Hort4EUGreen

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SUSTAINABLE USE OF PESTICIDES AND THEIR RESIDUES MONITORING

Residue analysis and monitoring

Volume 5

University of Agronomic Sciences and Veterinary Medicine of Bucharest
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"Enhancing practical skills of horticulture specialists to better address the demands of the European Green Deal"

Bucharest, 2025

Table of Content

Volume 5
Residue analysis and monitoring

Introduction

In the world we inhabit, the presence and impact of chemical residues – be it in food, soil, water, or air – are of great importance. The compounds we encounter, ranging from pesticides to pharmaceuticals, not only play a crucial role in our daily lives but also pose significant challenges in terms of their potential environmental impact and implications for human health. This course aims to bridge the gap between theoretical knowledge and practical application, enabling you to analyze and monitor these residues effectively.

Throughout this module, you will delve into advanced analytical techniques such as chromatography, mass spectrometry, and spectroscopy. You will gain experience about the work performed in state-of-the-art laboratories, learning to detect, quantify, and evaluate the risks associated with various chemical residues. The module will cover a broad spectrum of topics, including sample preparation, method validation, quality assurance, and the interpretation of analytical results in the context of international standards and regulations.

As future experts in residue analysis and monitoring, you will play a crucial role in safeguarding public health and preserving the environment. This course is not just an academic journey; it is a pathway to becoming a pivotal contributor to a more sustainable and health-conscious world. Welcome aboard, and we look forward to embarking on this enlightening journey together.

Volume 5
Residue analysis and monitoring

Summary

This Training Module is intended to provide the principles in pesticide analysis, and in the instrumental analysis of environmental samples. The module will consist of sessions to cover the following topics: sample preparation and treatment, sample analysis by chromatographic and immunoanalytical methods, basics of mass spectrometry. The course is comprised of simple (usual) and more advanced analytical methods which will develop the skills of the working understanding of MS and PhD students.

Learning outcome descriptors

By the end of the Module, the trainee should be able to:

- follow the analytical protocols;
- understand the goals of sample preparation steps;
- select the appropriate analysis method for a pesticide active ingredient (AI);
- consider the influencing parameters;
- understand the limitations that should be considered during the study design of monitoring;

General and transferable skills

1	Prepare the sample for specific analytical method;
2	Choose the appropriate analytical method for the target molecules;
3	Understand the analytical protocol;
4	Demonstrate the capacity to understand the obtained results in order interpret the information from a variety of analytical methods.

Knowledge, understanding and professional skills

1	Select the appropriate analytical method for determination of pesticide AI;
2	Gain knowledge in approach using different methods of chemical analysis;
3	Interpretation of the obtained data, limitations of the information.

Unit 5.1 Selection of appropriate analysis method

Mária Mörtl

The appropriate analysis method includes the sample preparation and the determination of pesticide active ingredients (Figure 5.1). There are very few cases, when the samples are measured directly without any sample preparation. At least filtration is necessary, but usually extraction of ingredients, removal of interfering matrix components (clean up) and in some cases concentration of sample or chemical modification of target components (derivatization) are required.

