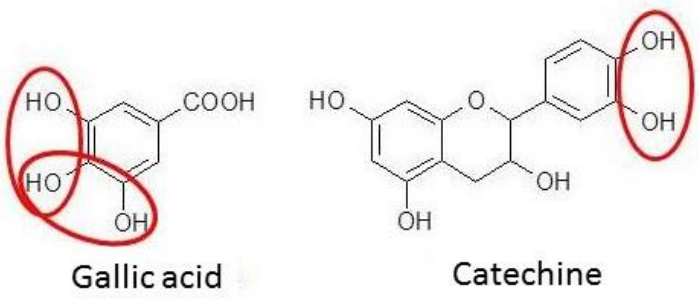

Fig. 12 Examples of phenolic compounds with an *ortho*-dihydroxy group (marked) that can be precipitated with Pb^{2+}

As a final type of purification, e.g., to remove undefined impurities after a chromatographic separation, activated charcoal is also used. Such undefined impurities are

often difficult to remove by chromatography or crystallization. However, it is necessary to set up the system in the way that prevents the adsorption of the purified substances.

Natural substances can be precipitated from a solution by adding a solvent in which the substance is insoluble. Compounds with rather small differences and similar polarities often dissolve in significantly different ways in various solvents. An example is the precipitation of some glycosides (e.g., hesperidin from *Citrus* spp.), which are insoluble in water. Hesperidin is readily dissolved in dimethylformamide, then easily precipitated by the addition of water, and finally isolated from the solution by centrifugation.

5.5 Crystallization

In the early days of phytochemistry, crystallization was one of the most frequently used methods of isolation. Most substances were isolated simply because they were easily crystallized from solution. At present, milligram amounts of a wide range of compounds are isolated in amorphous form by means of chromatography. Crystallization no longer plays an important role in the last step of their isolation. However, it remains an interesting, cost-effective, and fast technique for both laboratory and preparative scale work with some compounds. This method is sometimes used in cases requiring the separation of substances with similar structures that are difficult to separate by chromatography. It is an advantage that crystallization enables the unambiguous identification of a substance by X-ray crystallography (see Chapter 6.5) – if we are able to prepare a crystal.

A system of solvent vapor diffusion is commonly used to prepare crystals for X-ray crystallography. This method works with the substance dissolved in a solvent in which it is readily soluble (forming a nearly saturated solution) placed into a closed vessel. A second solvent in which the substance dissolves with difficulty then evaporates from the surrounding environment and diffuses into the solution of the substance (Fig. 13). As the composition of the solvent changes, the solubility of the substance declines and the substance crystallizes, preferably as a monocrystal.

Finding the appropriate system for a crystallization is rather a matter of empirical procedure. A solvent or solvent system in which the crystallized substance exhibits less solubility than the impurities is usually sought. An inverse system is possible, though less frequent.

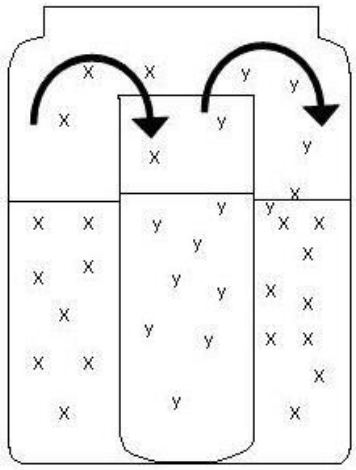

Fig. 13: Scheme of the apparatus used for vapor diffusion crystallization from a solvent X, in which the crystallized substance is less soluble, diffuses into the inner vessel. Y, a solvent in which the substance is highly soluble, diffuses out of the inner vessel.

5.6 Chromatographic methods

Prior to the discovery of chromatographic methods, the isolation of natural substances was quite a difficult matter based on multiple crystallizations. It was therefore possible to isolate (and subsequently identify) only crystalline substances obtained in large amounts. In 1950s, the routine introduction of chromatography and the development of sophisticated chromatographic methods significantly advanced the isolation of natural substances. Chromatographic methods can be used for both the isolation of secondary metabolites (in the preparative mode) and for identification and determination of the amounts of the analyzed substances (in the analytical mode). Thus, chromatography is the subject of the entire following chapter.

5.6.1 Principles of substance separation in chromatography

In chromatographic separation, molecules of the analyte are distributed between two immiscible phases effectively moving against each other (the stationary and the mobile phases). Chromatographic separations are based on the different affinities of the substances to be separated for the mobile and stationary phases as well as on the multiply repeated resetting of the phase equilibrium between these two immiscible phases of the mixture being analyzed. The stationary phase can be laid down as a layer (thin-layer chromatography) or placed into a column or capillary. Chromatographic methods are divided into liquid chromatography and gas chromatography according to the state of the mobile phase (supercritical chromatography also exists). Another classification can be based on the required use, i.e., analytical chromatography (quantitative and qualitative analysis) or preparative chromatography (isolation of substances from a mixture).

Chromatographic methods can also be classified according to the nature of the forces which cause the distribution of substances between the stationary and mobile phases. According to the nature of the separation forces, several types of chromatography are distinguished. Methods used in phytochemistry particularly exploit the various abilities of solid stationary phases to adsorb separated substances (adsorption chromatography) or the distribution of the separated substances between two phases on the basis of their different solubilities (partition chromatography). In partition chromatography, the stationary phase is a

thin liquid film adsorbed onto a solid carrier (applied particularly in gas chromatography). In liquid chromatography, this solution is not stable enough (adsorption of the liquid onto the carrier might not be firm enough to create a permanent film); it has therefore been replaced by so-called chemically bound phases (see Chapter 5.6.5.3). In this place, it is necessary to emphasize the difference between gas and liquid chromatography; the separation in gas chromatography is not affected by the mobile phase used (mostly an inert gas). On the contrary, the composition of the mobile phase in liquid chromatography is substantially important for the course of the separation. Liquid chromatography with normal phases exploits polar stationary phases (*e.g.*, silica gel or aluminum oxide) and relatively non-polar mobile phases (*e.g.*, benzene or toluene). In liquid chromatography with reversed phases, more often referred to as reversed-phase chromatography (often chromatography with chemically bound phases), the stationary and mobile phases are non-polar and polar (*e.g.*, water, methanol), respectively. Non-polar stationary phases are prepared, for example, by binding aliphatic hydrocarbon chains of various lengths (*e.g.*, C8, C18) to an appropriate carrier (*e.g.*, silica gel) (see Chapter 5.6.5.3 and Fig. 19). The most frequently used type of stationary phase in high performance liquid chromatography is the reversed phase.

Other mechanisms of separation can be used in liquid chromatography, i.e., ion exchange, effect of net charge, and biospecific reactions. However, the use of these methods is much less widespread in phytochemistry; thus, only the basic principles are mentioned here.

Ion exchange chromatography exploits stationary phases consisting of a solid carrier with a covalently bound anion (*e.g.*, $-SO^{3-}$) or cation (*e.g.*, $-N(CH_3)^{3+}$) functional groups. Separated ionic substances are attracted to the stationary phase with the opposite charge by electrostatic forces.

The effect of net charge is used in size-exclusion chromatography, which exploits porous gels with pores of various sizes as the stationary phase. Big molecules of dissolved substances cannot penetrate the porous stationary phase and pass through the system faster than the smaller molecules that do penetrate the pores of the stationary phase (this is why it is sometimes called reverse filtration). Size-exclusion chromatography is used mainly for macromolecular substances in polymer chemistry and biochemistry, but it can also be used in the phytochemical laboratory.

Affinity (biospecific) chromatography is based on the biospecific interaction of the analyte with complementary substances (ligands) that are anchored in the stationary phase. Examples are the interactions of enzymes with inhibitors or substrates or those between antigen-antibody complexes, etc. This method is used particularly in biotechnology and biochemistry.

5.6.2 Thin-layer chromatography (TLC)

Thin-layer chromatography is one of the most frequently used techniques for the analysis of secondary metabolites (and chemical substances in general). It is based on classical chromatographic principles and is carried out in a surface layout. The base is a thin layer of stationary phase applied on an inert solid matrix, such as glass or metal foil. The sorbent is usually anchored to the matrix with plaster. After the sample is applied at the start line, the plate is put into a vessel (cuvette) containing a mobile phase. Capillary forces cause the mobile phase to ascend evenly through the sorbent particles and the separated substances are carried along according to chromatographic principles (usually adsorption chromatography) (Fig. 14). The plate behaves as a big capillary as the mobile phase is ascending. So-called capillary elevation therefore takes place with the result that the front of the mobile phase then has the shape of a meniscus. This shows that the mobile phase moves faster at the edges of the plate with resultant broadening of the stains of the separated substances at the edges. This makes it difficult to compare chromatographic traces at the edge of the chromatogram with those in the middle. These so-called side effects can be minimized by removing a thin strip from both edges of the plate and cutting off the lower corners of the chromatogram before developing it.

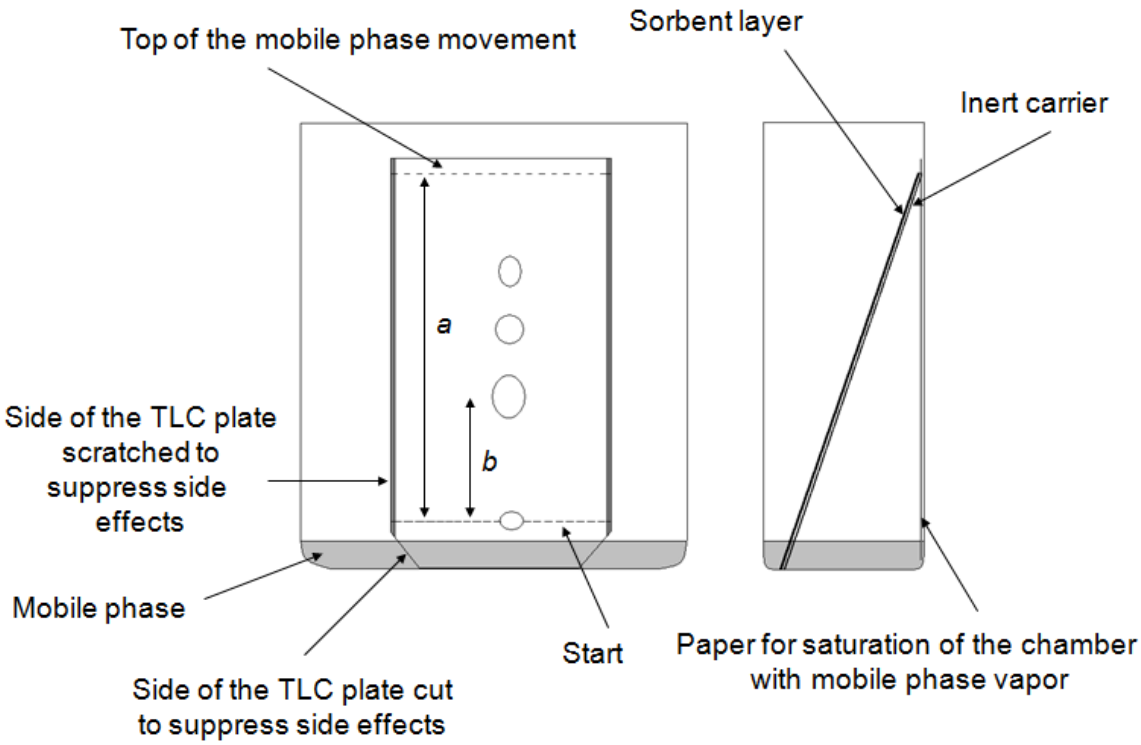

Fig. 14: Scheme of TLC apparatus (a – distance between the front and the starting line, b – distance between the center of the spot and the starting line)

Compared with column chromatographic methods, TLC has two basic differences. Whereas a column is open to the atmosphere and the stationary phase is dry at the start before being saturated with the solvent during the separation, a TLC chamber should be firmly closed and the inner atmosphere should be saturated with mobile phase vapor. TLC can be used for both analytical and preparative purposes. Preparative and analytical TLC use plates with a layer thickness of 500-2000 μm and 100-250 μm, respectively. At present, various stationary phases are commercially available, but classical silica gel is still most often used. The variant with silica gel is the cheapest and separations with silica gel are the most investigated, with the biggest number of its applications described for them. Silica gel is used to separate a really wide spectrum of substances, ranging from non-polar compounds of the terpene type to the rather polar alkaloids.

Modified silica gels are also available for TLC. The most commonly used is the C18 reversed phase. However, these sorbents are rather expensive, which limits their use, particularly for preparative purposes. So-called HPTLC (high performance TLC), in which the sorbent particles are smaller and of a more regular (spherical) shape, is used to enhance the efficacy of separation.

The mobile phase is selected according to the type of substances to be separated and the stationary phase. For the most commonly used silica gel, a mobile phase consisting of a two- to three-component mixture of organic solvents is selected. According to polarity, one solvent is more polar (causing stronger elution) and one is non-polar. The eluting power of the mixture is adjusted to meet the requirements of the separation by changing the solvent ratio. The third or, if necessary, fourth component of the mobile phase is mainly an acid-base or other modifier used to adjust the properties of the mobile phase in the sense of higher resolution or ”focusing“ of the spots. The solvents and modifiers can be selected according to their characteristics by using the Snyder Triangle (Fig. 15) or from the eluotropic series (Table 1).

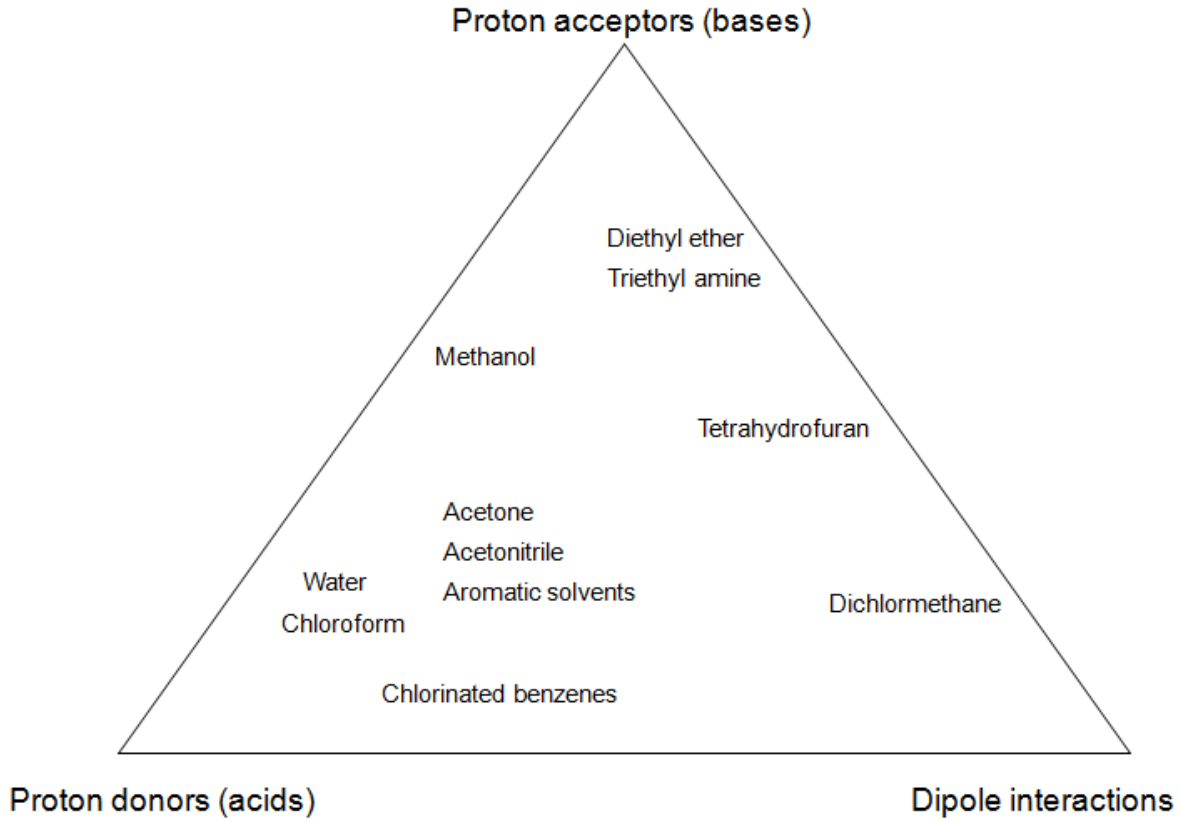

Fig. 15: The Snyder Triangle of solvent selectivity

The solvents are arranged according to their acid-base characteristics and capacity for dipole interactions. The correct selection of components of the mobile phase and their ratios is usually the result of a number of experiments using the method of trial-and-error.

Solvent	$E^0(Al_2O_3)$	Boiling point [°C]	Viscosity [mN.s.m^{-2} (20 °C)]	UV cut off [nm]
Pentane	0	36	0.24	210
Cyklohexane	0.04	69	0.98	210
Tetrachlormethane	0.18	77	0.97	265
Toluene	0.29	111	0.59	286
Diethyl ether	0.38	35	0.25	218
Chloroform	0.40	62	0.57	245
Dichlormethane	0.42	40	0.44	235
Tetrahydrofuran	0.45	66	0.55	220
2-butanone	0.51	80	0.32	330
Acetone	0.56	56	0.32	330
1,4-dioxane	0.56	107	1.44	215
Ethyl acetate	0.58	77	0.45	255
Diethyl amine	0.63	115	0.33	275
Acetonitrile	0.65	82	0.37	190
2-propanol	0.82	82	2.50	210
Ethanol	0.88	78	1.20	210
Methanol	0.95	64	0.59	210
Water	1	100	1.0	-

Table 1: Eluotropic series $E^0(Al_2O_3)$ – Snyder solvent strength parameter

2D thin-layer chromatography can also be used to enhance the separation. After the chromatogram is developed in one direction and dried, the plate is turned 90° and developed in a mobile phase with a different selectivity. It is sometimes possible to apply "3D" by yet another turning and development.

The rate at which the solvent ascends on the plate is not constant and slows with increasing distance from the starting line. Diffusion of the substances in the solvent increases and the zones (spots) of particular substances get bigger. The probability of inconsistency in the passage of the solvent through the sorbent increases with increased distance from the starting line. The enlargement of spots reduces the resolution and several techniques supposed to prevent this phenomenon have therefore been developed.

Rotation planar chromatography (RPC) exploits centrifugal force to transport the mobile phase across the plate. The sample is applied into the center of a round plate and the mobile phase is then also put there. Rotation of the plate spreads the mobile phase homogeneously outward to the edge of the plate where it can be caught along with the separated substances (this is why this method can be used for preparative TLC).

Optimum performance laminar chromatography (OPLC) exploits a plate with sorbent covered with a thin inert foil. The mobile phase is pumped into the sorbent layer. Unlike classical TLC, there is no contact with the atmosphere of the developing chamber, so the method is similar to HPLC. OPLC can be used for both analytical and preparative purposes.

In preparative TLC, 20 × 20 cm plates are usually used. Up to 100 mg of sample can be separated on one plate. Instead of applying drops with 5-10 mm diameter, the sample is applied in a line along the entire length of the starting line. An automatic pipette, a Pasteur pipette or an automatic applicator can be used to apply a narrow starting layer, which should not be wider than 10 mm. The starting layer can be narrowed by using a brief development in a mobile phase with high eluting power (e.g., methanol for silica gel). After development, the detection is carried out (see Chapter 5.6.2.1); the sorbent is scraped from the plate and the substances are extracted with an appropriate solvent. Preparative TLC has advantages and disadvantages compared to other systems of preparative chromatography. It is simple and does not require complex apparatus. The method used for analytical TLC is easily adaptable for preparative purposes. Quite large amounts of sample can be quickly separated with better selectivity and resolution than classical column chromatography (see Chapter 5.6.5). On the other hand, this method is costly and laborious for really big amounts of sample (approx. 10 g); decomposition reactions can take place on the silica gel and the separation is not exactly repeatable each time.

The behavior of substances separated by TLC is described by the so-called retention factor (Rf). This quantity is dependent on the character of the mobile and stationary phases used, the structure of the separated substance, the development path of the chromatogram, and other conditions of the separation. For specific conditions, it is constant for each substance analyzed. It is a dimensionless quantity, determined by the ratio of the distance from the starting line reached by the analyzed substance (center of the spot) and the distance reached by the front of the mobile phase. Thus, the maximum value of Rf is 1, if the substance of interest is not retained on the stationary phase and migrates up the chromatogram with the

face of the mobile phase; minimum values near 0 are reached by the substances which do not migrate at all on the selected mobile phase and are firmly bound to the stationary phase. Rf values of analyzed substances can be mutually compared and used for identification, following comparison with a standard. However, it is always necessary to consider the conditions of separation and perform analysis of a standard together with that of the sample (on the same plate). The Rf value can also be used to estimate the polarity and basic characteristics of the analyzed substance. Polar compounds are firmly bound to silica gel (polar stationary phase) whereas non-polar compounds migrate even in a ”weak“ mobile phase.

5.6.2.1 Detection methods for thin-layer chromatography

Most chromatographic plates are intended for qualitative or semi-quantitative purposes, i.e., for finding out if the analyzed substance is identical with the standard or if the analyzed fraction contains a sufficient amount of the target compound. Needless to say, there are also preparative plates; their detection will be mentioned separately.

TLC analysis can be performed without any visualization reaction only in cases where the analyzed substance itself is colored. Detection methods can be classified as non-destructive or destructive. Typically, non-destructive methods are used first, followed by those that destroy the sample.

The simplest non-destructive visualization is achieved by using a fluorescent indicator which is currently added to the majority of TLC plates. When such an indicator is added to a silica gel, cellulose and aluminum oxide, or C18-type sorbent which is then irradiated with UV radiation at the wavelength 254 nm, green or light blue fluorescence is observed. If the substances tested by TLC absorb radiation at the wavelength 254 nm (*e.g.*, aromatic compounds, or those with an α, β unsaturated carbonyl, or conjugated double or triple bonds), the UV radiation does not pass through the substance to the indicator and a dark spot occurs at the location of the substance. These substances are then detected with quite high sensitivity (around 1 μg). Detection can also be observed for irradiation at a wavelength of 366 nm. This detection is not universal as, in this region only substances with extended conjugated systems fluoresce. On the other hand, it is quite sensitive and enables detection of 0.1 μg of a substance. Because of its great sensitivity, this method must be taken with a pinch of salt; the

chromatogram may also contain the spots of substances present in very low concentrations. Typical substances detectable in this way include flavonoids and other plant pigments, *e.g.*, chlorophyll.

Destructive detection methods can be used after a chromatogram has been analyzed by UV-visible radiation. Destructive detection methods are based mainly on chemical reactions. It is usually possible to use a wide range of chemical reagents for universal (non-specific) and selective (specific) reactions; thus simple reagents and basic chemical glassware should be part of a phytochemical laboratory.

Typical non-specific reagents include, for example, sulfuric acid in diethyl ether, which dehydrates and oxidizes separated substances. The chromatogram is heated and colored spots subsequently occur at the locations of the substance spots. However, the chromatogram cannot be stored after detection and it decays. An example of a selective reagent is Dragendorff reagent, which reacts with the majority of alkaloids to form orange-colored complexes. Examples of other reagents are shown in the following table.

Detection reagent	**Compounds detected**
Anisaldehyde in sulfuric acid	Essential oils, terpenes, aromatics
Anthrone in sulfuric acid	Sugars, glycosides
Chloramine in trichloroacetic acid	Cardenolides
Dragendorff reagent ($[BiI_4]^{2-}$)	Alkaloids
Fast Blue B (3,3′-dimethoxy[1,1′-biphenyl]-4,4′-*bis*(diazonium) dichloride)	Phenolic compounds, for example aflatoxins
Neu′s reagent (2-aminoethyldiphenylester of boric acid)	Flavonoids
Ninhydrin	Amino acids
Potassium hydroxide in ethanol	Anthraquinones
Concentrated sulfuric acid in diethyl ether	Universal detection reagent
Vanillin in sulfuric acid	Essential oils, terpenes, aromatic compounds, sugars

Table 2: Detection reagents used in TLC and the substances they detect

Color detection on the chromatogram using non-destructive and, subsequently, destructive methods can be a good method for identifying a substance if comparison with a standard is used and a spot with the same Rf exhibits the same behavior.

Non-destructive and destructive methods of detection can also be used for preparative TLC. Preparative plates can also be equipped with fluorescent indicators. Following development and detection under UV radiation, a part of the plate is covered with a glass or with aluminum foil, the reagent is applied and the color reaction observed. After the spots have been observed and the sorbent with analyzed substance scraped off, UV radiation can again be used to confirm the visualization reaction.

5.6.2.2 Quantitative analysis by TLC

Quantitative analysis requires the application of an exact amount of sample on the TLC plate. This can be achieved by using an automatic pipette or an automatic sample injector. To determine the quantity, it is again possible to use both non-destructive and destructive techniques. The spots on the TLC chromatogram can be scanned by using a densitometer. The densitometer can usually measure both transmittance and reflectance. If a sophisticated device and a sufficiently large TLC plate are used, the TLC quantitative analysis method can serve as an alternative to HPLC, as it enables the processing and analysis of even several tens of samples.

A simpler variant, tracing and cutting out the spots and weighing then eliminates the use of densitometer. Naturally, the precision and reproducibility are not as high in this case.

5.6.3 Theoretical background of column chromatography

This chapter describes the general theory related to all forms of column chromatography. With proper modifications, this theory can be applied to planar chromatography (a stationary phase on a surface layer) as well.

The separation in column chromatography takes place in a separation column. A separated sample (a mixture of several substances) is applied at the start of the separation column. The separated substances repeatedly pass between the stationary and mobile phases. As the sample migrates through the column (carried by the mobile phase), particular dissolved substances are separated according to the varying physico-chemical affinities of substances of the stationary phase. If the strengths of the particular interactions with the stationary phase are sufficiently different, the dissolved substances are distributed into separate zones. The result of the chromatographic separation is recorded by a proper detector at the end of the column. A record of the intensity of the signal from the detector as a function of time or volume of the eluted mobile phase is called a chromatogram (Fig. 16). In an ideal case, one zone on the chromatogram represented by a Gaussian-shaped curve called a peak corresponds to each separated substance.

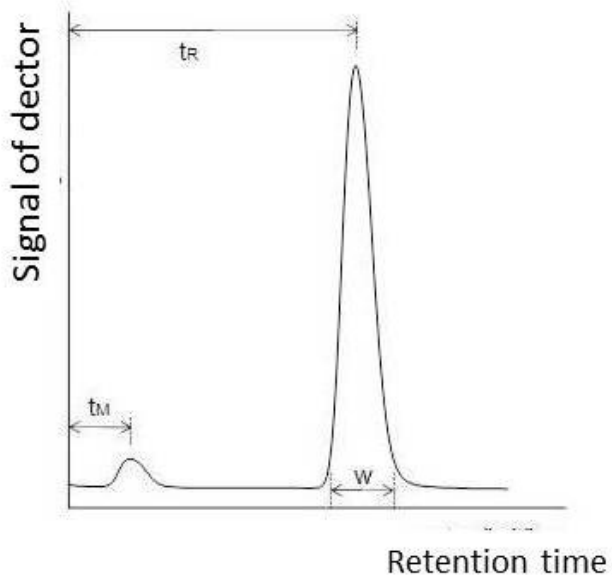

Fig. 16: Chromatogram with the main characteristics identified (t_R - retention time of the substance, t_M - dead time, w - width of the chromatographic peak at the baseline)

5.6.3.1 Basic characteristics of the chromatographic process

The retention time (t_R) is the time which elapses between the application of the sample on the column and the detection of the peak maximum of the particular substance. It corresponds to the total time during which the substance resides on the column. The dead time (t_M) corresponds to the time which the substance requires to pass through the column if it is not retained by the stationary phase (i.e., if the substance moves with the same rate as the mobile phase). The retention of the compound can also be measured indirectly as the volume of the mobile phase passing through the column within the time during which the separated substance resides on the column. This parameter is known as the retention volume (V_R). The dead volume (V_M) is the volume of the mobile phase which has to pass through the column to ensure that a substance which is not retained gets from the beginning to the end of the column. A frequently used retention quantity is the retention ratio $k`$. It expresses how much longer the molecules of sample reside in the stationary phase than in the mobile phase, i.e., after how many multiples of the dead time the substance elutes. It is also the ratio of the amounts of analyte in the stationary and mobile phases at equilibrium.

$$k` = \frac{t_R - t_M}{t_M} \qquad (3)$$

Another significant parameter is the width of the chromatographic peak at its base (w). The width of the peak is determined by the points of intersection of tangent curves with the points of inflexion on both sides of the chromatographic peak. The width of the chromatographic peak reflects the width of the zone of a particular analyte in the column. In addition to the width of the peak, another parameter is also used - the width of the peak at half of its height ($w_{1/2}$).

The retention time and volume are important qualitative parameters and are constant for a particular substance under specific conditions of separation. Thus they can be used to identify the substance by comparison with a standard. However, as similar substances have similar retention times, the concordance of t_R between a substance a standard is not sufficient to provide unambiguous identification. Further information is required, e.g., from detection of an additional parameter. The ability to identify a substance (comparison with a standard or, as the case may be, without one) thus depends on the detector or the combination of detectors used and the data obtained from them (UV/Vis spectrum, mass spectrum, etc.). For quantitative analysis the area of the peak of the substance to be determined is observed. This

area is proportional to the amount of the substance in the sample. The peak area is most frequently determined by the method of numerical integration. In modern devices connected with a computer, this calculation is performed by a suitable program specifically for the evaluation of chromatographic data. Quantitative analysis requires that standards of the substance to be determined be used to calibrate the system (most commonly, a calibration curve).

5.6.3.2 Efficacy and resolution of chromatographic separation

The aim of chromatography is to separate a mixture of substances into a series of individual components wherein each peak on the chromatogram corresponds to one substance. The efficacy of a chromatographic column decreases with increased broadening of the zones of the separated substances. This broadening is caused by several factors:

1. Turbulent diffusion - the molecules dissolved in the mobile phase traverse different distances while flowing around the particles packed in the column.

2. Longitudinal diffusion - simple diffusion of substance molecules which move from a place with higher concentration (chromatographic zone) to places with lower concentrations.

3. Resistance to mass transfer - processes within the mobile and stationary phases that are necessary to transport the substance to active sites in the stationary phase where interaction then takes place.

The quantitative expression of the measure of separation of two peaks is called resolution (R_S) and can be calculated from Equation 4. Better separated peaks are reflected in increased values of R_S; $R_S = 1$ is a sufficient value (the peak overlap is approx. 3 %); virtually complete separation of peaks arises at $R_S > 1.5$. Because they prolong the time of analysis, high R_S values are not beneficial. The resolution makes it possible to assess whether or not a change in chromatographic conditions has resulted in better separation of substances.

$$Rs = \frac{2 \times (t_{R2} - t_{R1})}{w_2 + w_1} \quad (4)$$

t_{R1} and t_{R2} are the retention times of two neighboring peaks ($t_{R1} < t_{R2}$), w_1 and w_2 are the widths of these respective peaks.

According to Equation 4, the resolution is related to the width of the chromatographic peak given by the efficacy of the chromatographic column (Equation 5). To quantitatively express the efficiency of column, we use the designation "a number of theoretical plates *N*".

$$N = 16 \times \left(\frac{t_R}{w} \right)^2 \quad (5)$$

The above mentioned factors are related to flow rate of the mobile phase. There is an optimal flow rate of the mobile phase to achieve the maximum efficiency of a chromatographic column. Increasing or reducing it results in diminished efficiency. The size and shape of the stationary phase particles also affect the broadening of the chromatographic zones. Reducing the particle size leads to better resolution and manufacturers make an effort to produce the smallest sphere-shaped particles possible. Moreover, smaller and regular particles in the stationary phase suppress the effect of the flow rate of the mobile phase on the resolution. This permits using higher flow rates and, shortens the time of analysis. At present, so-called monolithic columns formed of one block of chromatographic sorbents are being used. These columns allow the use of high mobile phase flow rates without an excessive increase in pressure and, at the same time, without the loss of separation efficacy. Another type of relatively novel stationary phase is represented by so-called core-shell particles, for which a layer of sorbent is sprayed on a solid core.

Knowledge of the number of theoretical plates can be used to estimate the number of dissolved substances which can be separated under specific conditions. This estimation is called the peak capacity n_C and expresses the number of peaks fitting into a chromatogram (6).

$$n_C = 1 + \frac{\sqrt{N}}{4} \times \ln \frac{V_{\max}}{V_{\min}} \quad (6)$$

where $V_{\min}$ and $V_{\max}$ are the smallest and the largest volumes of the mobile phase in which the dissolved substances can be eluted, respectively.

5.6.3.3 Optimization of the conditions of chromatographic separation

Based on the above mentioned facts, it can be said that better separation (increased resolution) of substances is achieved by either increasing the difference in the retention times of separated substances (thermodynamic aspect) or narrowing their chromatographic zones (kinetic aspect). The width of the peaks depends on the flow rate of the mobile phase, the column length, the size of the stationary phase particles, and the temperature (temperature affects diffusion coefficients); i.e., on the factors that determine the efficiency of the chromatographic column.

Changing the thermodynamic aspect requires a more substantial intervention into the chromatographic system and is affected by changes in the stationary and, in some cases also, the mobile (in the case of liquid chromatography) phase and by the temperature (temperature affects the distribution coefficients and thus the distribution of analytes between the stationary and mobile phases). Optimizing the composition of the mobile phase is frequently used in liquid chromatography, because it is easier and cheaper to change the composition of solvents than to acquire a new chromatographic column with a different type of stationary phase. Gradient elution is highly advantageous and frequently used. The composition of solvents is changed during the analysis and the eluting power of the mobile phase is increased gradually. This is hugely significant for complex samples containing substances with different polarities. With a lower mobile phase eluting power, substances that are less strongly retained on the column are eluted and gradually increasing the eluting power results in faster elution of the other retained substances. This procedure enables the separation of substances with very different polarities in one analysis. What would be a prolonged analysis using a mobile phase of constant composition (isocratic elution) is considerably shortened. Another parameter which can be used to optimize the chromatographic conditions is temperature. As already mentioned, temperature affects both kinetic and thermodynamic aspects and the effect of an increase or decrease in temperature on the resolution cannot be estimated unambiguously. Changing the temperature to optimize chromatographic conditions is used in both liquid and gas chromatography, although is much more significant for gas chromatography. Gas chromatography often employs a temperature program (temperature gradient) whereby the temperature of the column is gradually increased during the analysis. At lower temperatures, the substances that are retained less on the column are eluted. Gradually increasing the

temperature results in the faster elution of substances more strongly retained by the stationary phase and shortens the time of analysis.

In the framework of this chapter, it is necessary to mention the capillary columns used in gas chromatography (the complexity of the instrumentation prevents their widespread use in liquid chromatography). In capillary columns, the stationary phase is applied as a thin layer on the inner wall of the column. The absence of column packing means that the mobile phase can migrate through the column under significantly lower pressure. This allows capillary columns to be produced with much greater lengths than would be acceptable for packed columns. In capillary columns, turbulent diffusion is eliminated and the resistance to mass transfer is reduced. Compared with packed columns, the combination of these factors with the greater length of the column increases the number of theoretical plates by approximately two orders of magnitude.

5.6.3.4 Formation of asymmetric peaks

In previous chapters, the description of chromatography was based on the presumption that the substances elute from the column as Gaussian-curve-shaped symmetric peaks. This ideal behavior is observed on condition that the partition ratio K_D is constant for all concentrations of the analyte. The partition ratio is given by the ratio of the analyte concentrations in the stationary and mobile phases and characterizes the distribution of the analyte between these two phases. The higher the partition ratio, the more the analyte is retained by the stationary phase and elutes with greater retention time. In some situations, we can observe deviations from ideal behavior (the ratio of the analyte concentrations in the stationary and mobile phases is not constant) which results in asymmetric peaks. Fig. 17(A) shows a sample of “tailing“ which causes broadening of the descending part of the peak. This behavior can arise when some places in the stationary phase retain dissolved substances more strongly than other places or if the substance is poorly soluble in the mobile phase (incompatibility of the sample with the stationary and/or mobile phase). Other causes may include technical problems within the device, e.g., leakage of the injection apparatus or broadening of the chromatographic zone beyond the column (too long a path between the column and the detector – too much ”dead volume“).

Another type of non-ideal behavior is a peak with a broadened ascending part (a so-called ”fronting“ peak) (Fig. 17(B)). Most commonly, this behavior is a consequence of column overloading (excessive concentration of sample) or the use of a solvent with eluting power greater than that of the mobile phase. The formation of tailing or fronting peaks can also be caused by a contaminated or degraded column.

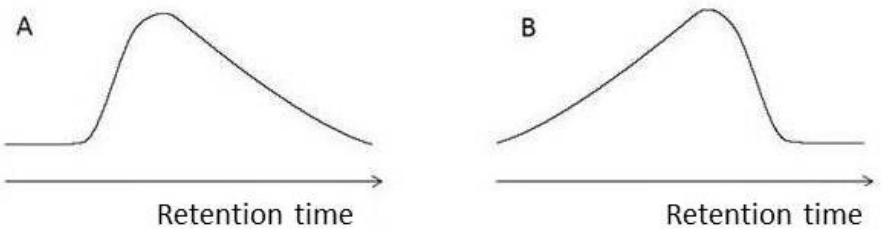

Fig. 17: Chromatographic peaks with broadened descending (A) or ascending (B) parts

5.6.4 Gas chromatography (GC)

Gas chromatography employs a stream of gas as the mobile phase which carries separated substances through the column. The mobile phase in gas chromatography is inert and does not participate in the chromatographic process (serving only as a carrier gas). The sample to be separated must first be introduced into the mobile phase. Gas chromatography is therefore suitable for the analysis of gases and volatile samples. The maximum temperature for the evaporation of volatile samples is 350 °C (which corresponds to the maximum molecular weight of analytes in the range of 600-800 D). However, there is also high temperature GC with operating temperatures up to 450 °C. The analysis of a non-volatile substance by GC is not possible unless it can be modified into a volatile substance by an appropriate chemical reaction (so-called “derivatization” of the sample). Gas chromatography is usually used for the qualitative and quantitative analysis of volatile substances, but it can also be used in a preparative mode.

The results of a separation are greatly dependent on the strength of the interactions of the analyte with the stationary phase. Another significant factor is the temperature, which determines the partial vapor pressure and thus the volatility of the analytes and also affects their partition ratios. These are also the main parameters of the chromatographic system which are optimized in an effort to separate a mixture of substances into particular peaks. For

the reasons described in Chapter 5.6.3.3, capillary columns are widely used in gas chromatography. Capillary columns are usually made of silica glass coated on the outside with an appropriate polymer to ensure resistance and flexibility. Packed columns are most often made of stainless steel or glass. See Table 3 for a comparison of the typical parameters of the capillary and packed columns used in gas chromatography. The greater efficiency of capillary columns enables them to separate analytes using a smaller variety of stationary phases, thus simplifying the process of optimization. The most important parameter in the development of a chromatographic method is selecting the appropriate stationary phase. Only professionally manufactured columns are currently in use; these can be prepared with high reproducibility and provide high thermal and chemical stability. Generally, there are two types of stationary phases in gas chromatography: those with adsorption or partition mechanisms of separation (see Chapter 5.6.1). Columns exploiting the adsorption mechanism are mainly packed with graphitized carbon, zeolites, silica gel, or aluminum oxide as the stationary phase. The most extensive use is seen in the columns exploiting the partition mechanism of separation, where in polydimethylsiloxane and polyethylene glycol are used as non-polar and polar stationary phases, respectively. These stationary phases are most common and their modification (e.g., exchange of the methyl groups of dimethylsiloxane for alkyl chains of various lengths) determines their resultant properties, e.g., stability, polarity, efficacy, and reproducibility of manufacture. The stationary phase for the partition mechanism of separation is applied onto the proper solid carrier with which the column is packed or is applied as a thin layer on the inner wall of the column in capillary columns.

Parameters	Packed columns	Capillary columns
External diameter	3.2 mm	0.40 mm
Internal diameter	2.2 mm	0.25 mm
Thickness of stationary phase film	5 μm	0.25 μm
Column length	1–2 m	15–60 m
Typical mobile phase flow rate	20 ml×min^{-1}	1 ml×min^{-1}
Number of theoretical plates N	6000	180000

Table 3: Comparison of typical parameters of capillary and packed columns used in gas chromatography

After the appropriate stationary phase has been selected, the second significant variable in gas chromatography is the temperature. The effect of temperature is so significant that temperature programs have become a common part of gas chromatography. A temperature program gradually increases the column temperature to accelerate the migration of the analytes through the column and reduce retention times. Fig. 18 shows the effect of a temperature program on the course of a separation. The first separation (A) shows the chromatogram of a mixture of aliphatic hydrocarbons at a constant temperature; the other one (B) shows the separation of the same sample with the use of a temperature program.

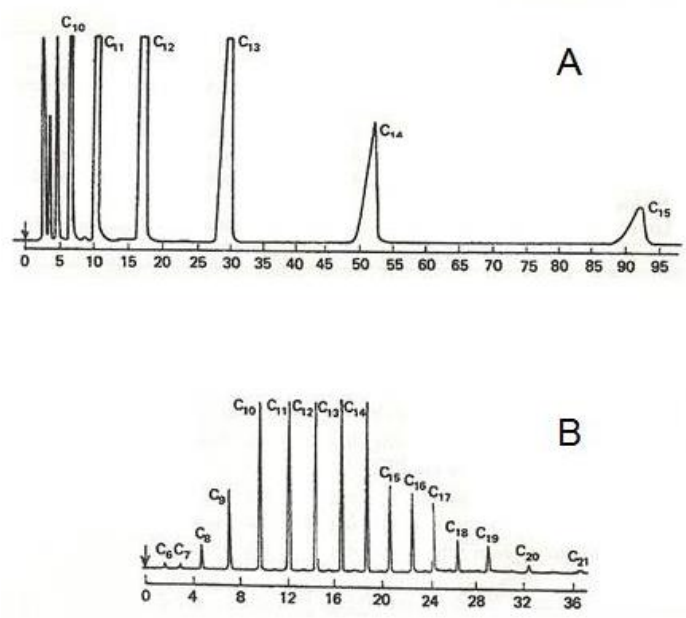

Fig. 18: Comparison of the gas chromatographic separation of aliphatic hydrocarbons at a constant temperature (A) and using a temperature program (B).

The most common mobile phases used in gas chromatography are helium, hydrogen, and nitrogen. These gases must be very pure for the purposes of gas chromatography. The use of helium is most common. Compared with nitrogen, helium exerts only a slight dependence of the column efficiency on the flow rate of the mobile phase. Higher flow rates of helium cause only a mild loss of efficiency, which simplifies the development of chromatographic methods and enables the use of higher flow rates in order to shorten the time of analysis. The properties of hydrogen are similar to those of helium with regard to the effect of the flow rate of the mobile phase on the efficiency of the column, but it has the disadvantage that safety provisions to prevent a potential explosion are needed.

Gases are compressible and the flow rate of the mobile phase is not constant along the entire length of the column; it increases from start to finish. To ensure constant inlet pressure and thus also the inlet flow rate, it is necessary to use special regulators in gas chromatography. Certain criteria must also be met as concerns the rate at which the mobile phase flows through the other parts of the chromatograph. The flow rate through the injection apparatus should be high so that the sample is washed out quickly to prevent its spreading into a greater volume. High mobile phase flow rates and a large amount of sample are not suitable for separation because the instrumentation contains a so-called splitter which carries away a part of the mobile phase containing the sample prior to their entering the column. To prevent the broadening of peaks in the detector caused by too long a residence of the analyte in the detector, a sufficient amount of the carrier gas is added before the detector to ensure the rapid elution of the sample from the detector.

The injection of a sample into the chromatographic system is most commonly carried out with a calibrated micro syringe, either by hand or by means of an automatic sample injector. The temperature of the injector is selected to affect the evaporation of the injected sample and prevent its subsequent condensation. That is why the temperature of the injector is usually 50°C higher than that of the column.

Besides qualitative and quantitative analysis, gas chromatography can also be used in a preparative mode; however, this application is complicated and requires a more complex device.

5.6.4.1 Detection methods for gas chromatography

Of all the separation methods, gas chromatography has the widest range of detection options and detectors with the greatest sensitivity because the mobile phase is an inert gas. The samples separated in phytochemistry are often mixtures of many substances of unknown origin. For this reason, it is beneficial to use detectors that provide structural information about the analytes, even at the cost of some sensitivity. Selected detection options are described in the following paragraphs.

5.6.4.1.1 FID - Flame ionization detector

FID is one of the most frequently used detectors for GC. It is used to detect organic compounds, including secondary metabolites. The principle of detection is based on combustion of the analytes coming out of the GC column in hydrogen flame. The combustion generates a stream of ions which can be measured. This detection method is very sensitive (with detection limits in picograms), universal for secondary metabolites, simple, and relatively robust. The disadvantage is that it does not provide any information on the structure of the substance detected; it is a destructive method of detection.

5.6.4.1.2 FT-IR - Fourier transform-infrared detector

Detection with FT-IR provides an on-line record of the infrared spectrum. Many substances with otherwise similar properties have different infrared spectra; for this reason FT-IR is advantageous for comparison with a spectral library and, at the same time, brings quite a lot of information for the identification of unknown substances. An advantage of FT-IR is that it is non-destructive and thus enables the on-line connection of an additional detector (e.g., GC-FT-IR-MS). Disadvantages include a rather high price, relatively low sensitivity and the fact that the IR spectrum of a substance in the gaseous phase differs from the spectra obtained for the solid phase or a solution of the same substance (special spectral libraries are necessary).

5.6.4.1.3 MS - Mass spectrometric detection

As a detection technique for GC, mass spectrometry (for a detailed description, see Chapter 6.3) combines universality, sensitivity, and specificity. A GC can be connected directly to an ion-source mass spectrometer by means of a simple interface. The most common means of ionization are electron impact (EI) or chemical ionization (CI). The ions formed are further analyzed, usually by means of a magnetic field, quadrupole, or ion trap. Mass spectra are usually very informative and, similar to FT-IR, they can be compared with a spectral library to identify a compound. They can also provide information about the structures of unknown substances. However, this method is destructive and can be used only at the end of a tandem on-line detection system. For preparative purposes, it is necessary to carry out so-called splitting of the gas stream coming from the GC.

5.6.5 Classical column liquid chromatography

Column chromatography in various configurations has been one of the most diverse separation methods. According to its configuration, it is a method where the stationary phase is placed in a glass tube closed with a sintered glass frit and with a shut-off valve at the lower end. The mobile phase usually passes through the stationary phase by means of gravity or, possibly, mild overpressure or vacuum. The size of the column where the separation takes place can differ from tens of centimeters in the length and several millimeters in diameter to hundreds of centimeters and tens of millimeters, respectively.

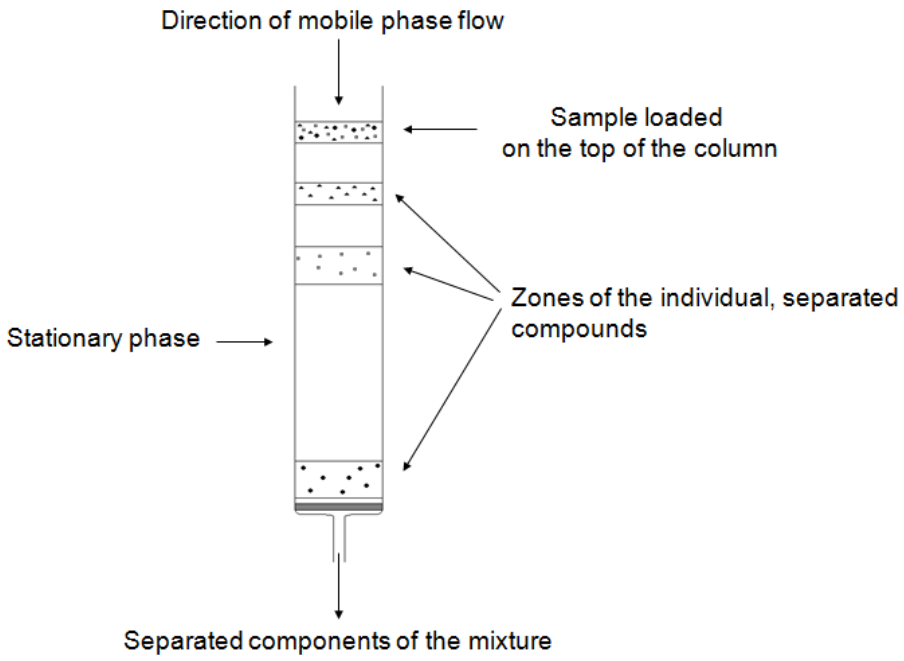

Fig. 19: Column chromatography

For economic reasons, silica gel, alumina, or Sephadex are used for large-size columns (see Chapter 5.6.5.3). Silica gel with a particle size 60-200 μm is the most commonly used. These columns are not pre-packed by the manufacturer; they are filled in the laboratory. Large-size columns are appropriate for the separation of large amounts of material.

The sorbents used are available at low cost but the quality of the separation is also lower mainly because of the large diameters of the sorbent particles. This type of chromatography is used for the first steps in the separation process, when it is necessary to process large amounts of material. Thus, with classical column chromatography, we usually obtain fractions as simple mixtures of substances which can then be processed using other types of chromatography.

The mobile phase for column chromatography is usually a two- or three-, or sometimes even four-component mixture of organic solvents. The usual way of elution used is isocratic elution (see Chapter 5.6.3.3). One of the components is a non-polar solvent, the other polar. Their component ratio sets the elution power of the mobile phase. The third component is an acid-base or other modifier that improves the separation. The appropriate composition of the mobile phase is verified by multiple TLC analyses in which a chromatogram with separate bounded spots with Rf values in the range 0.2-0.5, is sought.