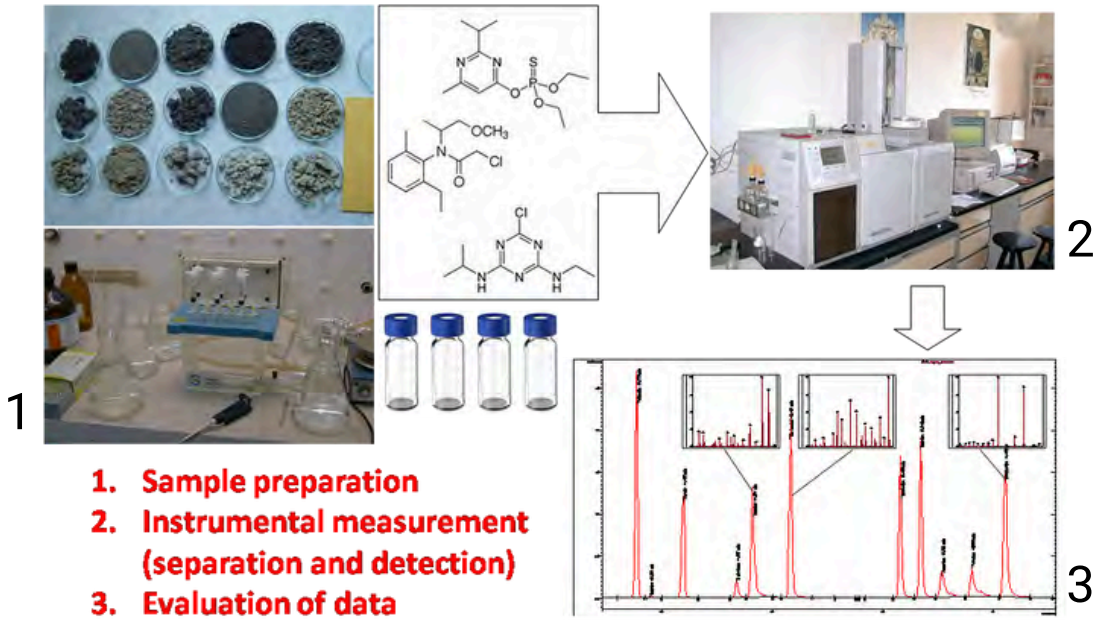


Figure 5.1. General protocol for pesticide residue analysis

The simplest method for the extraction is the liquid extraction by an organic solvent or solvent mixture and a separatory funnel or a Soxhlet extractor (See Unit 5.2.). Polarity and solubility of target compounds in the extraction solvent is a crucial point, therefore efficiency of this step is examined by determination of the recovery from the matrix. Many technical solutions are also commercially available to carry out liquid extraction (e.g. microwawe assisted). Pre-concentration of the extract is inevitable in the trace analysis, if the concentration is out of the dynamic range of the instrumental analytical technique. Also, extract chemical treatment might be required for analytical determination, e.g. derivatization. Among the goals of derivatization are either to make the target component suitable for the instrumental analysis (e.g. increase the volatility by lowering the polarity or increase the stability by substitution of labile/mobile moiety) or to improve the detectability (e.g. by a chromophore group).

If we have the concentrated extract, containing the pesticide residues, then we have to analyze the sample and identify the components. Determination of pesticide residues is usually performed by separation of compounds using a chromatographic method followed by their detection. There are a lot of pesticide active ingredients, which are currently used in the European Union, and due to their illegal use or in imported food, banned components have to also be measured. By applying the multi-residue methods, more target components

are determined by the selected instrumental analytical technique. The method is validated to a group of pesticide active ingredients (e.g. triazines, chloroacetamides or organophosphates), and the calibration is carried out regularly with the mixture of standard materials. Selection of the appropriate instrumental analytical technique depends on characteristics of target components. Volatile and thermally stabile pesticide active ingredients are measured by gas chromatography (GC), whereas less volatile and thermally labile components are more amenable to liquid chromatographic (LC) separation. There are several methods for the detection of separated components (e.g. flame ionization, FID), but identification of pollutants requires structure related information as well. Mass spectrometry is nowadays the most often applied detection mode as it allows selective and sensitive detection of components (Figure 5.2). In some cases other detection modes might be also suitable, if the matrix (e.g. soil or food) does not contain interfering components and/or the requirements regarding the limit of detection (LOD) is not so strict. There are also single methods, when only one target component will be measured. Immunoanalytical methods belong typically to this category.