There are two ways of applying the sample on the column. The simplest case occurs when the sample is highly soluble in the mobile phase used. It is then sufficient to apply the sample dissolved in a minimum volume of the mobile phase at the top of the column and let it ”soak“ into the sorbent and separate. However, because of the nature of the material being processed (a mixture of substances with different polarities), the solubility of the sample in a small volume of mobile phase is usually limited. Another method of application is adsorption of the sample on a small amount of the stationary phase (approx. 1:1). This is used in case the solubility of the sample in the mobile phase is poor. The sample is dissolved in a suitable and easy-to-evaporate solvent that is then evaporated after addition of the sorbent. Having been adsorbed, the sample is applied on the top of the column. The mobile phase is usually then brought from a reservoir directly to the column by gravity flow.

5.6.5.1 Flash chromatography

So-called flash chromatography is analogous to classical column chromatography. To improve separation, sorbents with particles of smaller diameter (around 40 μm) are used. However, the great density of these sorbents reduces the flow rate. To speed up the separation, we therefore use overpressure (usually a stream of air or nitrogen) at the input or create a partial vacuum at the output. Columns used for flash chromatography usually have smaller dimensions than those for classical column chromatography. They can be packed with a sorbent directly in the laboratory. To improve and/or speed up the adsorption, it is possible to obtain a device for flash chromatography that includes a mobile phase pump and a detector. These systems are fitted with columns packed by the manufacturer, which usually ensures their homogeneity and, consequently, higher quality separations.

5.6.5.2 Detection methods for classical column chromatography

The separation and purification of the substances contained in plant extracts are not themselves the final objective. They are usually followed by further steps. If a separation is carried out analytically, the following step is qualitative or quantitative analysis. Preparative or semi-preparative separations and purifications are followed by determination of the structure or biological activity. Naturally, the presence of the target substances obtained in different fractions during the process must be monitored. Just as there are whole range of methods for extraction, separation, and purification, there are also lots of ways to perform detection. We can define so-called on-line or off-line detection. On-line detection means measurement carried out directly during the process, e.g., immediately after the output from the separation process. This is used mainly in cases of analytical separation. An off-line technique is one for which some kind of sample transfer is necessary; it is usually used in preparative separation. An on-line technique has several advantages. It is fast, less labor intensive, and involves no danger of significant decomposition of the sample. On the other hand, it is usually more costly, less flexible, and technically more complicated. Moreover, it cannot be used in all cases. Some methods of detection can be used either on-line or off-line.

If a column chromatography system is fitted with an on-line detector, which is not very common, the detection is simple. We scan for a selected characteristic property of the

eluted substances (e.g., absorption of UV/Vis radiation) using an on-line detector. Based on the detector response, we then try to catch particular fractions to obtain simpler mixtures of the substances or the pure substances themselves.

If on-line detection cannot be performed, it is necessary to analyze the fractions obtained. The fractions - separated by volume or time - are obtained by periodic collection of the mobile phase flowing out of the system. The fractions obtained are usually subjected to TLC, analyzed, and compared or, as the case may be, mixed according to their mutual similarity. Besides TLC, other methods, e.g., HPLC, can be used to analyze fractions for similarity.

5.6.5.3 Sorbents for liquid chromatography

5.6.5.3.1 Silica gel

Silica gel is the most common sorbent used for the chromatography of natural substances. Its interaction with separated substances is based on its adsorption. Silica gel is derived from silica sol (polymerized silicic acid). Precipitation from an acid solution results in the formation of primary particles. Water is removed as these particles and a gel forms. In this phase, a three-dimensional net is formed under controlled conditions. It is washed and heated to 120 °C and a so-called xerogel or silica gel, i.e., amorphous porous material, forms. This is a solid substance with a large sorption surface. Free OH groups are exposed into the space in silica gel and grant it a polar character (siloxane groups). These groups can then interact with water. The polarity of silica gel corresponds to its degree of hydration. The hydroxyl groups expressed on the surface of silica gel particles can be classified as ”free, bound, or reactive“.”Free“ hydroxyl groups are accessible for adsorption, mainly *via* hydrogen bonding, and change into ”bound“ hydroxyl groups. ”Reactive“ groups are formed by the association of neighboring free hydroxyls. The ratio of particular types is changed mainly by thermal activation/deactivation (with the optimum at 200 °C) and affects the chromatographic properties of the silica gel (Fig. 20).

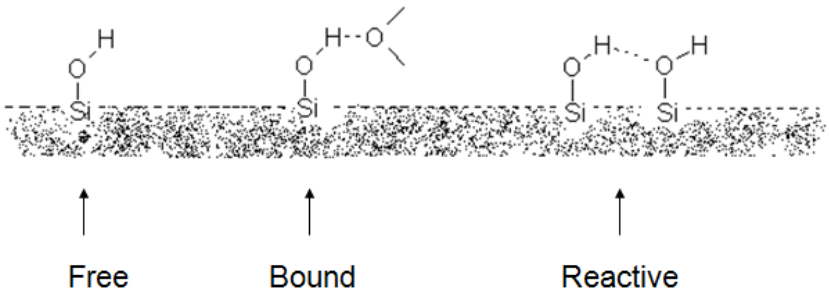

Fig. 20: Scheme of silica gel depicting the nature of silanol groups

Silica gel is universal and is suitable for the separation of the majority of substances; only strongly alkaline substances are irreversibly bound to it by its acid reaction. Under normal conditions, silica gel enables the separation of substances which differ in the presence and number of polar functional groups. Differences in the lipophilic parts of the molecule are less important for the separation.

The synthesis of silica gel allows for the formation of particles with controlled porosity and uniform size. In practice, chromatography employs silica gels with particle sizes under 100 μm, according to the type of application.

5.6.5.3.2 Alumina

Aluminum oxide (alumina) is a sorbent used as an alternative to silica gel in adsorption chromatography. It is a polar sorbent. It is particularly appropriate for the separation of less polar substances which differ sterically or in their functional groups, mainly those that enable the formation of intramolecular hydrogen bonds. The presence of double bonds in the structure of separated substances increases the interaction with this sorbent.

An idealized surface structure can be represented by a layer of aluminum cations and a layer formed of oxygen ions. Under normal conditions, there is sorption-bound water on the surface. When it is heated, this water is partially desorbed and surface hydroxyl groups are formed. However, hydroxyl groups on the surface of the sorbent are not the basis for the adsorption of separated substances. The presence of aluminum and oxygen atoms generates a strong electrostatic field which is the basis for the formation of three main adsorption centers.

A positive field is formed by centers with acid character. Centers of basic type have proton acceptor properties and are formed over oxygen atoms or by ionizable hydroxyls. The third type of centers have electron acceptor properties. The principle of adsorption is therefore different from that for silica gel.

Aluminum oxide can be used in acidic, alkaline, or neutral variants. In a mobile phase containing water, acid substances (phenols, carboxyl groups, or amino acids) are separated on acidic aluminum oxide. Amines and imines separate on alkaline aluminum oxide (alkaloids) and aldehydes, ketones, and lactones separate on neutral aluminum oxide. In an anhydrous environment, separations on basic aluminum oxide are common (alkaloids, carotenoids, aromatic hydrocarbons and steroids). This sorbent is more reactive than silica gel and this might cause problems such as the irreversible sorption of some samples. Al_2O_3 with particles smaller than 200 μm is commonly used.

5.6.5.3.3 Modified silica

The application of chlorodimethyl alkylsilanes, chloroalkoxy silanes, or other reagents on chromatographic silica gel transforms the polar stationary phase into a non-polar one by reacting with the free hydroxyl groups. Current technology ensures the formation of particles with a regular, single-layer lipophilic surface. C-2, C-4, C-6, C-8, or C-18 alkyls are used to prepare non-polar phases, but the spectrum can be broader.

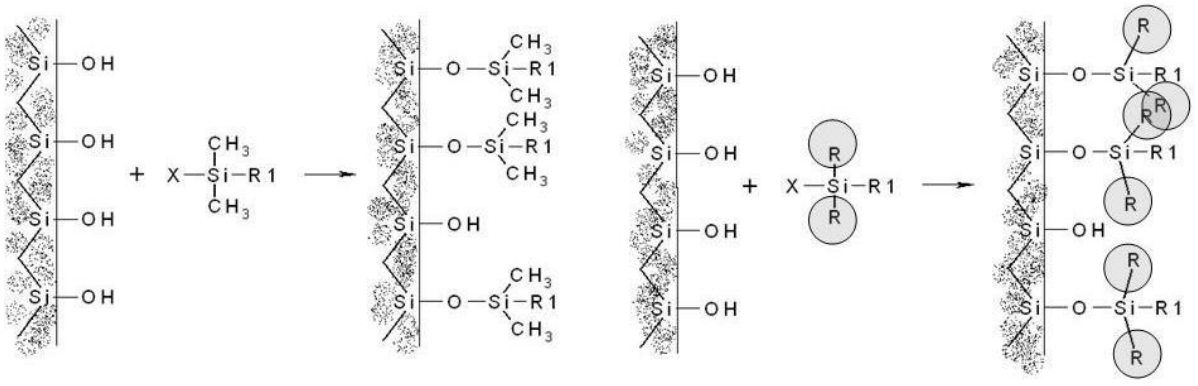

Fig. 21: Schemes for preparing modified silica gels: X – halogen, R1 – alkyl, e.g., C8, C18, aryl, etc., R – a voluminous substituent for the steric hindrance of free hydroxyls

The sorbents most commonly used to separate natural substances are C-8 and C-18 (octyl and octadecyl silica gels). Free hydroxyls of silica gel that have not reacted are subsequently removed by the process of end-capping, i.e., reaction with chloromethyl silanol. If the free hydroxyls on the surface of silica gel are not removed, some of the hydrophilic character of the sorbent remains and can cause complications in the separation, e.g., peak broadening or tailing.

For details of separation by modified silica gels, see Chapter 2.5.7. Modified silica gels have limited use in classical column chromatography. Compared with other sorbents, their limiting factor is a substantially higher price. Their exceptional separation capabilities and capacity for high resolution promote their use in flash chromatography.

5.6.5.3.4 Polyamides

Polyamides (polycaprolactam, polyhexamethylene diaminoadipate, polyamine undecanoate) are sorbents whose separation capabilities are based on the formation of hydrogen bonds. The number and strength of hydrogen bonds depends on the character of the separated substance (phenols, hydroxyls, carbonyls). Elution depends on the ability of the chosen mobile phase to break the hydrogen bridges that have formed. Typical separations are those of phenols, indoles, steroids, and other substances.

5.6.5.3.5 Cellulose

Cellulose is a sorbent used mainly in the chromatography of highly polar substances, such as sugars. In essence, the basics of chromatography on cellulose are the same as those for paper chromatography. The retention of analytes on a sorbent is based on the principle of partition chromatography wherein water anchored on the cellulose surface behaves as a stationary phase. Besides partitioning, adsorption and ion exchange also come through. Thus, the final retention of analytes is a combination of the effects of several factors.

5.6.5.3.6 Sephadex LH-20

Inert polymers formed by polysaccharides are particularly appropriate for the isolation of labile molecules. The polysaccharides can then be interconnected and form a three-dimensional network. Sephadex is a gel produced by the reaction of water-soluble dextran with epichlorohydrin. The water-insoluble polymer formed is networked with ether bridges. This material swells by absorbing a certain amount of water. The swollen particles are able to chromatographically separate different compounds according to their molecular weights. The degree of swelling determines the chromatographic properties of the gel - the thicker the gel, the smaller particles it can separate.

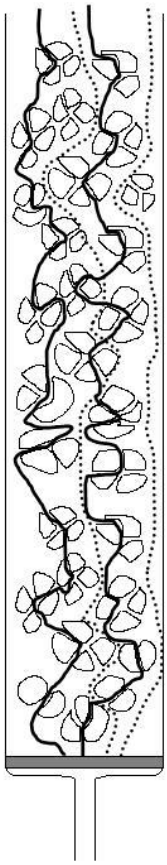

Fig. 22: Scheme of separation by means of so-called exclusion chromatography. Smaller particles (solid line) penetrate into the porous material more easily than bigger particles (dashed line) and are retained by the gel.

Sephadex LH-20 is a hydroxypropyl derivative of Sephadex G-25. The hydroxypropyl derivatization increases its lipophility while retaining its hydrophilic properties. This gel swells in polar solvents (water, methanol, tetrahydrofuran). The ability to interact with organic solvents and its lipophility make this gel advantageous for the separation of natural substances that are soluble in organic solvents. When a single-component mobile phase is used, the substances are separated on the basis of their molecular weights. Particles with molecular weights exceeding 4000 are not caught and elute without any interaction. When a mixture of solvents is used, the more polar one is preferentially absorbed by the gel. This phenomenon causes a two-phase system with different stationary and mobile phases to form and the gel filtration is then supplemented with distribution chromatography, resulting in a so-called mixed-mode separation. The best results are then achieved by optimizing the two-component mobile phase consisting of polar and non-polar solvents. Another unspecified mechanism has been described for the separation of phenolic compounds and heterocycles which are very strongly retained by Sephadex, particularly if the mobile phase is a low molecular weight alcohol.

The dextran gels are generally inert and no irreversible interaction with the separated substances occurs. Another advantage of these gels is that it is possible to reuse them without complicated regeneration.

5.6.6 High-performance liquid chromatography (HPLC)

Compared with classical column chromatography, HPLC is a highly efficient method of separation. This is achieved by using a stationary phase formed of small regular-shaped (spherical) particles (usually ≤ 5 μm) that homogeneously fill the column (see Chapter 5.6.5.3). Under these conditions, the flow of the mobile phase through the column is possible only under high pressures (usually units to tens of MPa) which require the use of high-pressure pumps. Compared with gas chromatography, the advantage of liquid chromatography is the possibility of influencing the separation by changing the composition of the mobile phase. HPLC also enables the use of a number of stationary phases based on various principles of separation (see Chapter 5.6.1), but chemically bound phases (mainly reversed phases, RP-HPLC) are the most significant for phytochemical applications. Thus, the following description will be focused particularly on stationary phases of this type.

The chromatographic columns used in HPLC are usually manufactured commercially to ensure the reproducibility of their production characteristics (size of particles, homogeneous packing of the column, etc.). The columns must resist the high pressures used in HPLC and are therefore made mostly from stainless steel. In addition to columns packed with stationary phase particles, so-called monolithic stationary phases wherein the column is packed with a polymer of defined porosity are sometimes used. Advantages of these stationary phases are that they fill the space in the column more compactly than other stationary phase particles, have high mechanical stability, are resistant to changes in the pH, and are very efficient in separations using high mobile phase flow rates (reducing the time of analysis). Despite the above mentioned advantages, traditional packed columns are still the most commonly used in practice, (though with improvement in the form of core-shell particles). The length and inner diameter of columns used for common analytical applications are 5-25 cm and 2-5 mm, respectively. The size of the column must be adapted to the mobile phase flow rate, which affects the efficiency of the column. Using high flow rates and small columns would result in a disproportionate increase in the system pressure. The flow rate of the mobile phase is usually about 0.2-1.5 $mL \times min^{-1}$. Preparative chromatography uses columns with lengths, inner diameters, and particle sizes in the ranges 25-30 cm, 1-5 cm, and 5-10 μm, respectively. The mobile phase flow rates are also higher, ranging in the interval 5-50 $mL \times min^{-1}$. Reversed phases are among the most frequently used in HPLC because they have a number of advantages over other types of stationary phase. They can separate analytes with a wide range of polarities and a sufficient supply of reversed phases modified to provide the required properties is available. Moreover, they can be used with relatively cheap and accessible solvents as mobile phases. The most common carrier for chemically bound phases (including reversed) is silica gel. The structure of silica gel contains free OH groups (Si-OH) which can be replaced by various functional groups with different properties (Figs. 20 and 21). The most commonly used chemically bound phase is silica gel with octadecyl chains (C18) or shorter alkyls such as octyl (C8) attached. A disadvantage of silica gel is that it is stabile only in a certain limited range of pH (usually 2-8). Another disadvantage is the presence of unreacted OH groups of the silica gel which results in undesirable adsorption. However, end-capped stationary phases in which some part of the free OH groups are deactivated are available.

As previously mentioned, the composition of the mobile phase significantly influences the process of separation. When reversed stationary phases are used, water (mainly a buffer or

a solution of a weak acid with a defined pH) with the addition of an organic solvent (e.g., methanol, acetonitrile, isopropanol, tetrahydrofuran) is used. The composition of the mobile phase can be altered by changing the ratio of the aqueous and organic components, changing the organic solvent used and, in ionizable analytes, also by changing the pH or by ion-pairing reagents. Reversed stationary phases are non-polar and thus the mobile phase used should contain at least 10% of the organic component (a purely aqueous phase does not wet a non-polar stationary phase). A mobile phase is characterized mainly by its eluting power (ε). The eluting power of the mobile phase increases (reducing the retention times) with decreasing polarity of the mobile phase (higher content of the organic constituent). The composition of the mobile phase can be constant for the entire time of the analysis (isocratic elution) or it can be continuously changed (gradient elution). In the course of gradient elution in RP-HPLC, the content of the less polar organic compound is increased at the expense of the more polar aqueous phase. Thus the eluting power of the mobile phase is continuously increased. Generally, gradient elution is used for samples with different affinities for the stationary phase. Figure 23 shows isocratic and gradient separations of the same mixture of substances. In the case of isocratic elution, the first peaks are not separated sufficiently and the last peaks are low and broadened because of long retention times. This suggests that the eluting power of mobile phase is strong for substances with short retention times and weak for substances with long retention times (i.e., strongly bound to the stationary phase). The solution to this problem is to begin the separation with a mobile phase of low eluting power (elution of the substances less bound to the stationary phase) and increase it gradually during the analysis. Generally, gradient elution results in narrowing of the chromatographic peaks, optimizing the selectivity, and reducing the time of analysis.

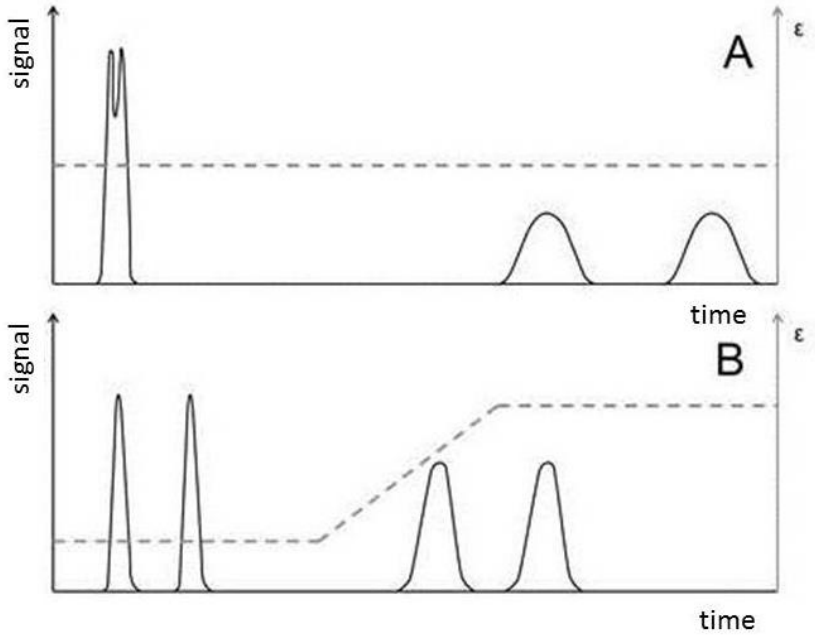

Fig. 23: Comparison of isocratic (A) and gradient (B) separations of a mixture of substances with different polarities; ε – eluting power of the mobile phase

In addition to the chromatography column selected (i.e., the stationary phase, length of the column, and size of the particles), the composition of the mobile phase and its flow rate, the temperature at which a separation is made can also influence the results. In any case, it is appropriate to use a thermostat for chromatographic separations to control the temperature of the column and ensure consistent separation conditions for analyses carried out on different days. A change in temperature does not influence the separation as significantly as in gas chromatography and the effect of increasing or decreasing it on the resolution cannot be determined unambiguously (see Chapter 5.6.3.3). To optimize the conditions of separation, it is appropriate to determine the influence of the temperature in order to perform the separation with best results. The range of temperatures used for chemically bound phases is approximately 10-80 °C.

Due to the high pressures of the mobile phase, the apparatus used for HPLC is much more complex than that for classical column chromatography (Fig. 24). To ensure the flow of mobile phase through the system, high-pressure pumps are used. Big demands are made on these pumps as they must ensure a constant, reproducible, and non-pulsating (use of a damper) flow of the mobile phase. The majority of commercial devices are fitted with a so-called mixing vessel where particular components of the mobile phase are mixed according to

a set program. The mixing vessel is a necessary part of the instrumentation in case we want to use gradient elution. Great demands are also made on these mobile phases, particularly for purity. Specially purified solvents are commercially available for HPLC applications. Air dissolved in the mobile phase can cause bubbles to form, increasing the noise at the detector. It is therefore necessary to degas the mobile phase prior to the analysis. A degassing device can be a direct part of the instrumentation (working on the principle of a vacuum) or the mobile phase can be degassed separately (e.g., by sonication or bubbling helium through it). The selected composition of the mobile phase must be compatible with the type of detector used (e.g., in UV/Vis detection, the components of the mobile phase may not absorb at the wavelengths measured). Other parts of the instrumentation include the sampler (high-pressure valve) that introduces the dissolved sample into the stream of mobile phase. The injection of sample is automated in some devices and takes place according to a set program. The injection of sample must be reproducible to ensure precision sufficient for quantitative analysis. The volumes injected into the system usually range in the interval 0.1-100 µl.

The injected sample is carried by the flow of the mobile phase into the chromatographic column and, subsequently, into the detector.

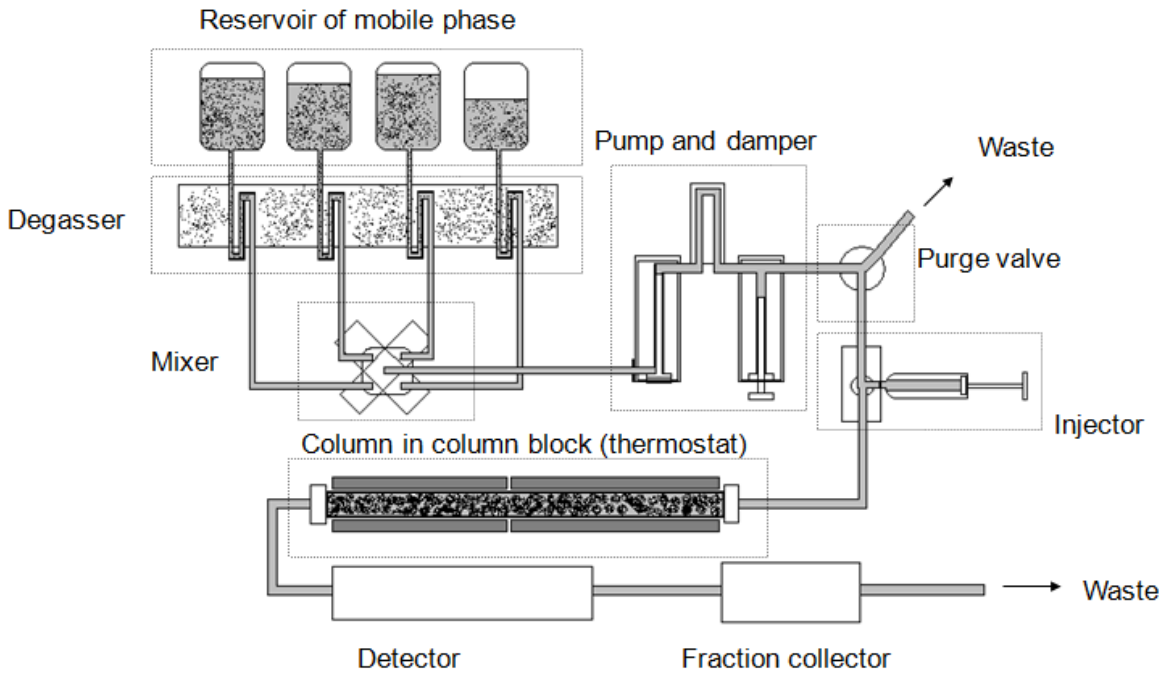

Fig. 24: Scheme of an HPLC device

5.6.6.1 Detection methods for HPLC

Only small amounts of samples are injected in high-performance liquid chromatography and detection therefore requires sensitive detectors which enable continuous observation of the substances emerging at the output of the column.

5.6.6.1.1 Spectrophotometric detectors

Spectrophotometric detectors are among the most common detectors used in HPLC because of their relatively low price and reliability, their ability to detect large numbers of substances (all substances that absorb radiation in the region 200-800 nm), and their compatibility with gradient elution. For the principles of the absorption of ultraviolet and visible radiation, see Chapter 6.2.2. Detectors capable of measuring the absorbance at one or more selected wavelengths or those that can record the entire spectrum in the UV/Vis region (multiple fast scanning of the solution passing through the measurement cell) are used. Recording the entire spectrum (by using a so-called diode array detector – DAD, Fig. 25) provides much more information which can be used to identify substances (by comparing the spectrum with a standard) or to determine the purity of a peak (changes in the spectrum in various parts of the peak suggest a mixture of unresolved analytes).

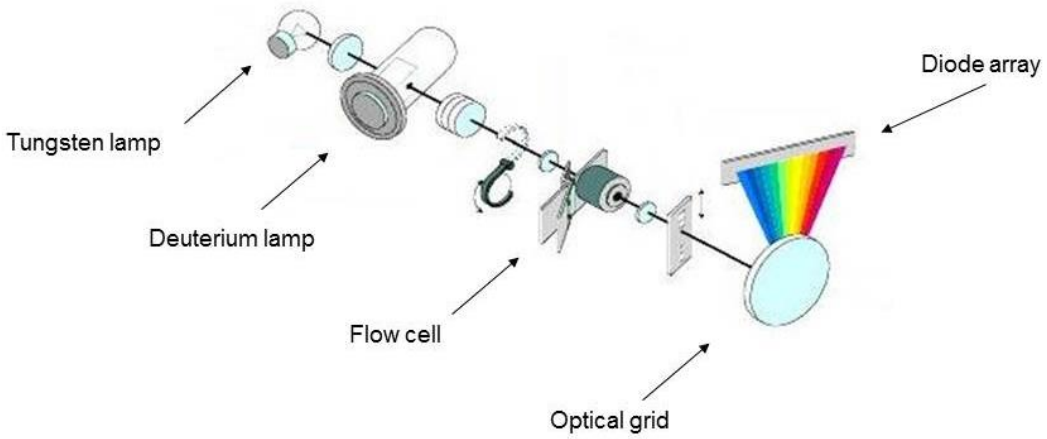

Fig. 25: Scheme of a diode array detector (copied and adapted from materials of HPST with agreement of the company)

5.6.6.1.2 Fluorometric detectors

Fluorometric detectors are selective for substances that exhibit natural fluorescence (or those that can be transformed into a fluorescent derivative by an appropriate chemical reaction). They cannot detect as many substances as spectrophotometric detectors, but the sensitivity of fluorometric detectors is greater by several orders of magnitude (100-1000times more sensitive). This great sensitivity is used mainly in trace analyses; its selectivity is used in the analyses of complicated matrices.

5.6.6.1.3 Electrochemical detectors

Electrochemical detectors measure an electric quantity (electrode potential or current) that is affected by the passage of the substance through a flow cell in the detector that contains electrodes supplied with the working current needed to bring about an electrochemical reaction. Electrochemical detectors can be used to detect substances that participate in an electrochemical reaction on a phase boundary electrode - water (mobile phase). These are selective and sensitive detectors. The most common are amperometric detectors (which measure the current caused by the passage of a reduced or oxidized substance) or coulometric detectors (which measure the charge necessary for the oxidation or reduction of the total amount of substance).

5.6.6.1.4 Refractometric detectors

Refractometric detectors are universally applicable. They measure the refractive index in an analytical cell of the detector. These changes are small and the sensitivity of a refractometric detector is therefore low. Another disadvantage is that they cannot be used with gradient elution. They are used particularly in cases when other detectors do not provide sufficient response (e.g., do not absorb in the UV/Vis region - the analysis of sugars).

5.6.6.1.5 Evaporative light scattering

Evaporative light scattering is based on the nebulization and subsequent evaporation of the mobile phase and the transformation of the detected substances into solid particles. These particles then pass through the beam of a laser in the detector and scatter its radiation. The scattered radiation is measured by means of a suitable photodiode. This detector is very widely applicable, but it cannot detect volatile substances which evaporate with the mobile phase. Another limitation is that it can work only with volatile mobile phases. Advantages compared with the refractometric detector are its greater sensitivity and the fact that it can be used with gradient elution. It is used to analyze substances for which other detectors do not provide sufficient response (e.g., analysis of sugars or fatty acids).

5.6.6.1.6 Mass detectors

A detailed description of the instrumentation and principles of mass spectrometry is presented in Chapter 6.3. These are sensitive detectors with high selectivity, which can detect large numbers of substances. Their disadvantage is their much higher price compared with the other types of detector mentioned. Another limitation of this type of detection is the need to reduce the presence of some buffering additives (e.g., phosphates) in the mobile phase. A basic precondition for detecting analytes by MS is the removal of the mobile phase prior to ionization. Spray ionization techniques of the electrospray type (ESI) are most often used. The ions formed are further analyzed, usually by means of a quadrupole, ion trap, or time-of-flight analyzer (TOF). Modern mass spectrometers are able to determine the molecular weight of the separated substance and carry out further fragmentation of the molecule. The fragment ions that are formed are further detected and the mass spectrum obtained can be used to characterize the structure of the substance that has been separated or isolated.

6 METHODS OF IDENTIFICATION OF PLANT SECONDARY METABOLITES

6.1 Introduction

The task of determining the structure of natural substance is based on knowledge of chemistry and physics and also on studying the literature. The determination of structure includes the number and identity of the individual atoms, their positions in the molecule, and the character of the bonds, their configuration and conformation. Each of these characteristics is determined by a different method; methods that contribute particularly to the identification of natural substances will be described in the next paragraphs. We will not deal with the theory of particular methods in detail, but rather with their use for the identification of natural substances.

6.2 Spectroscopic methods

6.2.1 Infrared spectroscopy (IR)

Infrared spectroscopy is based on the absorption of infrared radiation by a tested substance. For practical reasons, the infrared part of the spectrum is divided into three regions: far-infrared (1000-30 μm), mid-wavelength infrared (30-2.5 μm), and near-infrared (2.5-0.8 μm). The region most frequently used for structural analysis is the mid-wavelength region.

Charge is usually distributed in a molecule in a certain characteristic way. According to which atoms participate in a bond, a positive or negative charge prevails. Asymmetric distribution of the electric charge results in the formation of a dipole moment for the molecule. For a molecule of a substance to be active in the infrared region of absorption, the vibration of its bonds must involve a change in dipole moment. For symmetric molecules (e.g., N_2), no change in dipole moment occurs within the vibration of the bond and thus these molecules do not absorb in the infrared spectrum. In asymmetric molecules (e.g., CO_2), the vibration causes a change in dipole moment which results in absorption. Complex molecules

(such as natural substances) have a lot of bonds and we therefore observe a lot of absorption bands in their infrared spectra.

Molecules of substances absorb electromagnetic radiation at specific frequencies, corresponding to their structure. Under normal conditions, atoms in molecules are bound by bonds and oscillate around their equilibrium position. A part of the infrared radiation absorbed by the molecule corresponds to so-called resonance frequencies, i.e., the frequency of the absorbed radiation corresponds to the frequency of the vibration of the bond between atoms in the molecule being measured. The energy and, therefore, the vibration, depend on the masses of the atoms and the strength of the bond between them. Because the frequency of vibration depends on the type of bonds in the molecule, the wavelengths absorbed will be different for different molecules and will form characteristic vibration bands in the IR spectrum obtained for a particular molecule or functional group.

In addition to the valence vibrations (stretching) that change the length of a bond during vibration, so-called deformation vibrations (bending) that change the angle of the bond are also observed. Valence vibrations are further classified as symmetric or asymmetric. Deformation vibrations are further divided into scissoring, wagging, rocking, and twisting.

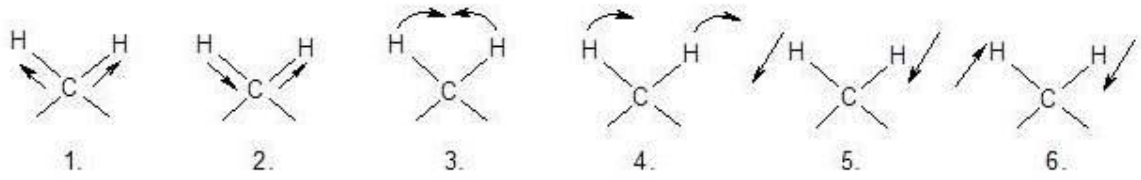

Fig. 26: Types of valence and deformation vibrations of the C-H bond: symmetric stretch (1), asymmetric stretch (2), in-plane scissoring (3), in-plane rocking (4), out-of-plane wagging (5), and out-of-plane twisting (6).

The mid-wavelength region of the infrared spectrum (30-2.5 μm) is used for the structural analysis of natural substances. For practical purposes, spectral bands are described not by their wavelength but rather by their wavenumber, which is the inverse value of the wavelength, with the unit cm^{-1}. In practice, the region 4000-600 cm^{-1} is used for the analysis. The mid-wavelength region can be divided into 3 parts: 4000-1300 cm^{-1} – the region of

characteristic strong vibrations, 1300-600 cm^{-1} – the fingerprint region from which the region 900-600 cm^{-1} – the region in which the benzene nucleus absorbs - is sometimes excluded.

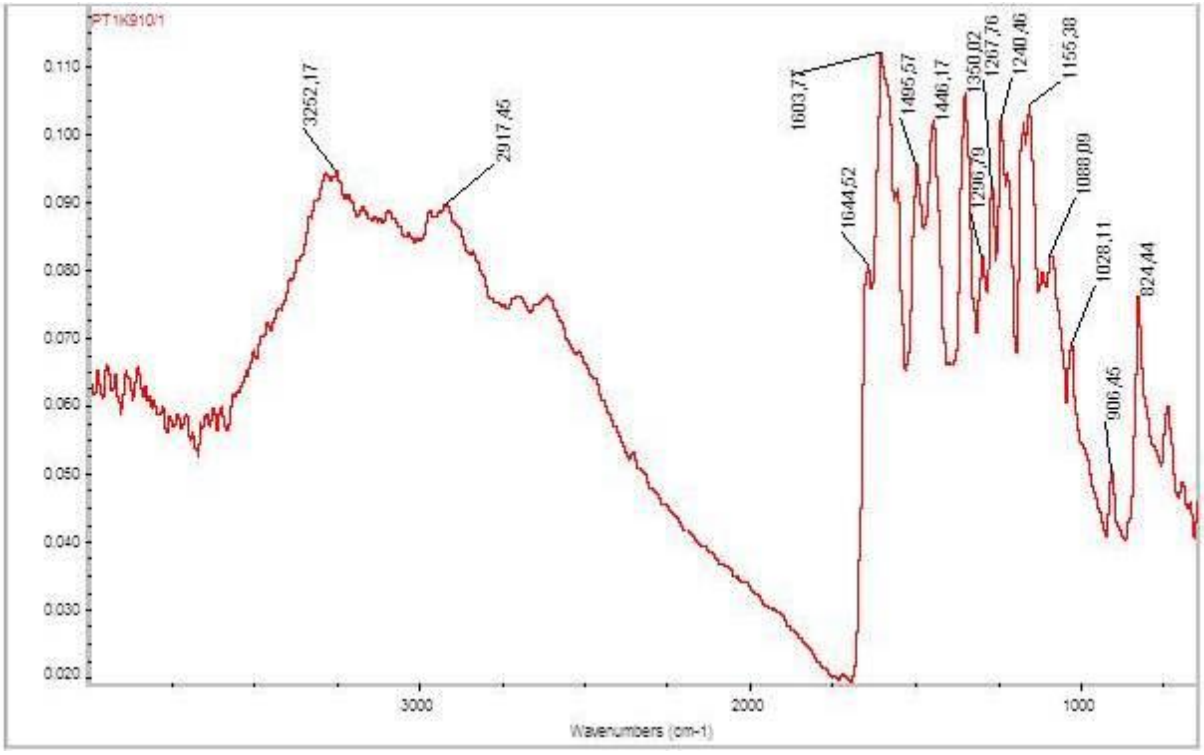

Fig. 27: Example of an infrared spectrum (a geranyl flavanone)

The region of characteristic vibrations typically contains intensive absorption bands with characteristic wavenumbers which can be quite reliably assigned to various functional groups. The functional groups consist of various atoms, usually with different electronegativities, and in the structure of a substance are “isolated from the skeleton” by a carbon-carbon bond. This causes a significant change in the dipole moment of the vibrations and, therefore, intensive absorption in the infrared spectrum. Natural substances usually consist of carbon, hydrogen, oxygen and nitrogen and we can limit description to the most basic examples of these functional groups (Table 4).

Type of bond	Type of compound	Range of characteristic frequencies, type of vibration
C-H	Alkanes	2960-2850 (s) stretching
		1470-1350 (w) scissoring and bending
	Alkenes	3080-3020 (m) stretching
		1000-675 (s) bending
	Aromatic compounds	3000 stretching
		1050-1020 *in-plane* bending
		1040 *out–of-plane* bending
	Aromatic compounds	1600-1400 stretching of the ring
C-O	Alcohols, ethers, carboxylic acids, esters	1260-1000 (s) stretching
C=O	Aldehydes, ketones, carboxylic acids, esters	1760-1670 (s) stretching
O-H	Primary alcohols	3640-3160 (s, br) stretching
	Alcohols and phenols that form a hydrogen bridge	3600-3200 (br) stretching
	Carboxylic acids	3000-2500 (br) stretching
N-H	Primary amines	3500 and 3400 stretching (w)
		1650-1580 bending (m-s)
	Secondary amines	3350-3310 stretching (w)
		1515 bending
		909-666 wagging (liquid samples)
C-N	Primary, secondary, and tertiary amines	1250-1020 stretching
	Aromatic amines	1342-1266 stretching

Table 4: Examples of typical bonds and frequency ranges (s – strong, m – medium, w – weak, br – broad)

The fingerprint region is interesting for comparing substances with spectrum libraries or with other substances. Determining structural characteristics responsible for an absorption band in this region is rather difficult, because of the number of intensive absorption bands and bands with significant overlap. The vibrations of carbon-carbon bonds and other skeletal vibrations appear mainly here. Every substance is characterized by its skeletal vibrations and concordance in this region of the infrared spectra means structural concordance.

The use of infrared spectroscopy as an extension of NMR analysis (see Chapter 6.4) has rather lost its significance in phytochemistry, but IR spectra can be interesting for structural analysis. Identifying characteristic functional groups in the region of characteristic vibrations by IR analysis can facilitate subsequent analyses. An unknown substance can be identified by comparing it with a known substance (standard). Analysis of the spectrum enables determination of the purity and reveals the presence of specific impurities. Observing the intensity of a selected absorption band also enables quantitative analysis, in a mixture or in a matrix. For quantitative analysis, the absorption band of the analyte must exist in a part of the infrared spectrum that is not covered by the absorption bands of other components of the mixture. A prior separation of the mixture is otherwise necessary (often by TLC). Observing changes in the infrared spectrum enables monitoring the course of simple chemical reactions (similar to UV/Vis spectrophotometry).

Spectra for the absorption of infrared light can be measured for gaseous, liquid, or solid samples, depending on the technical arrangement of the measuring device. Measurement in the gaseous state is little used with natural substances (except for online detection in GC), but solid and liquid samples are applicable.

Liquid samples can be used as they are or solid substances put into the form of a solution. Compared with spectra measured in the solid state, the infrared spectra of solutions usually reflect differences caused by the interaction of the substance with the solvent. Measurement can be performed in different solvents, with the precondition that the solvent absorbs in a different region of the IR spectrum than the sample. A short optical trajectory is required for the measurement and the measurement is therefore carried out in cuvettes of thickness 0.1 mm, or the sample is inserted between two silica plates, or the ATR (attenuated total reflectance) technique (see further) is used.

The most common techniques used for measuring samples in the solid state are the KBr pellet and the ATR technique. Measurement using a KBr pellet involves pulverizing the

sample into a fine powder and mixing and homogenizating this with potassium bromide (in approx. 1:200 ratio). After homogenization, the powder is pressed into a pellet and the infrared light that passes through the pellet is measured. In the ATR technique, the sample to be measured (approx. 1 mg) is mechanically pressed onto a crystal which is part of the device (mechanical pressing is not used for liquid samples). Multiple reflection of the radiation between the sample (multiple absorption) and the crystal is used to obtain a spectrum without the need to prepare a KBr pellet.

At present, the device most frequently used for infrared spectroscopy is the so-called Fourier transform infrared spectrometer (FTIR). Infrared radiation of the entire range of wavenumbers to be measured is directed on the sample. An interferometer divides the input radiation into two beams, one of the beams is phase shifted (the beams pass a system of mirrors with each traveling a different distance), and the beams are then united again. The phase shift causes constructive or destructive interference depending on the wavelength. The resultant modulated radiation is transferred to the sample and, subsequently, to a detector. The recorded signal is called an interferogram and represents the intensity of radiation as a function of the difference in path between the beams. A mathematical operation called Fourier transformation then converts these data into an infrared spectrum (absorbance or transmittance, depending on the wavelength).

6.2.2 UV/Vis spectrophotometry

UV/Vis spectrophotometry determines at which wavelengths and to what extent the sample absorbs ultraviolet (~200-400 nm) or visible radiation (~400-800 nm). Measuring the absorption of radiation in the wavelength range from 200 to 800 nm is the most frequently used method in phytochemical analysis (measuring the absorption of UV radiation with wavelengths <190 nm is not performed in practice because oxygen also absorbs at these wavelengths). UV/Vis spectrophotometry is a simple, popular, and cheap method. It is often used for detection, combined on-line with HPLC or capillary electrophoresis. HPLC in particular currently uses DAD detectors (described in Chapter 5.6.6.1.1).

UV/Vis spectrophotometry is based on the absorption of electromagnetic radiation, which causes valence electrons to transition to higher energy levels. These electronic transitions are accompanied by changes in molecular vibration (i.e., by the changes we

observe in infra-red spectroscopy). These absorption bands overlap leading to broad, little characteristic electron bands which are typical for UV/Vis spectra. For phytochemistry, intra-molecular transitions are especially important in UV/Vis spectrophotometry, i.e., bonding electron transitions from σ or π orbitals and non-bonding n electrons moving to anti-bonding σ* or π*orbitals, as are charge-transfer transitions, where an electron from one part of the molecule transfers to another part (the electron transition from the basic bonding π orbital or n non-bonding orbital to the anti-bonding orbital of a π* acceptor).

The absorption is caused by certain structural groupings, called chromophores, which enable these electron transitions. Types of intra-molecular transitions along with examples of chromophores are given in Table 5.

Transition	Area of absorption	Examples of chromophores
σ → σ*	up to 190 nm	Aliphatic compounds
n → σ*	UV radiation	Molecules containing an atom with a free electron pair (for example chloroform)
π → π*	UV/Vis radiation	Unsaturated compounds (an increase in the number of bonds involved in conjugation results in an increase in the wavelength of the absorbed radiation)
n → π*	UV/Vis radiation	Functional groups having an atom with a free electron pair in the double bond (e.g., nitro, keto, azo)

Table 5: Types of intra-molecular transitions

Another functional group involved in UV/Vis absorption is the "auxochromes", which influence the position and intensity of maximum absorption of the chromophores present in the molecule. Depending on the kind of auxochrome, spectra can be modified by the action of a so-called bathochromic shift (red) towards higher wavelength, a hypsochromic shift (blue) towards lower wavelength, a hyperchromic shift that increases the intensity of absorption, or a hypochromic shift that decreases the intensity of absorption. Typical auxochromes include various electron-donor or acceptor groups bonded to an aromatic ring (e.g., substitution by a hydroxyl or amino group). These shifts also occur, e.g., in the reaction of flavonoids with specific agents and they can be used to identify the substitution of flavonoids. Substances of natural character are commonly typified by the presence of systems of conjugated double-bonded chromophores, especially in aromatic compounds. This makes it possible to use the spectrum to determine whether the substance is of aromatic character and other details. However, the band spectrum cannot be interpreted exactly according to the functional groups

and numerous functional groupings within the structure of the substance being analyzed do not manifest unambiguously in the spectrum.

6.2.2.1 Qualitative analysis

This kind of analysis is especially applied in relation to separation methods, where we can compare the UV spectra obtained with those for each individual separated substance or with a library of standards. It is of course possible to use a classical spectrophotometer and perform a qualitative measurement and comparison with a standard in a cell. As mentioned, the structural information obtained from a UV/Vis spectrum is not so important, compared with that from other analytical methods. Analyzing the spectrum, usually gives us only an idea of the electronic status of the molecule and the basic structure of the substance.

6.2.2.2 Quantitative analysis

UV/Vis spectrophotometry can be used for quantitative analysis by the application of the Lambert-Beer Law.

$$A = \varepsilon \times c \times l \qquad (7)$$

A – absorbance

ε – molar absorption coefficient [$dm^3 \times mol^{-1} \times cm^{-1}$]

c – concentration [$mol \times dm^{-3}$]

l – cell pathlength (optical path) [cm]

It follows from this equation that the absorbance of a substance is directly proportionate to the concentration of the substance in the solution used for the measurement. The absorbance is usually measured at the maximum of the absorption band. If we know the absorption coefficient of the substance being analyzed, we can create a calibration curve by measuring a series of solutions of known concentrations of the analyzed substance and read

from it the concentration of the analyzed substance in the unknown solution. UV/Vis quantitative analysis is most frequently used in phytochemistry in connection with separation methods when we analyze the individual separated substances. We can perform such a determination using classical measurement in a cell only if the absorption band we have selected does not overlap with other bands originating from other substances present in the mixture being analyzed. As the samples analyzed in phytochemistry are usually complex mixtures of substances and the direct determination of individual substances is impossible, more or less selective agents which lead to group reactions that form colored products with typical absorption maxima suitable for analysis are sometimes used. Such determinations provide an approximation of the total content of the whole group of compounds which react with the specific agent used. Some examples are the Baljet agent used for cardio active glycosides, the Folin-Ciocalteu agent used for polyphenols, and aluminum chloride used for flavonoids.

6.3 Mass spectrometry (MS)

Mass spectrometry is a physicochemical method for determining the weight of atoms, molecules, and molecular fragments. Mass spectrometers are devices that convert atoms or molecules into ions and further divide these according to the ratio of their mass *m* to charge *z* (*m/z*). The principle of separation relies on the fact that the trajectory of an ion moving through a magnetic and electrostatic field depends on its mass and charge. Suitably interpreted results of the method provide very good information about the structure of the substances being analyzed. Mass spectrometry is probably the most sensitive technique used in the field of analysis and identification of natural substances. This method can provide information about the molecular weight and the major and minor structural fragments of molecules using only sub-microgram quantities of material. It is one of few methods usable for the identification of small structural elements of the molecular ”puzzle“. This so-called sequencing method, i.e. putting smaller structural fragments together, is still used for analyzing peptides, glycosides, oligosaccharides, etc. Fragmentation studies are still important, especially when combined with NMR analysis, and help to complete the picture of the elucidated structure.

It is necessary to emphasize the importance of mass spectrometry at high resolution (HRMS – High Resolution Mass Spectrometry). This method can measure a molecular weight with an accuracy of milli-Da. It enables the determination of the molecular formula and thus replaces elementary analysis, which requires significantly more of the material being analyzed to serve the same purpose (several milligrams).

The development of MS has focused on increasing the sensitivity and resolution and on developing ion sources able to obtain ions from non-volatile compounds and high-molecular weight substances. Mass spectrometers are often used in combination with high-performance separation methods, such as gas or liquid chromatography. A simplified diagram of a device for analyzing by means of mass spectrometry is given in Fig. 28. The following chapters give a brief overview of the ionization techniques and methods of execution of mass analysis.

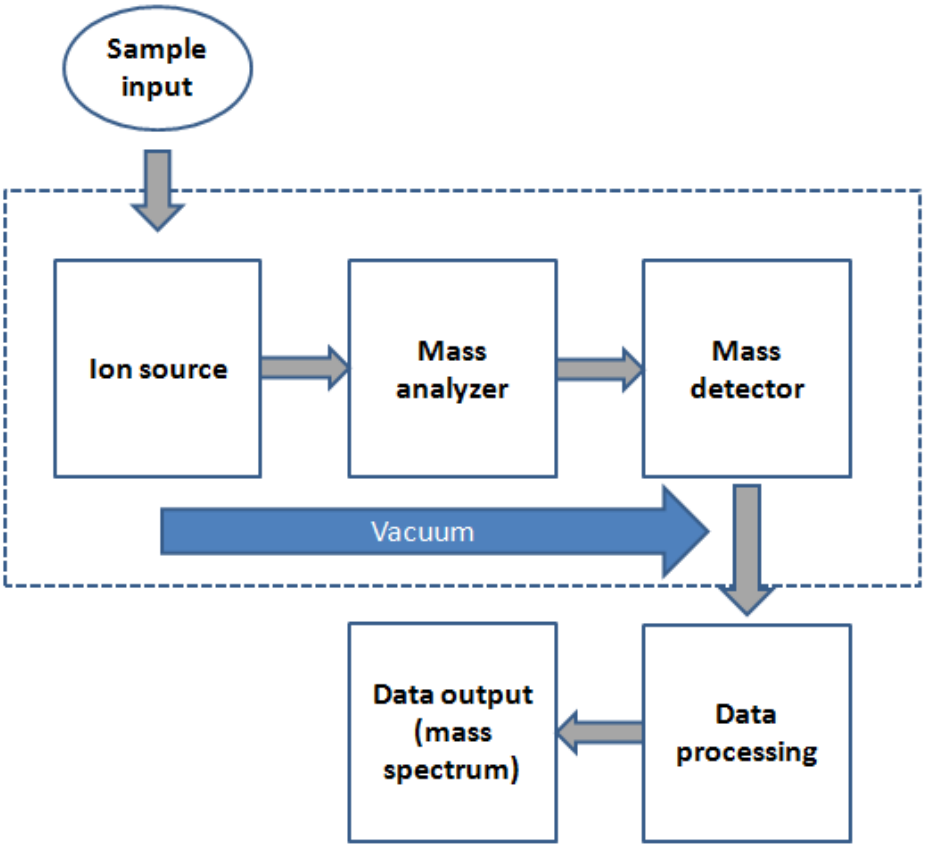

Fig. 28: Schematic diagram of a mass spectrometer

6.3.1 Ion sources

The ion source is a basic part of a mass spectrometer. All the information we obtain about substances derive from particles that have a charge; electro-neutral particles cannot be detected in mass spectrometry. Ionization is an energy-demanding process. The quantity of energy necessary for ionization differs depending on the type and structure of the compound. For common types of compounds, it ranges between 7 and 16 electron volts (*e*V). The quantity of ionization energy that is delivered influences the manner of ionization and thus the applications for which the technique is used. As mentioned before, current ionization sources are able to release ions even from non-volatile materials and high molecular-weight substances, thus extending the applications.