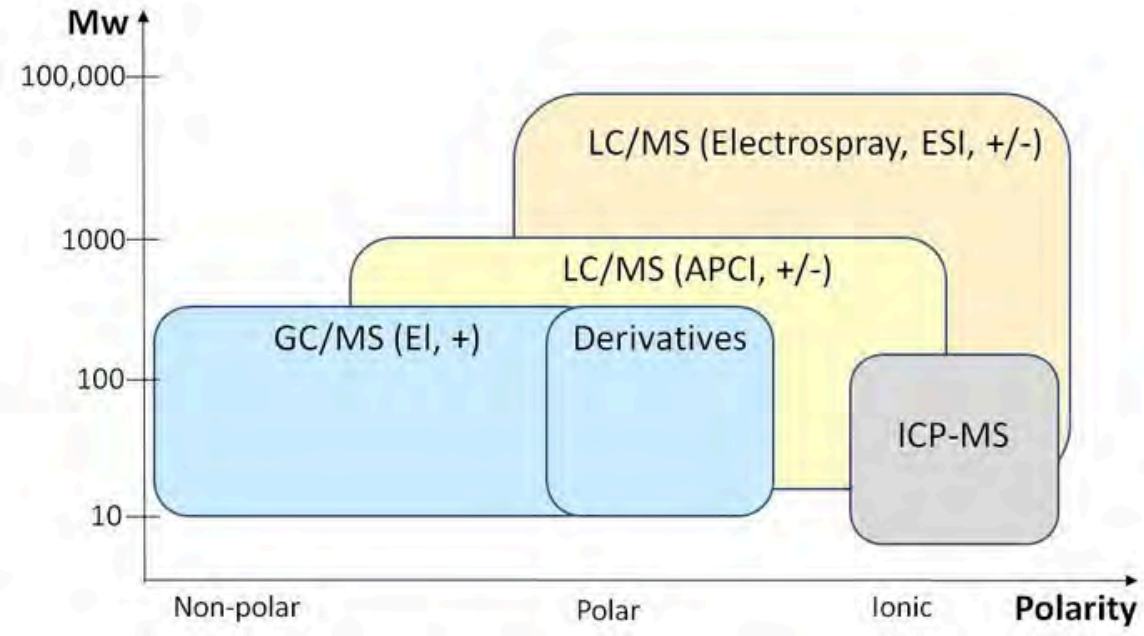


Figure 5.2. Application fields of mass spectrometry coupled to different instrumental analytical techniques. Molecular weight vs. polarity of the analyte.

Unit 5.2 Sample preparation

Mária Mörtl

Direct analysis of the sample rarely occurs, as at least the filtration prior to LC measurements is mandatory and prior to GC measurements is strongly recommended in most of the cases. The routine analytical techniques are generally suitable for the measurement of homogeneous samples (gases or solutions), therefore we have to extract the compounds of interest from the solid samples (e.g. soil, food). In addition, extraction of a liquid sample (e.g. surface water) by an organic solvent may concentrate the pollutants due to their better solubility in the organic phase, and a further increase in the concentration can be achieved by evaporation of the organic solvent. This is important as the analytical instruments have a dynamic range for each component, which means that the detector signal is linear between some concentration limits, out of this range the reliability of the measurements is poor. Below the linearity range (at low concentrations) the signal (detector response) is low, near to the noise, and at high levels the detector becomes saturated (it cannot measure the real amount). Separation techniques are applied during the sample preparation including physical (filtration, decantation, centrifugation) and/or physicochemical (distillation, extraction) methods.

5.2.1 Preparation of air samples prior to determination of pesticide residues

Low amounts of pesticide active ingredients can be found in the air due to their volatilization and due to pesticide drift. Appearance of pesticides in the air is also facilitated by soil erosion and wind, but in this case compounds are adsorbed to the surface of the small solid particles (aerosols). It is quite difficult to measure the concentration of pesticides in the air, as it is very low and to determine the signal vs concentration (=calibration curve) is also complicated. First of all, it is not so easy to prepare a series of known concentrations in the air, and then we have to concentrate the sample to achieve the dynamic range of the instrumental analytical method. Thus, two main sampling strategies (active and passive sampling) are used in determination of pesticide residues in the air to solve the problem.

In the active sampling, controlled volume of the air is pumped through a tube containing the sorbent phase (Figure 5.3). Usually lower volumes are required to determine the occupational exposures and larger volumes for environmental monitoring. Active sampling is typically shorter (1-7 days) compared to passive sampling, but the goal is the same, to enrich the analytes of interest until the LOD or better the limit of quantification (LOQ) of the

instrumental analytical procedure is achieved. Sometimes two consecutive tubes are used to avoid analyte losses due to the saturation sorbent phase (Figure 5.4). The air is filtered prior to the tube, which removes particulate matter that may clog the system. As the filtered fraction (aerosols) may contain significant amounts of pollutants, it is worthy to measure them as well.

Typically the less polar and less volatile compounds are adsorbed on the surface of particulate matter. Worthy of note, that only the vapors pass through the sorbents, from which the adsorbed compounds are usually solved and measured after the sampling.

The most frequently applied sorbent materials for this purposes are the polyurethane foams (PUF), which are not suitable for volatile components, but chlorinated compounds, non-polar (e.g. polycyclic aromatic hydrocarbons, PAHs) and polar compounds also are adsorbed.

In contrast, the other often used sorbent, XAD resin (a styrene divinylbenzene co-polymer) has hydrophobic, non-polar adsorptive properties. Thus, selectivity of the sorbent determines, which contaminants are retained.