According to the quantity of energy delivered, we distinguish ”soft” and ”hard” ionization techniques. For soft techniques, the energy delivered, and especially its surplus, is low, which means a low probability of fragmenting the primary ion formed. These techniques

offer a high probability of preserving the parent molecular ion and are therefore used to determine the molecular weight. Hard techniques deliver a significant surplus of energy for ionizing (> the bond energy) and thus can decompose the structure into fragments. Another distinction can be made between gas-phase ionization, wherein the substance is evaporated in a vacuum, and condensed-phase ionization, which is suitable for some non-volatile substances. The principle of various types of ionization will be briefly explained below.

6.3.1.1 EI (Electron Impact)

Electron impact ionization is a hard gas-phase ionization technique. It is one of the oldest, and therefore most studied and most frequently used means of ionization in MS. The principle a heated rhenium or tungsten filament provides a flow of electrons, which are accelerated to impact on the substance being analyzed. This impact knocks an electron out of the valence shell of the target molecule M and creates a radical cation $M^{\bullet+}$.

$$M + e^- \rightarrow M^{\bullet+} + 2e^-$$

Disregarding the negligible weight of an electron, the weight of the ion formed is identical with the weight of the parent molecule. The formation of a radical anion (i.e., the capture of an electron) is unlikely and can be considered only in the presence of a highly electro-negative element in the structure of the substance being analyzed. An electric field then directs the flow of ions to the anode and they are pushed from the area of ionization towards an ion analyzer by another electric field (repeller) (see Chapter 6.3.2).

Because of the energies delivered to the particles of the substance (usually 70 *e*V), this type of ionization very often leads to smaller fragments and the molecular peak is often suppressed. The method is therefore used especially in the structural analysis of small molecules, most frequently in connection with gas chromatography.

6.3.1.2 CI (Chemical ionization)

Like EI, chemical ionization is a gas-phase technique, but it is included among the soft ionization techniques. The primary source of energy is again a rapid flow of electrons, but the energy is transferred to the substances being analyzed by means of a reaction medium (methane is most frequently used) which is forced to ionize in an ionization chamber under pressure. Radical cations are formed from the reaction medium and in turn form secondary reaction cations which then react with the substance being analyzed to form quasi-molecular ions $[M+H]^+$. The energy surplus is not highly significant, the substance does not fragment as in EI, the molecular peak is not significantly suppressed, and the method can even be used to determine the molecular weight. Like EI, chemical ionization is used especially in connection with gas chromatography.

Formation of primary ions: $CH_4 + e^- \rightarrow CH_4^{\bullet+} + 2e^-$

Formation of secondary ions: $CH_4 + CH_4^{\bullet+} \rightarrow CH_5^+ + CH_3^{\bullet}$

Formation of ion products: $M + CH_5^+ \rightarrow CH_4 + [M+H]^+$

6.3.1.3 FAB (Fast atom bombardment)

Fast atom bombardment, the ionization of a substance to be analyzed by bombarding it with accelerated atoms of a noble gas (xenon, argon) is a soft condensed-phase ionization technique. The impact of these atoms on the matrix material containing the substance (e.g., glycerol) results in desorption and ionization of the substance. Quasi-molecular ions $[M+H]^+$ are formed. A relatively small amount of fragmentation enables easy reading of the molecular peak and thus determination of the molecular weight of the substance being analyzed. The method is used especially for analyzing non-volatile thermally labile compounds.

6.3.1.4 MALDI (Matrix assisted laser desorption/ionization)

MALDI is one of the most modern ionization techniques. It is a soft condensed-phase technique that uses laser radiation of an appropriate wavelength to desorb a substance from the matrix and ionize it. The presence of the matrix, usually a weak aromatic acid, ensures that large molecules of the analyte are desorbed and ionized without being significantly fragmented. The matrix absorbs the energy of the laser pulse and carefully hands it over to molecules of the analyte with significantly lower inner energy than would be the case for laser ionization without such a matrix. An important advantage of forming only molecular ions is the method that can be applied to the analysis of mixtures of compounds to obtain results that are not overly complicated by the presence of multiple signals. This method is often used with a TOF (Time of Flight) mass analyzer. MALDI is used especially in biochemistry, biotechnology, and phytochemistry to analyze proteins, polymers, such as polysaccharides, and complex natural substances.

6.3.1.5 ESI (Electrospray ionization) and TSI (Thermospray ionization) spray techniques

ESI and TSI are currently widely used, especially in conjunction with liquid chromatography. They are soft liquid-phase ionization techniques. The principle of ionization is the fast spraying and evaporation of the liquid phase (Fig. 29). In TSI, the liquid phase is heated to a temperature of 200-300 °C and spraying occurs as violent boiling. The gradual evaporation of the mist that forms, increases the surface charge until ions dissociate and enter into the gaseous phase. Ions formed in this way are transferred to the mass analyzer by means of a repeller. ESI is probably the most frequently used ion source for the LC-MS combination. The liquid phase is sprayed when it passes through a capillary to which a high voltage is supplied. Very small droplets with a high surface charge density are formed in this process and are quickly dried by a counter-flow of hot inert gas (150-200°C). Dissociation and transition to the gaseous phase occur and the ions that form are subsequently transferred to the mass analyzer. It is possible to work in a positive $[M+H]^+$ or negative $[M-H]^-$ mode, depending on the polarity of the voltage supplied. Electroneutral particles of the solvent are removed by vacuum in both of the spray techniques described.

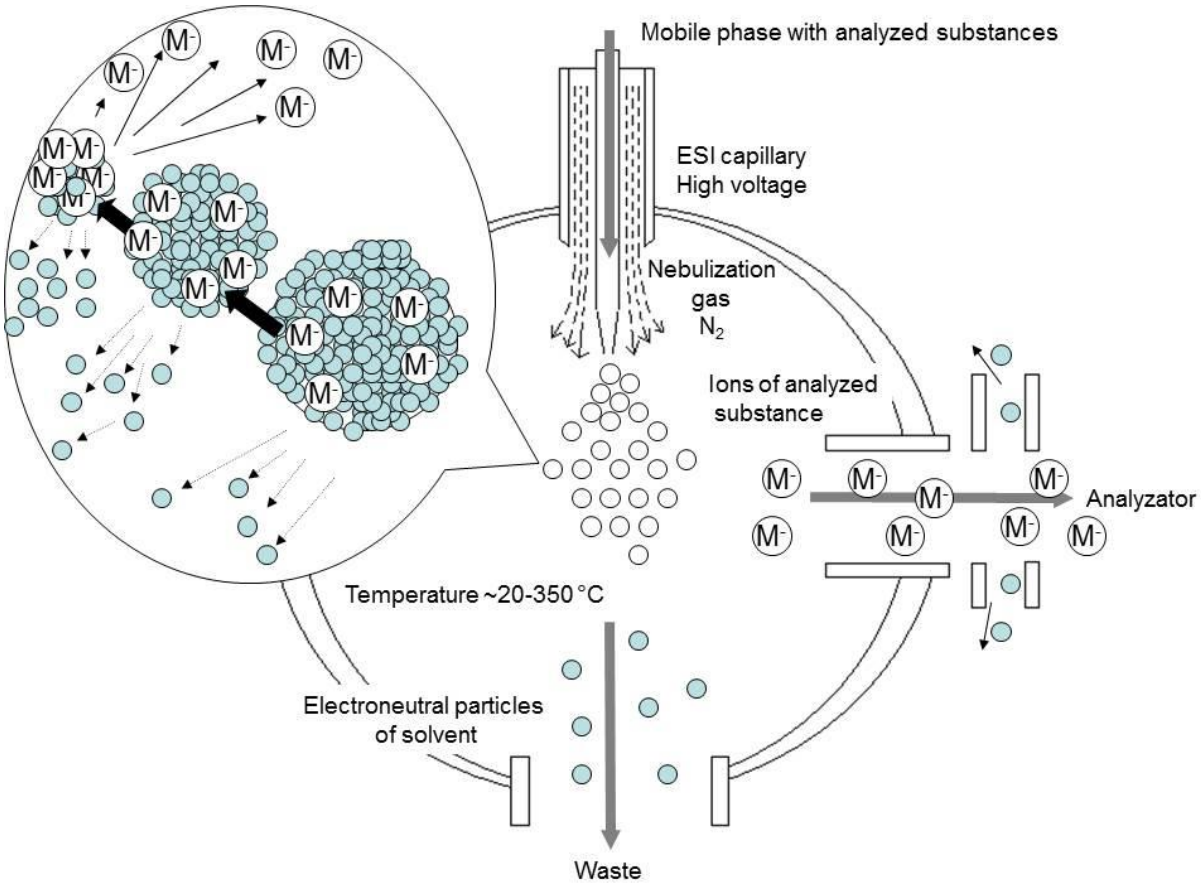

Fig. 29: Schematic diagram of ESI equipment operating in negative mode

6.3.2 Mass analyzers

A mass analyzer forms an integral part of a mass spectrometer. It filters and separates ions according to their ratio of mass to charge *m/z*. If *z* equals one (the most common case), *m/z* expresses the molecular weight of the ion. As far as design is concerned, the separation of ions can be addressed in various ways. In fact, all methods use the movement of ions in an electric and sometimes also a magnetic field. Obtaining quality mass spectra necessitates a high vacuum inside the equipment. Special pumps are used for this purpose.

6.3.2.1 Magnetic mass analyzer (sector electromagnetic analyzer)

The magnetic mass analyzer is one of the oldest types of mass analyzer and was widely used in the past. The design is that of an electric magnet with extended poles between which ions can pass (Fig. 30). After acceleration in an ion source, the ions have kinetic energy E_K

$$E_K = mv^2/2 = zV \qquad (8)$$

where m is the mass of the ion, v is its velocity, z is its charge and V is the accelerating voltage of the ion source. The ions move on a curved trajectory in a homogeneous magnetic field where a radial Lorentz force acts on them

$$F_L = BzV \qquad (9)$$

(B is the magnetic induction) which is balanced by the centrifugal force (r is the radius of the trajectory of the ion).

$$F_O = mv^2/r \qquad (10)$$

It therefore applies that

$$BzV = mv^2/r \qquad (11)$$

By combining and modifying these equations we reach the following relation:

$$m/z = B^2r^2/2V \qquad (12)$$

It follows from equation 12 that ions with different ratios of m/z describe trajectories with different radii and for this reason ions can be sorted by magnetic field scanning. To improve the resolution, ions are repeatedly separated by some focusing in the sector influenced by the magnetic field and they then fly on to the detector.

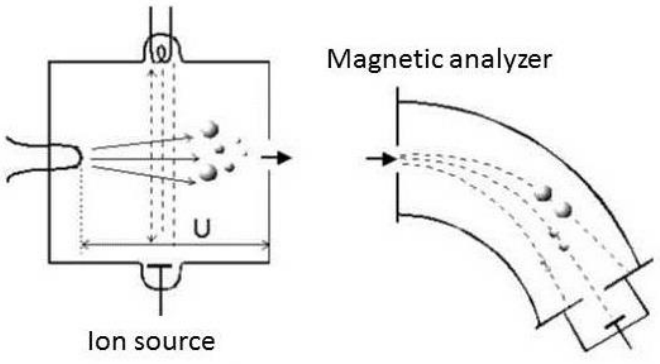

Fig. 30: Schematic diagram of a magnetic analyzer

This type of detection is in decline due to the wider use of TOF, but it is still commonly used in combination with GC, EI, or CI ionization.

6.3.2.2 Quadrupole mass analyzer

The quadrupole mass analyzer is one of the most widespread types of mass analyzer. It is used in practice independently or connected in series with several other quadrupole devices (Triple-Q) or with a different type of mass analyzer (ion trap, TOF). A quadrupole filter is usually a part of low resolution mass spectrometers suitable for combination with gas chromatography and HPLC. It is called quadrupole according to its structural layout. It consists of four metal rods of hyperbolic or circular cross-section connected to sources of both direct and alternating voltages. After ionization, ions fly into the area among the rods, enter the alternating electrical field, and start oscillating. The ”filtration of ions“ is based on changes in the direct and alternating voltages because ions that have a certain *m/z* pass through only at specific values (Fig. 31). By changing the voltage, it is possible to select ions with a certain *m/z* while the others are trapped by the rods. Typically, several quadrupoles are combined, or a so-called octopole is used.

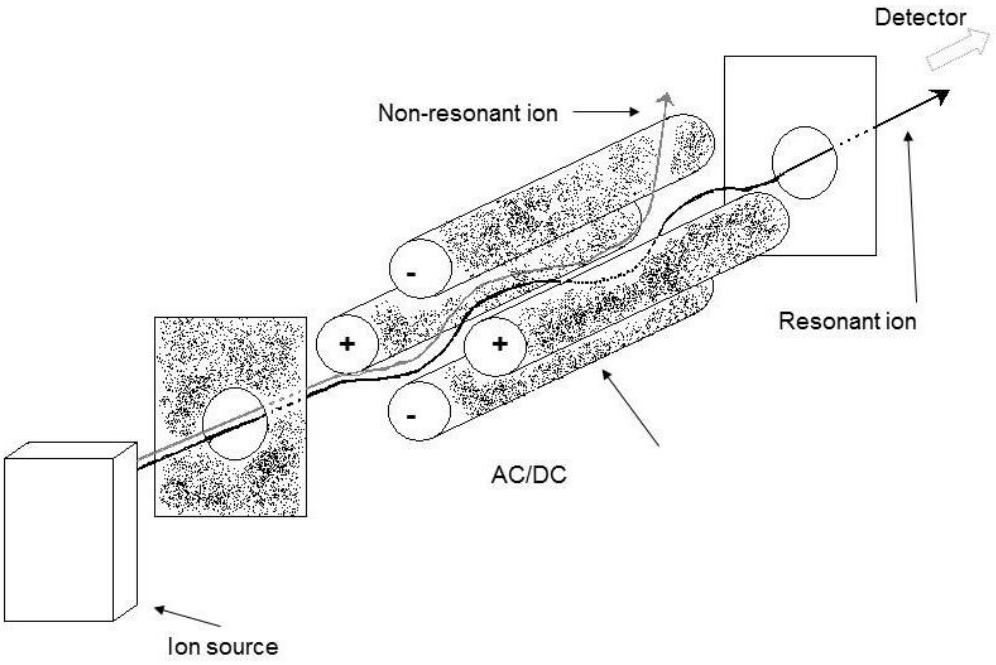

Fig. 31: Schematic diagram of a quadrupole mass analyzer

6.3.2.3 Ion Trap

An ion trap is a device which uses the effect of an electromagnetic field to enclose ions in a confined space (Fig. 32). There are several types of ion traps. Mass spectrometry uses especially the Penning trap. The ion trap consists of an input and output electrode and a central electrode in the form of a ring. Ions get into the trap through the input electrode and are forced by the set electromagnetic field, to circle inside the trap in closed trajectories with minimum collisions. Changing the amplitude of the voltage supplied to electrodes, changes the trajectories of the ions and they can be ejected towards the detector through the output. The device enables accumulating ions in the trap up to a certain number or within a certain time, which increases the likelihood of them detecting and thus the sensitivity of the device. The ion trap is especially used in combination with LC-MS.

Other types of trap are represented by the Orbitrap system or linear trap.

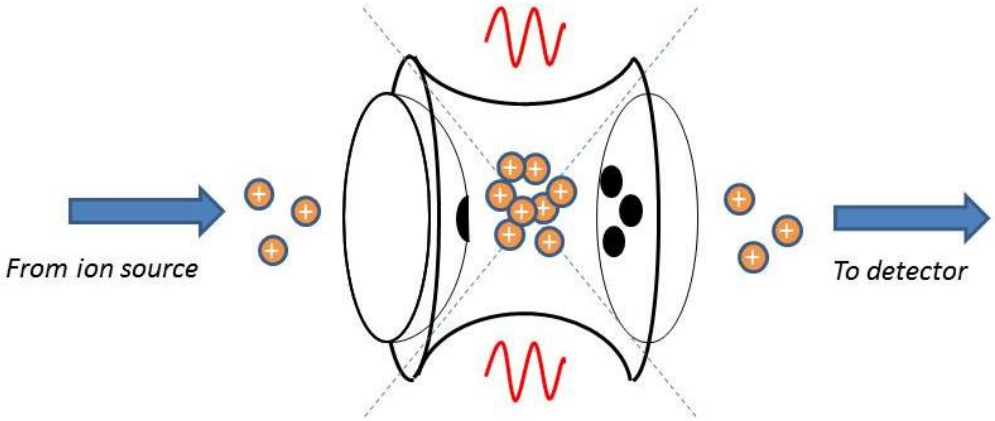

Fig. 32: Schematic diagram of an ion trap

6.3.2.4 Time of Flight Analyzer (TOF)

One of the simplest and most precise mass analyzers is the TOF. In principle, it consists of only an evacuated tube into which ions that are fed from an ion source are accelerated by a repeller (Fig. 33). The velocity of each ion depends on its *m/z* ratio. The time required by the particle to traverse the tube and reach the detector is measured. Heavier particles have a lower velocity and thus require a longer time, which can be measured with high precision. We can calculate the value of *m/z* from these data using a computer. Modern TOF is used especially in combination with ESI or MALDI ionization and for high resolution measurements, i.e., to determine molecular formulas.

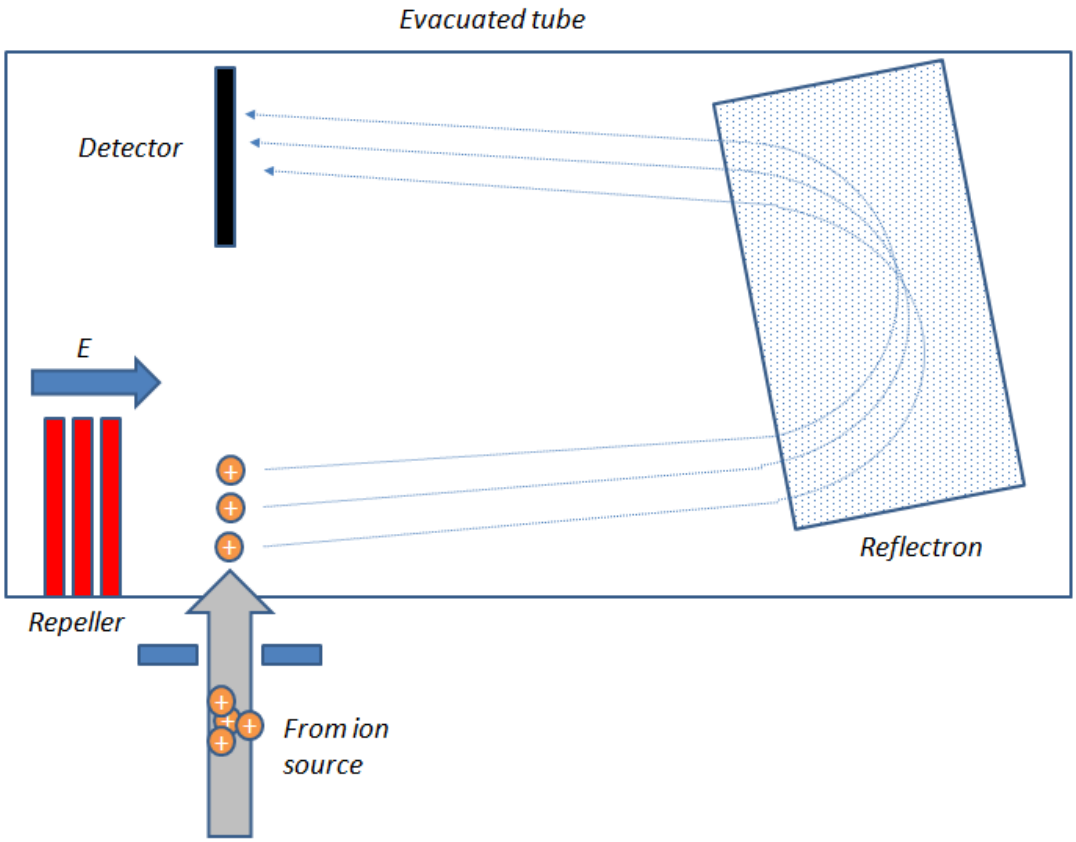

Fig. 33: Schematic diagram of TOF analyzer

6.3.3 Detectors used in MS

The ions separated in a mass analyzer must then be detected and counted. There are two types of detectors: **detectors for direct measurement** which detect the electrical flow caused by the direct impact of the ion being determined and **multiplying detectors** which use the effect of the multiple electrons released from a conversion dynode upon the impact of an ion. The electric current generated is then used to show the intensity of the signal for that particular value of *m/z*. Multiplying detectors are the most frequently used type of detectors in MS.

6.3.4 Use of mass spectrometry

The output of a mass spectrometry is a mass spectrum which represents the dependence of the response of the detector on *m/z*. In a standardized form, the most intensive

peak is given a response value of 100% and all other responses are expressed as proportionate values. In the spectrum, we can observe the molecular peak of the analyzed substance and its potential adducts with solvent or sodium ions, along with fragments of the substance, depending on the method used. We can observe only those fragments which preserve the charged character of an ion, i.e., neutral losses cannot be seen.

Our example is the analysis of the mass spectrum of a flavonoid glycoside. The test compound was isolated from *Pulicaria crispa* by means of preparative HPLC on a reversed phase column (Fig. 34). The substance was analyzed by means of spectrophotometry in the ultraviolet region and a match with the spectrum library confirmed the character of a flavonoid. The substance was then further analyzed by means of LC-MS ESI in the negative mode (suitable for phenolic compounds of acidic character) to obtain more data. The parent ion *m/z* $[M-H]^-$ at 563 was identified in the mass spectrum first and from it a molecular weight of 564 was determined for the compound. This is quite a high weight for a flavonoid. Combining it with the retention time obtained from HPLC leads to the deduction that the substance has glycosidic character. *O*-glycosides (sugar unit bound to oxygen) are relatively labile compounds and they often split under the ESI conditions used in the experiment. In the mass spectrum, it is possible to calculate losses which correspond to the split-off of the whole hexose or pentose molecule and the typical fragments corresponding to the ions of aglycones *m/z* $[M-H-180]^-$ or m/z $[M-H-150]^-$ are then found. No fragments typical for *O*-glycosides were present in the mass spectrum. MS/MS was used for further work. In this technique, the ion trap (in our case *m/z* $[M-H]^-$ 563) is used to select a mother ion, which is then accumulated and subsequently, after it has been supplied with a certain amount of energy, it splits into daughter fragments derived from certain functional groups present in the structure of the analyzed substance. We first analyzed the fragment of *m/z* $[M-H-18]^-$ 545. The loss of 18 units typically corresponds to the split-off of a hydroxyl group. Unfortunately, the origin of this hydroxyl and the position of the hydroxyl substitution cannot be easily identified. We also analyzed fragments of *m/z* $[M-H-90]^-$ 473 and *m/z* $[M-H-120]^-$ 443. The *m/z* 473 fragment corresponds to the split of *C*-glycosidic-bound hexopyranose, the *m/z* 443 fragment to the split of pentofuranose. The *m/z* $[M-H-60]^-$ 503 fragment also corresponds to the split of a sugar unit. It is impossible to identify the position of the split here because both hexopyranose and pentofuranose could possibly fragment. The presence of such fragments is thus typical for *C*-glycosides. Whole sugars do not split off from *C*-glycosides, but fragmentation of the sugar unit in them often occurs due to the stability of the C-C bond

between the sugar and the aglycone. We could derive the following conclusions from the combined analysis of the UV/Vis spectrum and MS/MS: the compound isolated as an aglycone is a flavone with three hydroxyl groups, probably apigenin. We searched the literature, identified MS/MS fragments typical for C-6 and C-8 diglycosides, and identified the substance as probably apigenin 6-*C*-β-D-glucopyranosyl-8-*C*-β-D-apiofuranoside. These conclusions were subsequently confirmed by ^{1}H and ^{13}C NMR analysis. As seen from the previous explanation, MS analysis can usually provide data on the molecular weight of the substance being analyzed along with information about any functional groups. Determination of the complete structure required the addition of ^{1}H and ^{13}C NMR analyses, which confirmed our assumptions.