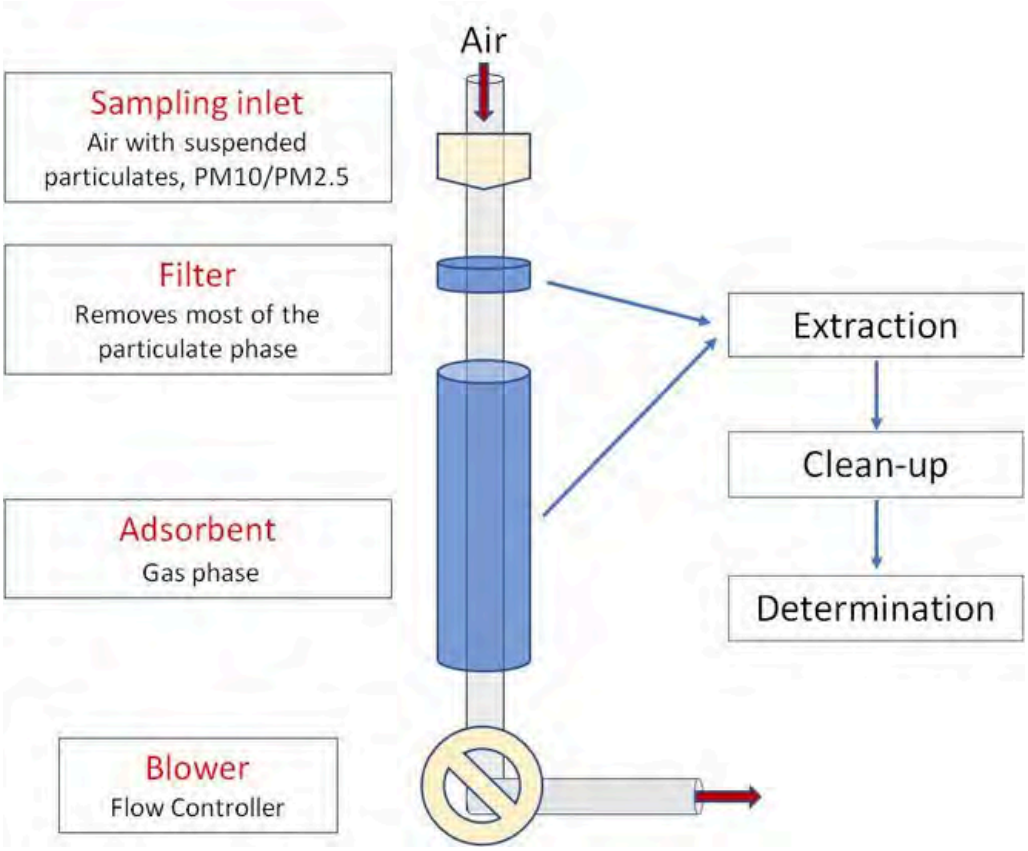


Figure 5.3. Typical procedure in active sampling of air pollutants. Removal of PM10/PM2.5 fractions depend on pore size of the filter.

Figure 5.4. Two consecutive tubes containing sorbent.

The second sampling method is the so called passive sampling, when a sorbent is placed into the air for a longer period of time (e.g. one month) and the pesticide active

ingredients are adsorbed on the surface of the sorbent (Figure 5.5). The partition of compounds between the two phases (air and sorbent) depends on the temperature, which influences the amounts of the adsorbed compounds as well. The most often applied sorbents are the same (PUF and XAD resin), as for the active sampling. Selection of the sorbent determines the selectivity and also the range of adsorbed compounds, which can be monitored. The PUF sorbent can be applied for monitoring of pesticides with low or medium polarity, but the zwitterionic glyphosate and its metabolite (AMPA) are adsorbed only by more polar polyester (PEF) phase. The adsorbed compounds are usually removed by an organic solvent and analyzed.

Calibration is a special issue, as the amount of adsorbed compound can be determined, but we do not know what is the corresponding concentration in the air. Partition coefficients between air and the selected sorbent material are not known for all pesticide active ingredients. To eliminate this problem, determination of sampling rates is performed by active sampling methods. In addition, the partition coefficients depend on temperature as well, which varies during the long sampling period. Therefore these passive sampling methods provide a good possibility a) to detect pesticide active ingredient in the air with low detection limit, b) to follow the long-term trends of pollution pattern c) to have a good estimation of the levels using the active sampling results.