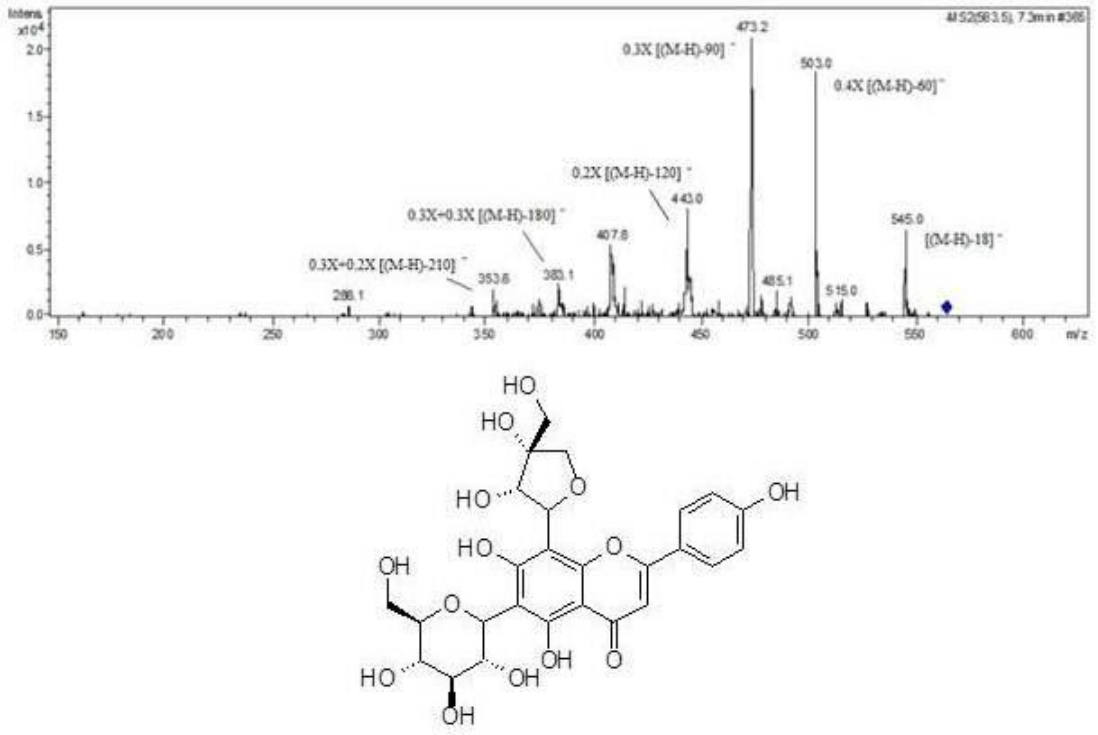

Fig. 34: Example of an ESI MS/MS mass spectrum in the negative mode with the structural formula of the substance being analyzed

6.4 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance (NMR) is one of the main techniques used to analyze the structures of natural substances, in particular of secondary metabolites. Thanks to the significant development of NMR technology brought about by the introduction of FT-NMR high-resolution spectrometers, the role played by NMR spectroscopy has become highly significant in identifying structures of natural substances. However, massive spreading of NMR spectroscopy has been limited by the high costs of acquisition for NMR spectrometers, on the order of tens of millions of Czech crowns (hundreds of thousands of euro or dollars).

6.4.1 Principle of NMR spectroscopy

A basic principle of nuclear magnetic resonance is the interaction of atomic nuclei situated in a strong magnetic field with radio-frequency radiation (RF). Only nuclei which have a non-zero value of the spin quantum number *I*, i.e., they can manifest a magnetic moment (behaving as miniature magnets) interact. For natural substances, NMR is associated mainly with protons ^{1}H and the ^{13}C carbon isotope (I = 1/2), and to a lesser extent, with the nitrogen isotope ^{15}N (I = 1/2). ^{15}N has been preferred recently despite its low natural abundance (0.37%) because the isotope ^{14}N (99.63%; I = 1) that was previously used provided very broad NMR signals and could not resolve non-equivalent nitrogen atoms in a molecule. Oxygen NMR spectra can be measured only to a limited extent (low molecular weight compounds). However, the naturally occurring isotope ^{16}O (I = 0) is invisible in NMR and only the magnetically active isotope ^{17}O (I = 5/2) can be measured. It has a very low natural abundance (0.037%), which makes detection difficult, with broad signals and low sensitivity.

In the absence of an external magnetic field, the magnetic moments of nuclei with non-zero spin are randomly oriented and without differences in energy. When these nuclei are inserted into a magnetic field, the energy levels of their nuclear spins split apart. The number of energy levels formed is determined by the spin quantum number *I* (number of energy states = $2I$+1), e.g., the nuclear spins of isotopes with I = ½ (^{1}H, ^{13}C) will take one of two energy levels – the α-spin state (the magnetic moment of the nucleus is identical with the orientation of the external magnetic field, characterized by magnetic quantum number m = +½) or the β-spin state (the magnetic moment of the nucleus is directed against the external magnetic field,

characterized by the magnetic quantum number m = -½). The energy difference between these two states is very small and therefore there is always only a small surplus of nuclei (< 10^{-5}) in the lower energy α-spin state.

Nuclei can be excited to the higher energy state (β-spin state) by the absorbing electromagnetic radiation supplied at the so-called resonance frequency (the energy of the radiation equals the energy difference between the α- and β-spin states). The populations of these states balance when such RF radiation is absorbed. The frequency of the electromagnetic radiation necessary for resonance depends on the strength of the induced magnetic field and the kind of nucleus being observed. The greater the intensity of the external magnetic field, the greater the energy difference between α- and β-spin states and also the more nuclei are in the lower-energy α-spin state. More RF energy is absorbed and the sensitivity and resolution of measurements with the device are increased (see Fig. 35).

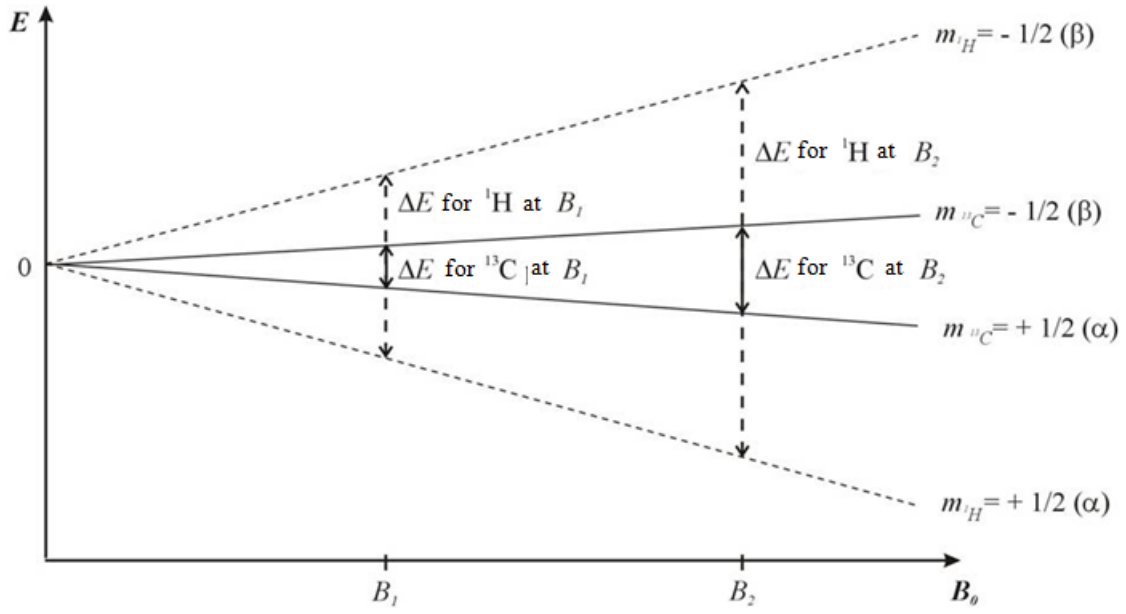

Fig. 35: The energy difference between α- and β- nuclear spin states for ^{1}H and ^{13}C nuclei depending on the magnetic induction B_0. (^{1}H nuclei have a higher resonance frequency and are more sensitive than ^{13}C nuclei in a magnetic field of the same strength.)

6.4.2 Chemical shift

According to the aforementioned facts, the resonance frequency in a constant inducted magnetic field should be identical for all nuclei of the same isotope. However, we do not measure the atomic nuclei themselves. The electrons around the nuclei also circulate around other nuclei in the external magnetic field, which then form their own induced magnetic fields. If this field is oriented against the direction of the external field, shielding - a slight weakening of the external field - occurs. In contrast, if it is aligned in the same direction, the external field is strengthened slightly - deshielding. The resonance frequency of a specific nucleus therefore depends on the electron configuration in its surroundings and differently shielded nuclei (so-called chemically non-equivalent nuclei) resonate at different frequencies. The absolute values of these resonance frequencies are on the order of MHz but the difference (shift) in resonance frequency caused by shielding is on the order of Hz. To state absolute values of resonance frequency would be highly impractical and the chemical shift of a specific nuclei is therefore expressed as the difference between the absolute value of the resonance frequency of the nucleus and the absolute value of the resonance frequency of a standard. The chemical shift obtained in Hz depends on the operating frequency of the spectrometer. To be able to compare data measured with different spectrometers, the δ chemical shift scale with dimensionless ppm units (parts per million of the operating frequency of the spectrometer) was created:

$$\delta = \frac{\nu - \nu_{st}}{\nu_0} \times 10^6 \qquad (13)$$

ν = resonance frequency of the measured nucleus

ν_{st} = resonance frequency of the standard

ν_0 = operating frequency of the spectrometer

For ^1H and ^{13}C NMR spectroscopy, tetramethylsilane (TMS; $(CH_3)_4Si$) was selected as the standard and its chemical shift δ equals 0 ppm according to the aforementioned relationship. TMS was selected because of its spectral and physical-chemical properties. It gives a single highly intensive signal in the ^1H NMR spectrum (it has 12 equivalent

hydrogens) and can therefore be added to a sample in very small quantities. Due to the high shielding of the protons (the bond of methyl groups to the electropositive silicon atom), this signal is shifted to an area of the magnetic field with minimum resonance for other nuclei. It is also chemically non-reactive and boils at 26.5°C, allowing it to be easily removed from a sample.

6.4.3 NMR instrumentation and measuring procedure

The original CW (continuous wave)-NMR spectrometers consisted of a permanent magnet or an electromagnet with an iron core, an RF-transmitter, a receiver registering the energy absorbed by the sample, and a recorder. The sample was placed between the poles of a magnet with the static magnetic induction B_0 and radiated with RF-radiation of continuously changing frequency (or the intensity of magnetic field was continuously changed under the action of RF-radiation with constant frequency). The individual resonance frequencies of chemically equivalent nuclei thus were found continuously and the resulting spectrum was a record of the intensity of absorption depending on the frequency.

Modern pulse NMR spectrometers with Fourier transformation (FT-NMR) are highly complicated devices. The FT-NMR spectrometer is based on a superconducting magnet (Fig. 36) formed by a solenoid coil made of superconducting material (most frequently an alloy of Nb_3Ti or Nb_3Sn). Superconductivity is achieved only at very low temperatures and therefore the coil is situated in a cryostat filled with liquid helium. The external casing of the cryostat is filled with liquid nitrogen to reduce the evaporation of helium. The cryostat is egg shaped and there is a measuring probe in the channel along the axis of the magnet into which a glass sample tube containing a solution of the substance to be measured is inserted. The main part of the probe is the electrical circuitry that serves to supply energy to the sample in the form of RF-pulses and to detect the frequencies emitted by the sample at relaxation. The probe and the magnet are connected with a console containing various electrical circuits (transmitting and receiving circuits, converters, sources for the supply of correction coils, modulators, RF pulse amplifiers, etc.). The console of the spectrometer is connected with and controlled by a central computer. In FT-NMR spectrometry the test sample is irradiated with a short high-frequency pulse containing the whole range of resonance frequencies. All nuclei of the observed isotope are then excited at the same time. The return of nuclei to equilibrium can then be observed as

so-called FID (free induction decay), which is a record of the dependence of the signal intensity on time. FID is saved in the computer and subsequently converted into an NMR spectrum - the dependence of signal intensity on frequency - using a mathematical operation called Fourier Transformation. An advantage of this method is the possibility of rapidly repeating individual measurements and averaging the resulting series of FID signals, thereby eliminating noise and increasing the sensitivity of the measurement. FT-NMR spectrometers have higher resolution thanks to several times stronger magnetic fields. The operating frequency of the spectrometer is usually stated as the resonance frequency of the ^{1}H nucleus. While previous CW-spectrometers could achieve only relatively low operating frequencies (30-100 MHz), FT-NMR spectrometers operate at 200-900 MHz. In 2009, Bruker Inc. introduced the first 1GHz NMR spectrometer.

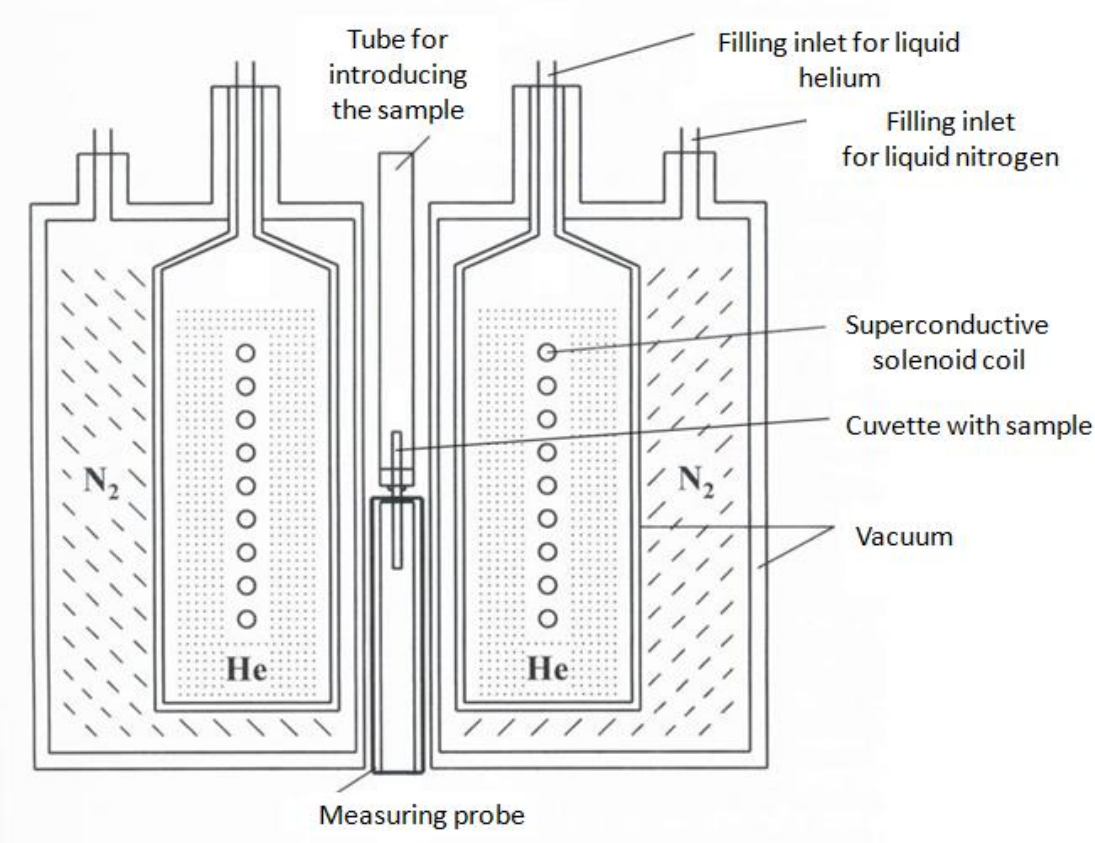

Fig. 36: Schematic cross-section through a superconducting magnet, (taken from [Friebolin, H.: Basic One- and Two-Dimensional NMR Spectroscopy. Wiley-VCH, 1998.] and modified)

Before measurement, the sample (on the order of a milligram for ^{1}H-NMR or tens of milligrams for ^{13}C-NMR) is dissolved in a deuterated solvent (e.g., $CDCl_3$, CD_3OD, DMSO-

d_6, D_2O). Solvents containing hydrogen atoms would interfere with measurement during the NMR analysis (the signal from ^1H of the solvent would be much more intense in the ^1H-NMR spectrum and could overlap or "swamp" the signals of the test substance). Deuterium also serves for tuning the spectrometer. The sample solution (0.5-0.7 mL) is filtered, if necessary, and transferred to a special tube made of borosilicate glass (a thin-wall tube with a diameter of 5 or 10 mm and a length of approximately 20 cm). This sample tube is inserted into a rotary adapter which is put into the measuring probe of the magnet on a cushion of compressed air. To increase the homogeneity of the magnetic field, the tube is rotated in the device. The processes of tuning the device - finding the resonance condition (locking) and optimizing the homogeneity of the magnetic field (shimming) by changing the value of the magnetic field B_0 using correction coils follow. During the measurement, the sample is irradiated with an electromagnetic pulse or pulses according to the type of experiment selected and the absorbed energy re-emitted by the sample is recorded. The measuring process is repeated many times (for ^{13}C-NMR spectra even up to 10000 times). The FID signals obtained are then converted into a classical NMR spectrum using Fourier transformation. The final step is interpreting the spectrum obtained.

6.4.4 One-dimensional ^1H NMR

One-dimensional ^1H NMR provides very fast information about the chemical nature and purity of the test substance. From the hydrogen NMR spectrum we can read how many kinds of chemically non-equivalent hydrogen atoms the molecule contains and, according to the positions and shapes of the peaks in the spectrum, we can distinguish the chemical nature of these protons and their immediate surroundings. Using the relative intensities of the peaks we can determine the numbers of equivalent protons.

The chemical shift δ of protons in ^1H NMR spectroscopy usually ranges from 0 to 13 ppm. Table 6 gives the chemical shifts of protons according to which particular structural grouping the signal can be attributed. Chemical shifts depend especially on the surrounding electron density. More shielded nuclei can have lower values of δ, the chemical shift (e.g., alkanes). Increasing the electronegativity of the neighboring atom or group causes a decrease in shielding and increases the value of the chemical shift and the presence of a multiple bond also causes a shift to higher δ values. The highest values of chemical shift are achieved by the

least shielded protons (e.g., the proton of an aldehyde group). The chemical shifts of protons bound to heteroatoms (NH, OH, SH) depend strongly on the solvent used, the temperature, and the concentration of the sample. Unlike protons bound to carbon, these can be substituted. Hydrogen is displaced by deuterium in the presence of a suitable solvent (e.g., D_2O, CD_3OD) and the respective signal in the spectrum grows weaker and can disappear completely.

Chemical shift δ [ppm]	**Proton type**	**Specific examples (δ [ppm])**
0.8–1.7	protons in a saturated hydrocarbon chain	primary alkyls (R-CH_3; 0.8–1.1) secondary alkyls (R-CH_2-R; 1.2–1.4) tertiary alkyls (R_3CH; 1.4–1.7)
1.5–3.0	protons on a carbon atom next to the double bond	allyls (R_2C=CR-CH_3; 1.5–2.2) ketones (RC=OCH_3; 2.1–2.6) benzyls ($C_6H_5CH_2R$; 2.2–3.0)
2.5–5.0	protons on a carbon atom bound to electronegative atom(s)	alcohols, ethers (RCH_2-O; 2.5–5.0) alkylamines (RCH_2-N; 2.5–3.5) alkyl halides (RCH_2-X; 2.5(I)–4.5(F))
4.5–6.5	protons on a carbon atom involved in a double bond	vinyl groups (R_2C=CH_2; 4.5–5.0) (R_2C=CH-R; 5.2–5.7)
6.5-8	aromatic and heteroaromatic protons	benzene (C_6H_6; 7.26) pyridine (C_6H_5N; 7.2–8.6)
9.5-13	protons in strongly electron-accepting groups	aldehydes (RCH=O; 9.5–10.5) carboxylic acids (-COOH; 11.0–13)

Table 6: Approximate ranges of the chemical shifts of selected protons in organic compounds

Chemically equivalent hydrogen atoms (homotopic hydrogen atoms) provide the same signal in the NMR spectrum. These are hydrogen atoms bound in the given molecule in the same way, i.e., they have the same electron surroundings and resonate at the same frequency. Chemically non-equivalent hydrogen atoms (heterotopic hydrogen atoms) differ in their electron surroundings and thus provide different signals in the NMR spectrum. A substitution operation can serve to assess the equivalence of individual protons in a molecule. We can gradually reduce each hydrogen in a molecule (always only a single one) with a hypothetical substituent and compare the structures formed. If identical compounds are formed, the

hydrogen atoms are equivalent; if a new compound is formed, the hydrogen atoms are non-equivalent and they will have different signals in the NMR spectrum. For example, if any hydrogen in a molecule of ethane is replaced, only a 1-substituted ethane will always be formed and there will be a single signal for ethane in the spectrum. In the case of propane, a 1- or 2-substituted propane will be formed and there will be two different signals in the spectrum, one for the methylene group and the other for the two methyl groups (Fig. 37). Another possibility for assessing the equivalence of individual protons is the introduction of a symmetry operation (rotation, mirroring). Atoms which are mutually related by the operation of symmetry are equivalent. For example, with regard to the symmetry of the molecule, all hydrogen atoms in *p*-dichlorobenzene are homotopic, but *o*-dichlorobenzene already contains two types of hydrogen atoms and *m*-dichlorobenzene contains three types.

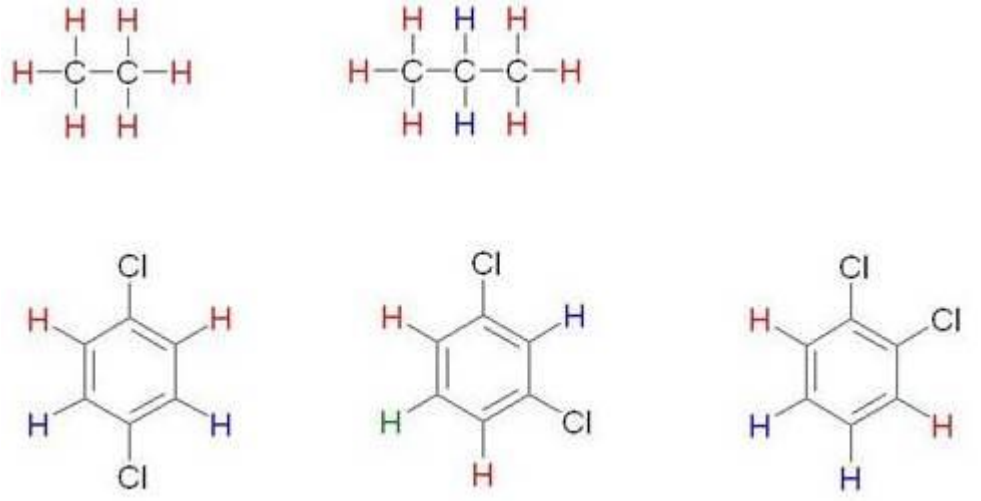

Fig. 37: Examples of compounds with equivalent atoms of hydrogen marked (atoms of hydrogen of the same color are homotopic in the given compound)

The intensities of the individual signals in an ^{1}H-NMR spectrum correspond to the number of equivalent hydrogen atoms involved in the given signal. The area under the signal is therefore directly proportionate to the number of protons. Quantitatively, this is determined by integration. In the case of propane, the mutual ratio of the areas of the two signals will be 6:2 (or 3:1).

Signals in the ^{1}H-NMR spectrum are not usually formed of only a simple peak, rather they are split into more lines, forming so-called multiplets. This split is caused by spin-spin interaction (spin-spin coupling) with the neighboring non-equivalent nuclei (usually the

maximum distance for splitting is three bonds). For magnetically active nuclei in general, the peak for the observed nucleus (or equivalent nuclei) neighboring an n other mutually equivalent nuclei with the spin quantum number I, is split into $2nI+1$ lines, specifically for hydrogen atoms ($I = ½$) and there are then $n+1$ lines. The intensity of the lines in the multiplet corresponds to the possible combinations of the spins of the neighboring nuclei (see Table 7).

Number of neighboring mutually equivalent protons	**Type of multiplet of the observed proton or equivalent protons (symbol)**	**Ratio of intensities**
0	singlet (s)	1
1	doublet (d)	1:1
2	triplet (t)	1:2:1
3	quartet (q)	1:3:3:1
4	quintet (qi)	1:4:6:4:1
6	septet (sep)	1:6:15:20:15:6:1

Table 7: Types of multiplets and ratio of the intensities of the individual lines

A quantitative measure of the magnetic interaction of nuclei is the coupling constant J, which represents the distance between the individual lines of a multiplet as given in Hz. The spin-spin interaction is mutual: the group of equivalent hydrogen atoms A splits the signal of the group of hydrogen equivalent atoms B and *vice versa* with the same coupling constant J (see Fig. 38). If protons neighboring the observed nucleus (or equivalent nuclei) are mutually non-equivalent, combined multiplets will be formed (e.g., doublets, doublet of triplets). Unless the structure of the signal is obvious, we call it generally a multiplet (m).

The multiplicity of signals thus provides valuable information about the nuclei surrounding hydrogen atoms and is key to the structural analysis of organic compounds.

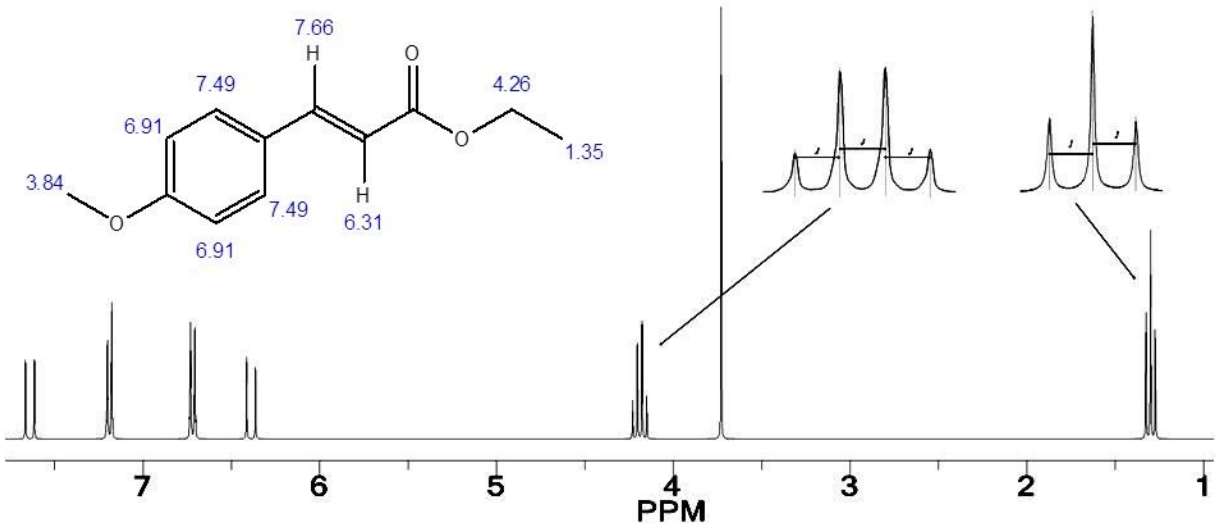

Fig. 38: ^{1}H NMR spectrum of 4-methoxy cinnamic acid ethyl ester:

^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.66 (d, 1H, J = 16.0 Hz), 7.49 (d, 2H, J = 8.8 Hz), 6.91 (d, 2H, J = 8.8 Hz), 6.31 (d, 1H, J = 16.0 Hz), 4.26 (q, 2H, J = 7.2 Hz), 3.84 (s, 3H), 1.35 (t, 3H, J = 7.2 Hz)

Allocating multiplicity of signals is usually very difficult in more complex natural substances because of considerable overlapping. A NOE (Nuclear Overhauser Effect) experiment can then be a good source of information about protons that are close to each other in space. The Nuclear Overhauser Effect results from dipolar interaction between two nuclei. It occurs because of direct interaction acting freely through space and in contrast to indirect spin-spin coupling, it is not influenced by chemical bonds. The Nuclear Overhauser Effect decreases with the sixth power of the distance between nuclei and is therefore very often used to measure the distance between nuclei, especially in 2D-NMR spectroscopy (see NOESY).

6.4.5 One-dimensional ^{13}C NMR

NMR spectroscopy of the carbon atom is possible thanks to the natural occurrence of the ^{13}C isotope (the most common isotope ^{12}C has I = 0, and it therefore provides no NMR signal). However, the occurrence of the ^{13}C isotope is relatively low (1.11%), and its resonance frequency is about four times lower than the resonance frequency of the hydrogen

atoms (e.g., when measured with a 200 MHz spectrometer, the resonance frequency of carbon atoms will occur at approximately 50 MHz). More sensitive spectrometers (FT-NMR) are required to measure carbon spectra, and the measuring time can be on the order of hours (depending on the amount of sample and quality of the device). As for hydrogen spectra, the chemical shifts in ^{13}C NMR spectra depend on the electron density around the given nucleus, but they provide a greater range of results, most frequently 0-220 ppm (relative to δ TMS = 0 ppm). Examples of chemical shifts for specific types of carbon atoms are given in Table 8.

Type of carbon (bond type)	**Chemical shift δ [ppm]**	**Type of carbon (bond type)**	**δ [ppm]**
primary alkyls (R-CH_3)	0-30	alkenes (C=C)	100-150
secondary alkyls (R_2-CH_2)	10-50	arenes (aromatic rings C=C)	110-160
tertiary alkyls (R_3-CH)	15-50	nitriles (C≡N)	115-130
Alkyl halides (RCH_2-X)	10 (I)-75(F)	amides (R_2NC=O)	150-180
amines (C-N)	40-70	carboxylic acids, esters (ROC=O)	160-185
alcohols, ethers (C-O)	40-80	aldehydes (RCH=O)	190-200
alkynes (C≡C)	60-90	ketones (R_2C=O)	200-220

Table 8: Approximate ranges of the chemical shifts δ of selected types of carbon atoms in organic compounds

Unlike ^{1}H NMR signals, the intensity of an individual signal in a carbon spectrum do not always correspond with the number of equivalent carbons involved in the given signal. For example, quaternary carbons cause weaker signals than carbons with hydrogen atoms.

As in hydrogen spectra, carbon signals can also be split by neighboring non-equivalent magnetically active nuclei. Because of the very low probability of the occurrence of two nuclei of the ^{13}C isotope in close proximity, ^{13}C-^{13}C homonuclear coupling cannot be observed under common conditions. However, heteronuclear ^{1}H-^{13}C spin-spin coupling can occur and to determine the multiplicity of a signal, the aforementioned n+1 rule can be used, where n is the number of hydrogen atoms coupled to the given carbon atom. The signal of a quaternary carbon will thus be a singlet; the signal of the carbon in a methine group will be a doublet, in a methylene group a triplet, and in a methyl group a quartet. This phenomenon should facilitate the interpretation of the measured spectrum. In practice, however, these interactions both reduce the sensitivity of the measurement (splitting the signal into individual

lines diminishes its intensity), and signals in spectral regions where more signals occur can overlap. Therefore, so-called **broadband heteronuclear decoupling** is used to measure routine carbon spectra. During the measurement of the ^{13}C spectrum the sample is irradiated concurrently with an RF pulse in a frequency band identical with the resonance frequencies of the hydrogen nuclei. This eliminates the hydrogen-carbon splitting and each non-equivalent carbon atom has a single signal in the spectrum (singlet) - see Fig. 39.

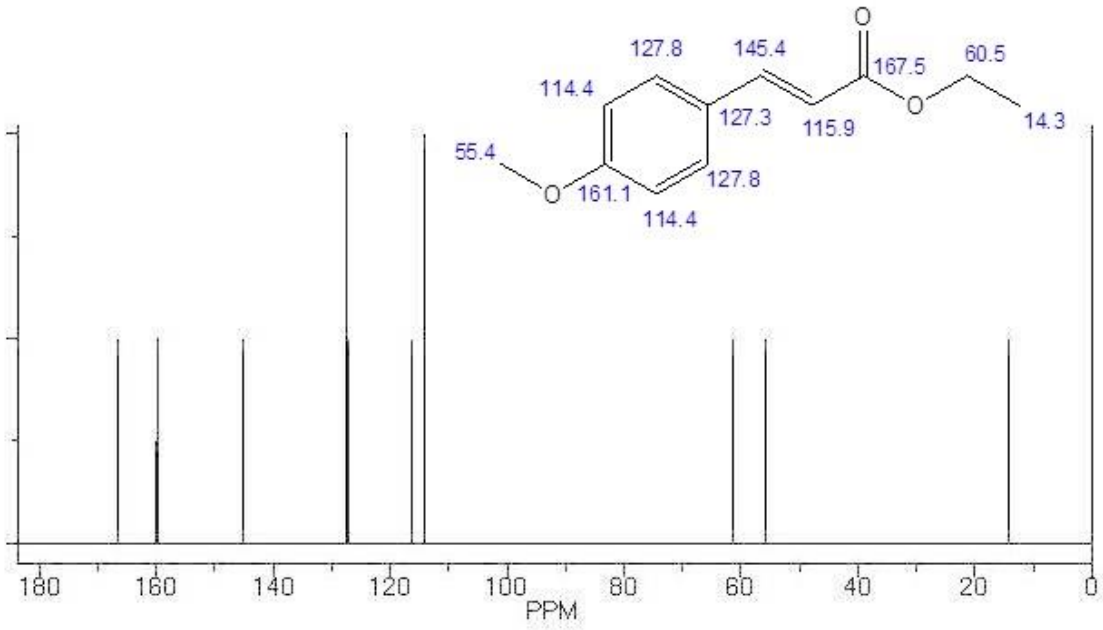

Fig. 39: The ^{13}C NMR spectrum of 4-methoxycinnamic acid ethyl ester (measured with broadband heteronuclear decoupling)

For some 1D ^{13}C-NMR experiments, broadband proton radiation need not be applied for the whole period during which the carbon spectrum is measured, but only during some sections. Carbon nuclei can also be irradiated using various pulses that serve to more easily allocate signals to individual carbon atoms of the measured compound.

The APT (Attached Proton Test) experiment uses a multipulse sequence, to distinguish between the signals of the carbons of the methine (>CH-) and methyl groups ($-CH_3$), for which the signals show negative intensity (i.e., the peaks are directed downwards under the zero baseline) from those of the carbons of the methylene group ($-CH_2-$) and quaternary carbons (>C<) which have signals with positive intensity and where >C< provides significantly less signal intensity than $-CH_2-$.

The DEPT (Distortionless Enhancement by Polarization Transfer) experiment distinguishes between the signals of primary, secondary, and tertiary carbon atoms. A DEPT spectrum is most frequently obtained using a series of partial experiments - DEPT-45 (all carbons bearing hydrogen are displayed), DEPT-90 (only >CH- is displayed), and DEPT-135 (the displayed signals of >CH- and $-CH_3$ are positive signals, the signals $-CH_2-$ are negative). Subsequent mutual reduction of the spectra yields a spectrum which consists of sub-spectra for the individual types of carbon. The signals of quaternary carbons are suppressed and must be identified by comparing them with the ordinary ^{13}C-NMR spectrum.

6.4.6 Two-dimensional ^{1}H and ^{13}C NMR

It is usually sufficient to use one-dimensional NMR spectroscopy for the structural analysis of a known substance, possibly also comparing with spectra in various databases. In the case of an unknown natural substance, 1D-NMR experiments are used for the basic analysis and to determine the character of the test substance, i.e., to find out whether the substance belongs among terpenes, aromatic compounds, alkaloids, glycosides, etc. The methods of two-dimensional NMR spectroscopy are then used to precisely determine the structure.

6.4.6.1 Basic principle of 2D NMR spectroscopy

Unlike 1D NMR, where FID provides a record of the dependence of the signal intensity on time, 2D spectra record the dependence of the intensity on two independent time variables. The general process of a 2D experiment can be divided into four phases. In the preparatory stage, the sample is irradiated with the first pulse, the development stage follows for the evolution time t_1, the second pulse represents a mixing stage and a detection stage then follows for the period t_2. The evolution time t_1 changes during the measurement and the detection time t_2 is constant. A three-dimensional spectrum is obtained by a subsequent double Fourier transformation. A panoramic 3D display (stacked plot) includes complete information about intensities, but it is often unclear, hard to interpret, and data demanding. A contour plot is a simplification corresponding to horizontal cuts at a certain height (level)

of signal intensity. It is possible to select a level that eliminates noise, but if it is too high, there is a risk of losing small signals.

Based on the correlation of types of nucleus, 2D experiments are divided into homonuclear (i.e., they correlate a single kind of nucleus, e.g., ^{1}H-^{1}H) and heteronuclear (these correlate two different kinds of nucleus, e.g., ^{1}H-^{13}C).

2D NMR spectra can be further divided into **2D NMR *J*-resolved spectra** (*J*,δ-spectra) - on one axis are the chemical shifts δ_C or δ_H and on the other axis are the respective coupling constants $J_{(H,H)}$ = homonuclear, $J_{(C,H)}$ = heteronuclear (older methods, not used at present) and to **2D NMR chemical shift correlation spectra** (δ,δ-spectra) - homonuclear or heteronuclear - there are chemical shifts δ on both axes.

At present there is a large number of 2D experiments and the following overview describes only some selected, most frequently used procedures.

6.4.6.2 Homonuclear 2D NMR experiments

One of the most frequently used 2D experiments is COSY (Correlation Spectroscopy). This is a homonuclear correlated ^{1}H-^{1}H spectrum which has a square character in the contour plot. On both sides there are chemical shifts of hydrogen atoms δ_H and the spectrum is therefore diagonally symmetric - peaks displayed on the diagonal correspond to the original ^{1}H-NMR spectrum. If non-equivalent nuclei interact, a spectrum of off-diagonal peaks appears - so-called cross-peaks. Interacting nuclei can be found in the spectrum by drawing horizontal and vertical lines from the cross-peak. At the point of intersection of the diagonal, there must be peaks for the corresponding hydrogen nuclei (see Fig. 40). Numerous COSY experiments have been created with the goal of increasing the resolution and quality of the correlation charts. These improved COSY experiments are often especially useful in cases where coupling constants are very low (J < 3 Hz) and often cannot even be distinguished in a 1D spectrum. An example is delayed COSY (= long-range COSY). In this experiment, it is possible to observe interactions among protons where the coupling constant is up to 2 Hz (allyl groupings, structural groupings separated by a quaternary carbon - long-distance interaction over more than three bonds).

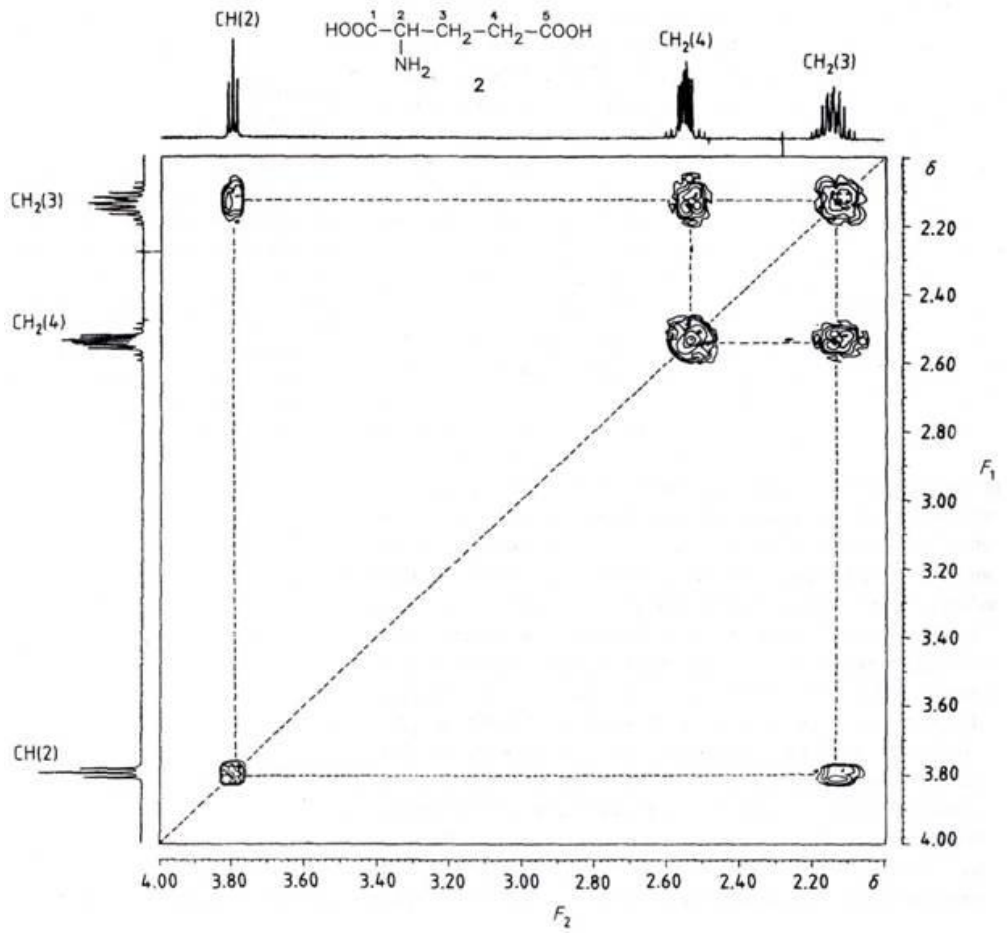

Fig. 40: ^{1}H-^{1}H COSY spectrum of glutamic acid; taken from [Friebolin, H.: Basic One- and Two-Dimensional NMR Spectroscopy. Wiley-VCH, 1998.]

A newer method of homonuclear correlated 2D-spectroscopy is NOESY (2D-NOE - Nuclear Overhauser Effect Spectroscopy). It enables measuring NOE among all hydrogen atoms of a molecule in a single experiment. In the spectrum there is a classical 1D spectrum on the diagonal, cross-peaks indicate through-space dipolar couplings in space and have a phase opposite to that of the signals of the diagonal peaks. The intensity of these signals is proportionate to the NOE intensity. It is possible to determine inter-atomic distances directly from the intensities of the cross-peaks by calibrating the spectrum using the known distances of the protons. NOESY spectra are therefore used especially in conformational analysis to determine the 3D structures of biomolecules. Data analysis, especially for large molecules, requires high-performance computers and sophisticated software for processing.

The TOCSY (Total Correlation Spectroscopy) experiment, also called the HOHAHA (Homonuclear Hartmann Hahn) experiment, uses a so-called spin-locking technique of

measuring (special composite pulses ”lock“ magnetization around the y axis, the energy levels of individual spins get closer, and magnetization can freely pass from one to another). The experiment results in a spectrum in which all of the nuclei of the spin system are correlated. In an ideal case, the spectrum contains cross-peaks with all of the other protons of the spin system for every proton. The method is used especially in the structural analysis of biomolecules of the peptide and oligosaccharide types.

The 2D-INADEQUATE (Incredible Natural Abundance Double Quantum Transfer; also C-C-COSY) experiment provides a map of connected carbon atoms on the basis of the existence of coupling constants J (^{13}C-^{13}C). The experiment requires the molecule to have two mutually bound ^{13}C atoms. Due to the very low abundance of the ^{13}C isotope, the probability of such a grouping is very low (approximately 1/10000). It is a demanding experiment and one of the peaks of the present NMR technology. The experiment requires a large quantity of sample (about 200 mg) and the measurement period is quite long. Recent technical improvements (nitrogen- or helium-cooled cryogenic probe, improvement in data collection) have enabled working with only a smaller quantity of substance.

6.4.6.3 Heteronuclear 2D NMR experiments

The most common heteronuclear alternative among correlated 2D spectra is the ^{13}C-^{1}H COSY = HETCOR experiment (Heteronuclear Correlation Spectroscopy). On one axis are the chemical shifts of the hydrogen atoms δ_H and on the other are those of the carbon atoms δ_C. Because the chemical shifts are different in both dimensions, HETCOR spectra are not diagonally symmetric. The spectrum shows cross-peaks, which result from correlations of the ^{1}H-^{13}C spin-spin interactions. From the spectrum, it is easy to see which protons are bound to a specific carbon atom by drawing horizontal and vertical lines from the cross-peak; the intersections of the axes determine the positions (chemical shifts δ) of the interacting nuclei (see Fig. 41). This method can be used for observing only carbons with directly bound hydrogen atoms; the signals of quaternary carbons do not appear in the spectrum. By adjusting the pulse sequence of the HETCOR experiment, the COLOC (Correlation via Long-range Coupling) experiment enables the interactions of a given carbon atom with hydrogen atoms *via* more than one bond (a long-range C-H interaction) to be displayed. When

identifying these spectra, it is best to always compare them with HETCOR spectra because residues of single-bond interactions may appear in COLOC spectra.

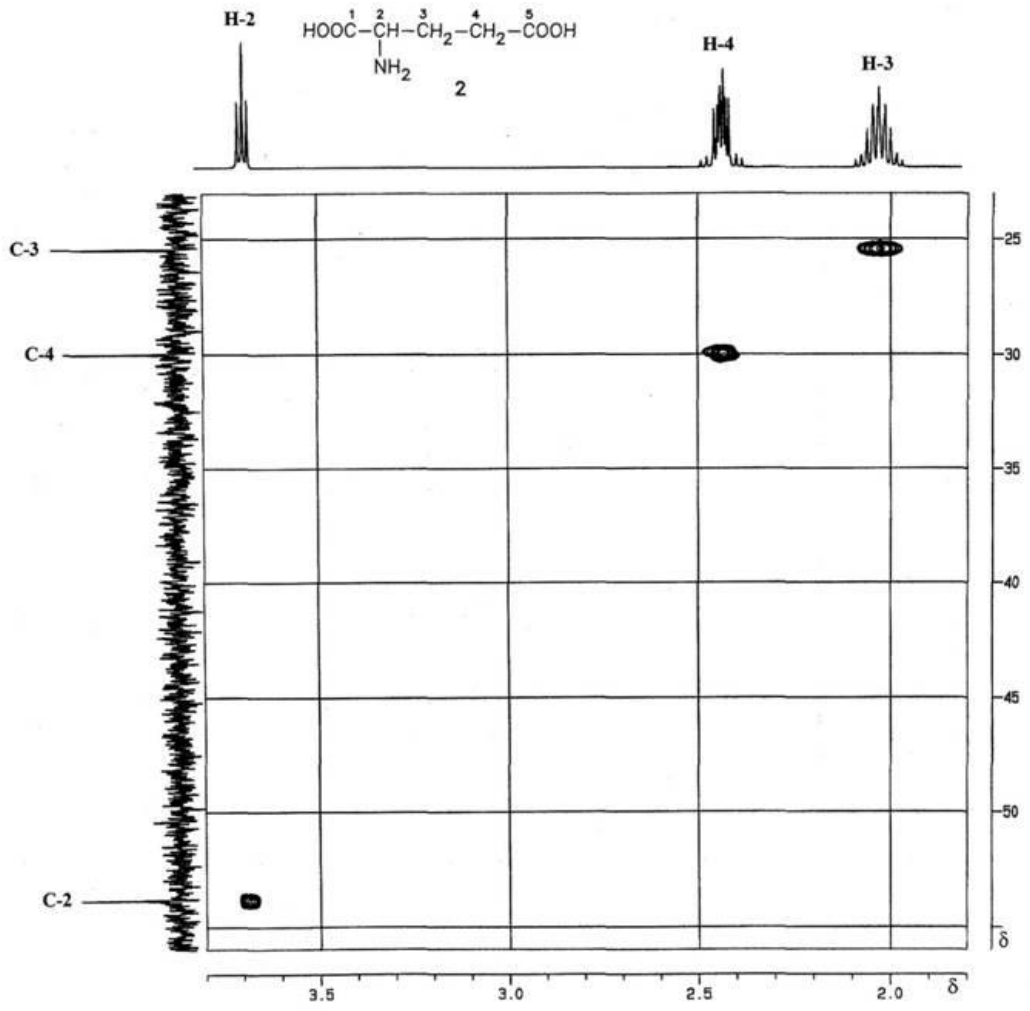

Fig. 41: ^{13}C-^{1}H COSY (HETCOR) spectrum of glutamic acid; taken from [Friebolin, H.: Basic One- and Two-Dimensional NMR Spectroscopy. Wiley-VCH, 1998.]

Currently, structural analysis often uses 2D heteronuclear experiments with inversion detection (HMQC, HSQC, HMBC). These are more sensitive than experiments with direct detection (HETCOR, COLOC). Greater sensitivity can be achieved by suitably arranging the experiment with regard to the excited and detected nucleus. An identical nucleus is excited and detected in direct detection. Inversion techniques involve exciting nuclei with a high sensitivity (^{1}H) and the subsequent transfer of their polarization to nuclei with low sensitivity (^{13}C, ^{15}N) and then back again for the detection of the nuclei with high sensitivity. The information obtained about a nucleus with low sensitivity by indirect detection is shown in the

resulting spectrum. Inverse experiments demand more sophisticated instrumentation and require a special inverse broadband NMR probe.

HMQC (Heteronuclear Multiple Quantum Correlation) and HSQC (Heteronuclear Single Quantum Correlation) experiments provide correlations between ^{1}H and ^{13}C nuclei directly bound to them, i.e., the magnetization is transferred *via* the single-bond coupling constant $J_{H\text{-}C}$. The spectra do not show the atoms of quaternary carbons, but only those carbon nuclei with a directly bound ^{1}H atom. The resulting spectrum provides information similar to HETCOR, but with greater sensitivity.

In contrast, the HMBC (Heteronuclear Multiple Bond Correlation) experiment correlates ^{1}H nuclei with ^{13}C (or ^{15}N) nuclei *via* two or three bonds (long-distance interactions). It serves especially to identify nuclei which do not have a directly bound ^{1}H atom, e.g., quaternary carbons. HMBC is also used to characterize alkaloid structures and to detect ^{15}N and ^{1}H correlations.

6.5 X-ray diffraction

X-ray diffraction is a method used to determine the arrangement of the atoms in a crystal. X-ray radiation that falls on a crystal is diffracted (reflected) in many directions. Using specialized programs, we can determine a 3D image of the electron density in the crystal from the measured angles and intensities of the diffracted beams of the rotating crystal and then determine the positions of the atoms in the crystal (a 3D model of the molecule), their chemical bonds, and other information.

X-ray diffraction includes two basic methods, namely single crystal diffraction and powder diffraction. X-ray powder diffraction is used especially as a control method for characterizing a crystalline material (industrial applications). Its main advantages include: the high speed of measuring, no need to prepare a single crystal, and the ability to analyze mixtures of substances. Powder diffraction is not suitable for the complete structural analysis of crystals with complicated structures. In contrast, the method using the diffraction of a single crystal enables the complete structural analysis of a substance (crystal) and is therefore one of the main methods for characterizing the atomic structures of new substances.

X-ray radiation is electromagnetic radiation in the wavelength range 0.001–10 nm. X-ray diffraction uses especially wavelengths around 0.05–0.25 nm, i.e., those comparable with the inter-atomic distances in crystals. The radiation is diffracted when the wavelength of the radiation is close to the distance between the atoms in the crystal lattice with which it interacts. X-ray diffraction uses the so-called flexible (coherent) scatter of the radiation that occurs when the scattered beams have the same energy and therefore the same wavelength as the original (primary) X-ray radiation.

Following the impact of X-ray radiation on the test material, non-flexible (incoherent) scatter or absorption of the radiation may occur. A photon loses part of its energy upon incoherent scattering of X-ray radiation and the scattered radiation that has a longer wavelength than the impacting radiation. When X-ray radiation is absorbed, all of the energy of the photon is transferred to an electron and the photon becomes extinct. If the energy of the photon is sufficient for incoherent scatter or absorption of the X-ray radiation, an electron can be released from the inner orbital of the atom and an electron from a higher orbital then falls to the vacated place. The surplus energy is radiated as secondary (fluorescent) X-ray radiation (Fig. 42). Fluorescent radiation is undesirable in the X-ray diffraction method because this

radiation increases the background during the measurement and it can be suppressed by using primary X-ray radiation of a suitable wavelength.

Fluorescent radiation can also be used for analytical purposes in a method called emission X-ray spectroscopy. The wavelength of the fluorescent radiation is longer than that of the primary X-ray radiation and is characteristic for individual atoms. Absorbed X-ray radiation interacts with inner electrons (not influenced by the atomic bonds in the molecule), and the resulting fluorescent X-ray radiation is largely independent of the chemical status of the elements. This phenomenon can be used to identify and quantify the chemical elements in the sample.

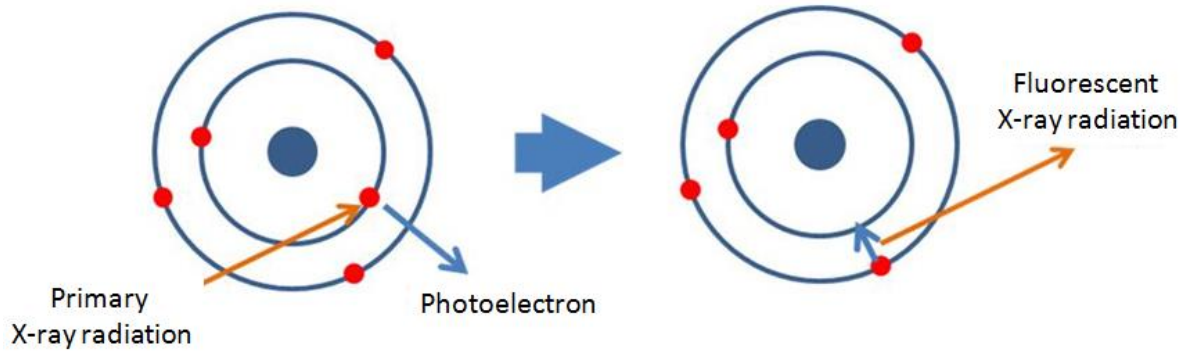

Fig. 42: Schematic diagram of the origin of fluorescent X-ray radiation

6.5.1 Origin of diffracted radiation

The diffraction of X-ray radiation results from the flexible scatter of radiation impinging on the electron shells of the atoms in molecules or crystals and its consequent interference, which results from the differences in the lengths of the trajectories between the various scattering atoms and the point of detection. Diffraction forms a diffraction pattern with characteristic maxima and minima of diffraction spot intensities. Analyzing diffraction spots (crystal patterns in reciprocal space) led to the discovery that crystal behaves as if it contained a large quantity of planes situated at regular distances from each other (Bragg`s description of diffraction). A diffracted beam is formed by ”reflection“ from this system of parallel planes where the atoms in the crystal are localized. The impacting and the diffracted beams both form the same angle θ with the system of planes being considered, which corresponds to the Diffraction Law (Bragg´s Law) (Fig. 43). Each plane reflects only a small

quantity of radiation, but the diffraction resulting from a large number of planes provides intensity sufficient for observation.

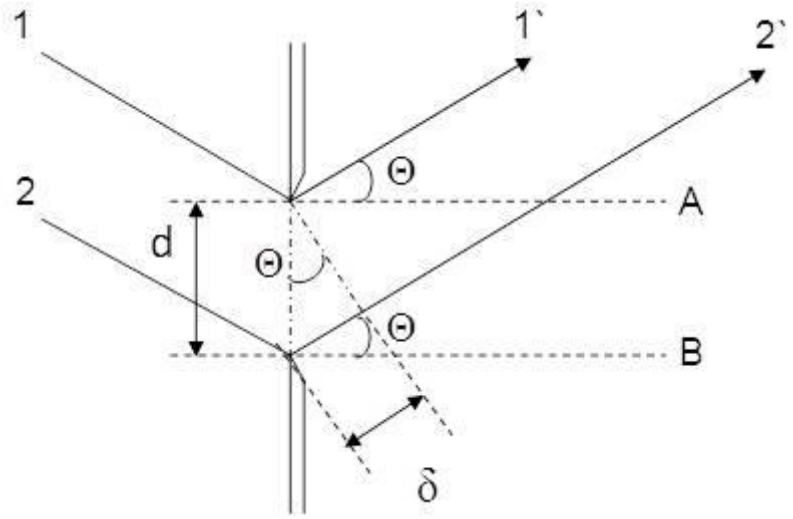

Fig. 43: Schematic diagram of diffraction on a crystal lattice; θ –angel of X-ray radiation impact; d – interplanar distance; δ – path difference

Structural information about the positions of atoms in a crystal can be derived from the positions and intensities of the diffraction maxima formed by the diffraction of X-ray radiation. X-rays are diffracted by every crystalline substance, depending on the type of atoms of which the crystal of the given substance consists, and their layout. When an X-ray impacts a sample and it is diffracted, it is possible to measure the distance between individual planes of the atoms according to Bragg`s Law (Equation 14). If we select any two parallel planes with a separation distance of *d* and have a bundle of parallel X-rays with the wavelength λ fall on these planes, the impacting rays are in phase. Ray 11` is diffracted by plane A and ray 22` is diffracted by plane B (Fig. 43). For both rays to be in phase after the diffraction, the distance covered by ray 22`, compared to ray 11` (the path distance δ) must equal a whole multiple *n* of wavelength λ of the incident radiation (Equation 14). We can detect the interference maximum of the diffracted rays only under this condition. The path difference of rays 11` and 22` equals $2d \times \sin\theta$, where θ is the angle between the incident ray and the plane of the crystal. By combining the aforementioned relations, we arrive at Bragg`s Law:

$$2d \times \sin\theta = n \times \lambda \qquad (14)$$

Bragg`s Law is the basic equation for X-ray diffraction. If we know the wavelength λ of the X-ray radiation and measure the angle θ, we can determine the distance *d* between the planes of the crystal.

Only a single set of planes was considered in the description of the aforementioned principles. By rotating the crystal and changing the angle of the incident radiation, various sets of planes can be caused to meet Bragg`s Law and we can obtain a point diffraction pattern of a single crystal (Fig. 44).

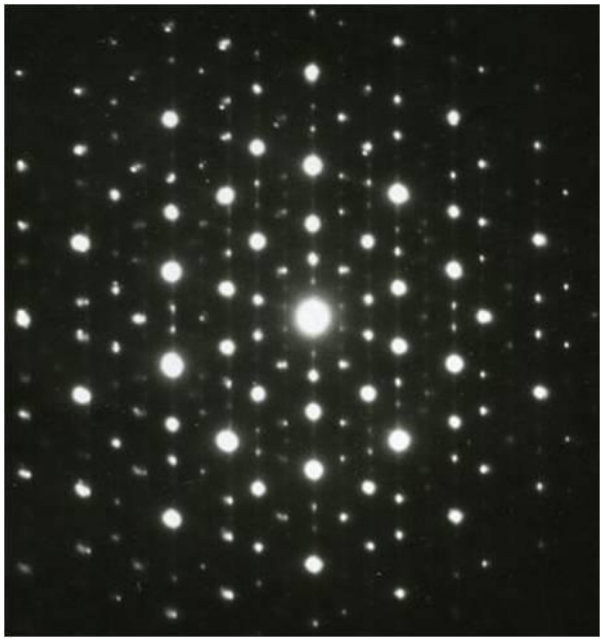

Fig. 44: A point diffraction pattern of a single crystal

6.5.2 Techniques for measuring and presenting results

In order to be diffracted, X-ray radiation must fall on a crystal at the angle θ (Bragg`s Law, Equation 14). This condition can be met by suitably orienting the crystal towards the X-ray radiation source. Observing the radiation diffracted from the crystal being analyzed at a single position is not sufficient to obtain the crystallographic structure. To obtain the necessary information, the crystal must be gradually rotated and the diffracted radiation must be continuously recorded. To determine the positions of the atoms in the crystal, we need to know the positions of the diffraction maxima and their intensity. The device that rotates the crystal in space and detects the diffracted radiation is called a diffractometer. The crystallographic analysis of single crystals of unknown substances is usually performed using

four-circle diffractometers (Fig. 45). This layout enables rotating the crystal around the common axes of ω and θ rotation along with rotation in two other directions, χ and Φ. The crystal rotates around the Φ axis which moves with the crystal inside the χ circle. This χ circle rotates around the ω axis. The position of the rotating crystal is then expressed by three angles – ω, χ, Φ. The detector then rotates in the plane of the goniometer according to the angle 2θ. The settings of the crystal and detector are usually controlled by a computer.

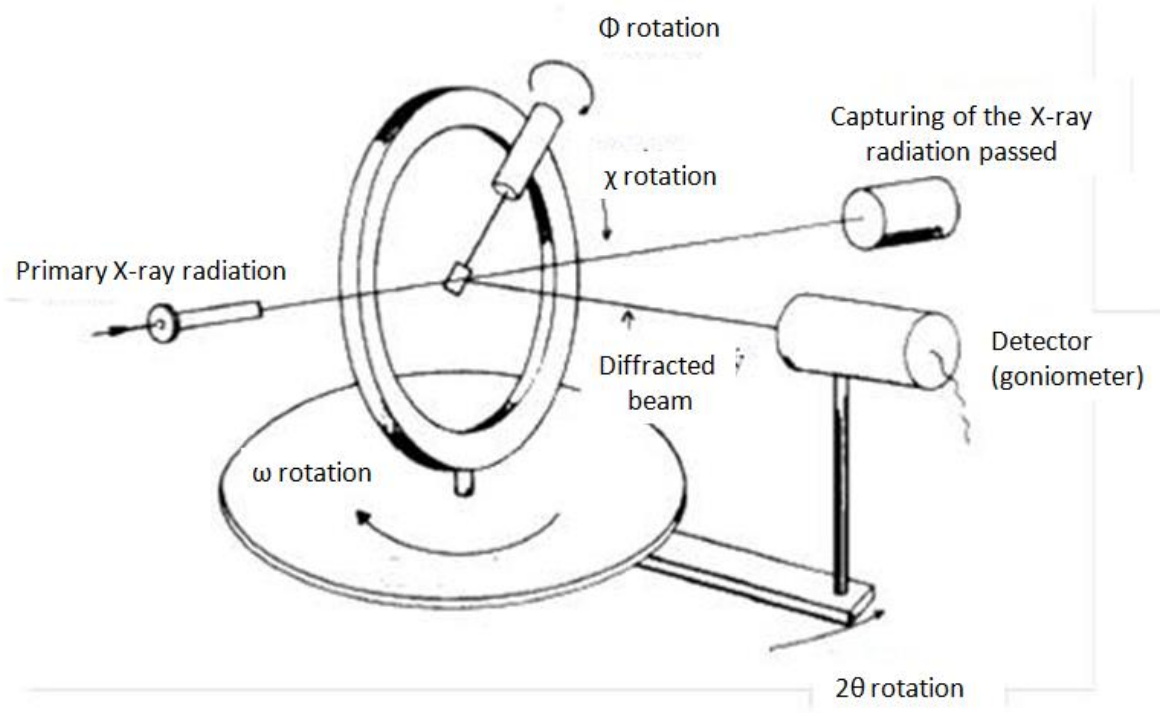

Fig. 45: Schematic diagram of a four-circle diffractometer

The most difficult step in structural analysis using single crystal diffraction is the preparation of a single crystal with the size of 0.1–1 mm. This step is also the main limitation of the method because it is not always possible to prepare such a crystal. If a suitable crystal is available, the necessary data are measured (lattice parameters, positions of diffraction maxima and their intensities) and used to calculate the electron density. A model of the structure is designed to map the electron density obtained in this way and optimized by a computer. Because of the large number of calculations, the measured data are evaluated exclusively by means of specialized computer software. The resulting structure is most frequently displayed by means of suitable software in the form of an Ortep pattern (Fig. 46).

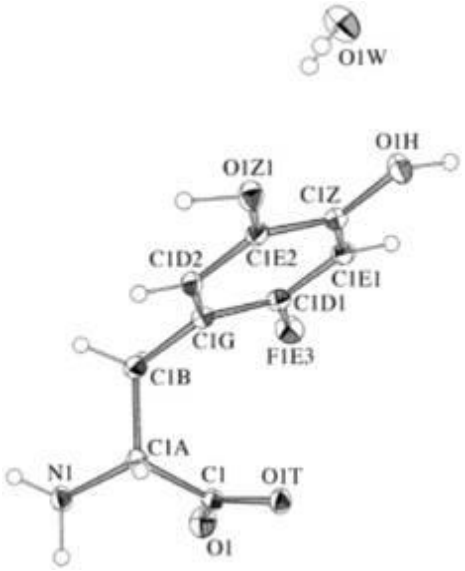

Fig. 46: An Ortep pattern showing the molecular structure of F-DOPA

6.5.3 Sources of X-ray radiation and its detection

X-ray radiation is generated by the impact of highly accelerated electrons on the atoms of a mass. X-ray lamps, or Röntgen-ray tubes, operate on this principle. They are evacuated ceramic tubes with two sealed electrodes between which there is a high voltage of 20 – 60 kV. A tungsten filament heated to a very high temperature serves as the cathode. The cathode produces electrons which are accelerated in the electric field and impact on the anode, the so-called anticathode, with high energy. Upon impact most of the kinetic energy of the electrons is converted into heat (an X-ray lamp needs to be intensively cooled down) and only about 1% of their energy is emitted as X-ray radiation. Continuous monochromatic radiation is generated. The monochromatic radiation is generated in such a way that the electrons are accelerated to a sufficient energy to penetrate into the sub-valence electron levels of the anode (electron levels K and L) and push out an electron from there. An electron with a higher energy then drops to the vacated place from a higher electron level (more distant from the nucleus) and the surplus of energy is radiated as X-ray radiation. The wavelength of the X-ray radiation induced by this transfer is given by the difference in energy between the two electron levels. Therefore, this wavelength is characteristic for the material of which the anode is made. The selection of an X-ray lamp (material of the anode) depends on the nature of the material being analyzed. Excessive absorption of X-ray radiation by the sample

(environment) is undesirable. The most commonly used materials for anodes are copper, cobalt, or molybdenum.

Another source used to produce X-ray radiation is the synchrotron. The X-ray radiation in a synchrotron circle is created by the curved path of a relativistic electron. The X-ray radiation is radiated in the direction of the particle (electron) along the tangent to its path and covers a large part of the electromagnetic spectrum (a continuous spectrum). This radiation is typically of high intensity. As the cost of this equipment is significantly higher than that of common equipment, it is used mostly for analyzing material with a short lifetime which could degrade under the influence of X-ray radiation (e.g., crystallographic analysis of enzymes). The analysis of these materials is possible thanks to the high intensity of synchrotron X-ray radiation, which enables short exposure and detection times.

Upon interaction with the sample the X-ray radiation is detected on the basis of several principles (the ionization of gases, the luminescence of certain materials, a change in conductivity, or the exposure of photographic film). While the method of saving the pattern on a photographic film prevailed in the past, digital and other methods are used today.

7 TESTING THE BIOLOGICAL ACTIVITY

Solubility needs to be considered when preparing material for testing the biological activity. All of the solvents which can be considered for dissolving the samples must first be tested for activity. Their activity must be zero or at least not interfere with the activity of the test material. Another condition is the compatibility of the solvent with the testing environment and its miscibility with any other solvents used. (This is very often a problem in testing lipophilic substances in an aqueous physiological environment.) The precipitation of substances must be avoided when the sample is mixed into its new environment. The most commonly used solvents for testing include dimethyl sulfoxide (DMSO), water, ethanol, acetone, and mixtures of these. It is also possible to use solubilizers, such as Tween or Cremophor.

At present, a very wide spectrum of *in vitro* tests that simulate numerous physiological or pathophysiological processes can be used to determine biological activity. In fact, it is possible to test anything, depending only on the equipment of the laboratory, the financial resources, and, of course, the abilities of the workers. Testing can be performed in almost any manner, but it is best to follow a rational selection of tests based, e.g., on an ethnopharmacological survey or chemical relationships of the substances.

At present, any testing of biological activity requires the use of standards as positive controls. Substances commonly used in practice to treat diseases or influence the activity to be tested, or model experimental compounds, e.g., model enzyme inhibitors, are usually selected for use as standards of a biological activity. In any case, the parameters of the effect of the reference substance should be well described and characterized.

In general, the prevailing trend is to test newly isolated substances *in vitro* (with no need for laboratory animals) for economic reasons and also because *in vitro* tests require smaller quantities of the isolated substances than *in vivo* experiments on laboratory animals. When the conditions of an *in vitro* test are established, an effort is made to approximate the conditions of an *in vivo* tests, e.g., by using cell cultures. The more precise the simulation of the natural environment, the better. However, it is obvious that if, after a series of *in vitro* tests, a substance is considered to be a promising candidate for practical use, it is necessary to perform *in vivo* tests on animals before any pre-clinical and later clinical evaluation. An overview of some common methods and their bases is given in the following table (Table 9).

Biological activity	Basis of assay
Antibacterial	Bacteria-inoculated culture medium, turbidimetry
Antifungal	Fungi-inoculated culture medium, turbidimetry
Enzyme inhibition	Colorimetry, radioactive marking, Western Blot
Anti-tumor, cytotoxic	Cell line, vital staining (MTT, WST)
Toxicity	Model organism – e.g., *Artemia salina*
Antiparasitic	Model organism – e.g., respective parasite
Binding to receptors	ELISA, chemiluminiscence, fluorescence

Table 9: Overview of the most common methods for testing biological activity

Bioactivity-guided isolation is a modern approach to obtaining bio-active compounds. In this process, biological activity is monitored at every step of the isolation of a natural substance. All fractions obtained by separation are tested with regard to both the substances contained and their biological activities. By selecting the bioactive fractions, the phytochemist gets to an active substance. To determine the active fraction, the activity must be quantified, at least approximately, usually by testing a diluted series of the individual fractions. In this way, the most active fractions are assayed and it can be determined if the initial activity detected in the extract has not been broken into many fractions. During separation, the activity of a fraction can be reduced or even disappear if an extract contains more than one active substance and the substance with the greatest activity has not been identified or the active ingredient has been degraded, an error has occurred during the separation leaving the active ingredient trapped on the column, or an active compound is distributed in more fractions.

The following examples of compounds that are in the last stages of clinical trials, or medicines based on natural substances which have recently been introduced into therapy show that the isolation of bioactive compounds can be successful and that testing for activity identifies new substances.

B-Arteether (Artemotil^{TM}) – antimalaric. Artemisinin is a sesquiterpene lactone present in *Artemisia annua* plants (traditional Chinese quinghao medicine). Its concentration

in plants is typically about 0.2%, which is a relatively small quantity. Artemisinic acid, present in greater quantities (0.8%), can easily be converted to artemisinin. Artemether, arteether, and artesunate can also be obtained from artemisinin by semi-synthetic means.

Galantamine (Reminyl™) – effect on Alzheimer`s disease. Galantamine is an alkaloid obtained from snowdrops (especially *Galanthus woronowii*), some daffodils, and snowflakes. This alkaloid has been known and used for quite a long time in the therapy of myasthenia gravis, but only recently has it been used as a nootropic against the symptoms of Alzheimer`s disease.

Leptospermone – fluoroglucine derivative isolated, e.g., from *Leptospermum* spp. It shows interesting antimicrobial activity against resistant bacterial species. Another activity is the inhibition of plant 4-hydroxyphenylpyruvate dioxygenase. This effect can be used as a new class of herbicides. **Nitisinone** (Orfadin™) is a synthetic derivative of leptospermone. It is used in the therapy of tyrosinemia, type I (HT-1), a genetic metabolic disorder characterized by the lack of fumarylacetoacetate hydrolase, an enzyme which is involved in the last step of the catabolism of tyrosine. In this disorder, the intermediate products of tyrosine metabolism which inhibit porfyrine metabolism are accumulated. Nitisinone is a competitive inhibitor of 4-hydroxyphenylpyruvate dioxygenase, an enzyme which is involved in the catabolism of tyrosine. By intervening in the catabolism of tyrosine, it prevents the accumulation of toxic intermediate products.

Tiotropium (Spiriva™) – a semisynthetic derivative of scopolamine (*Atropa belladonna*) with parasympatholytic properties. It does not have the full selectivity in relation to muscarinic receptors, but when applied locally (by inhalation), it affects mainly the M_3 receptors localized in the smooth muscles of the bronchi. It causes bronchial dilatation and is used in chronic obstruction lung disease.

Morphine-6-glucuronide – the main active metabolite of morphine (*Papaver somniferum*). It is potentially a very interesting compound for short-term post-surgical analgesia or local and peripheral analgesic therapy with fewer adverse effects than morphine.

Vinflunine (Javlor™) – belongs in the group of substances called vinca-alkaloids (*Catharanthus roseus*); it is a semisynthetic fluorinated derivative. By binding to tubulin, the substance prevents mitosis and suppresses the division of tumorous cells. It is used for the therapy of advanced or metastasizing carcinoma of the urinary tract.

Exatecane – a semisynthetic water-soluble derivative of camptothecine (an alkaloid of *Camptotheca acuminata*). It inhibits topoisomerase I by stabilizing the topoisomerase I-DNA complex that inhibits the replication of DNA and starts apoptosis. Exatecane does not require enzymatic activation and is more active than camptothecine and its analog.

Calanolide A – prenylated coumarin isolated from the *Calophyllum lanigerum* tree. It belongs to the NNRTI group (non-nucleoside reverse transcriptase inhibitors). It can potentially be useful in the therapy of viral diseases (HIV).

Betulinic acid – pentacyclic triterpene, present e.g., in *Betula pendula*. It shows anti-retroviral, anti-malarial, and anti-inflammatory effects. It inhibits topoisomerase and especially its analogs are being tested as potential anti-cancer substances.

Silvestrol – isolated from *Aglaia folveolata*, is a derivative of cyclopenta[b]benzofuran. It is a compound potentially usable in cancer therapy. It increases the level of apoptosis, inhibits tumorous angiogenesis, and inhibits the translation of mRNA related to the malignity of cells.

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Title:	Laboratory Methods of Experimental Phytochemistry
Department:	Department of Natural Drugs, Department of Chemical Drugs, Department of Pharmaceutics
Počet stran:	143
Edition:	1.
Publisher:	University of Veterinary and Pharmaceutical Sciences Brno

ISBN 978-80-7305-796-1