There are also some other less commonly applied methods. Diffusion based devices contain a semipermeable membrane and their selectivity depends on the polarity of the liquid filled into the membrane. These types of passive sampling is also applied for monitoring of water.

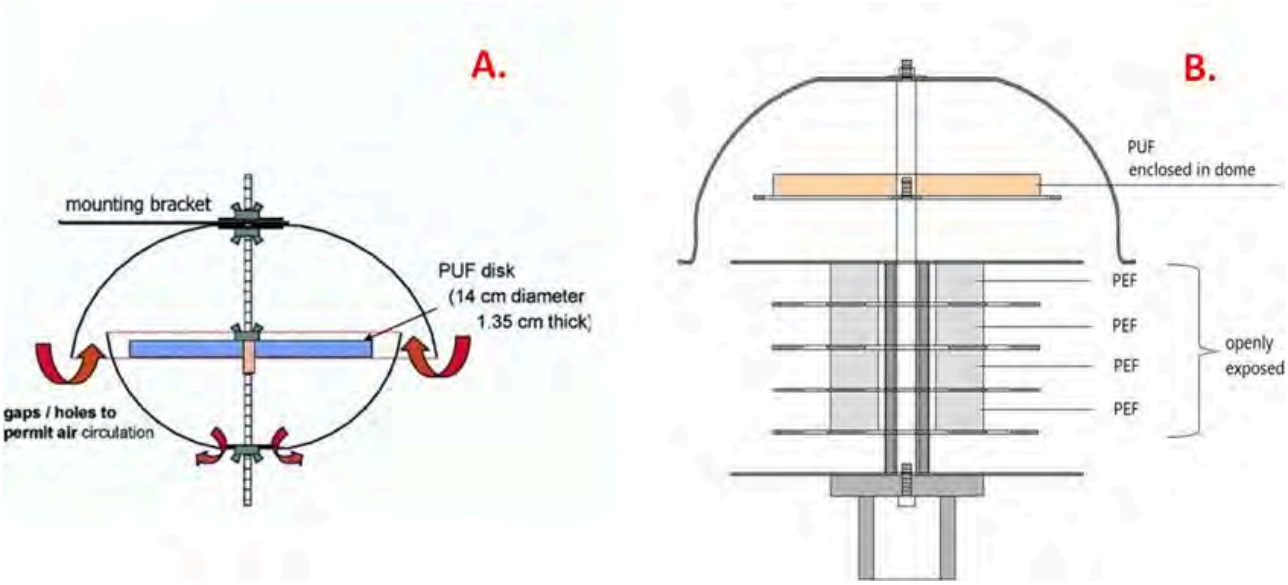


Figure 5.5. Passive sampling devices (source: Milukaitė, A. et al. 2008 and Kruse-Plas, M et al. 2021, see references).

5.2.2 Preparation of water samples prior to determination of pesticide residues

Water samples generally contain more contamination of agricultural origin compared to the air, but it is still recommended to increase the concentration of pesticide pollutants. There are usually other compounds in the sample (matrix components), which cause difficulties in the determination of target components and concentration of the extract results in simultaneously concentration of these matrix components as well. Therefore removal of interfering matrix components (clean up) is often inserted into the sample preparation protocols. This may involve washing steps and/or solvent exchange.

The simplest way to perform extraction in a separatory funnel (Figure 5.6), when the liquid sample (e.g. ground water) is shaken with an immiscible organic solvent or solvent mixture. There are also machines for the longer shaking, then the phases are separated in the funnel. Density of the organic solvent determines which is the upper or the lower layer. Chlorinated solvents are denser than the water (bottom), but hydrocarbons (e.g. hexane) are the upper layers. Non-polar pollutants are not solved in the water phase, but adsorbed on the suspended particulate matter.

The amount of the suspended particulate matter is variable, but it is often present in the environmental water samples (e.g. ground water or surface water). According to the law for water protection in Europe (Water Framework Directive, 2000/60/EC), the whole water body should be analyzed for pollutants of concern. The liquid extraction also removes non-polar components adsorbed to the particles, and the contamination of whole water body will be determined. If filtration of the environmental water sample is necessary, it removes partly or entirely the less polar contaminants as well, then we measure only the concentration of contaminants dissolved in the water.

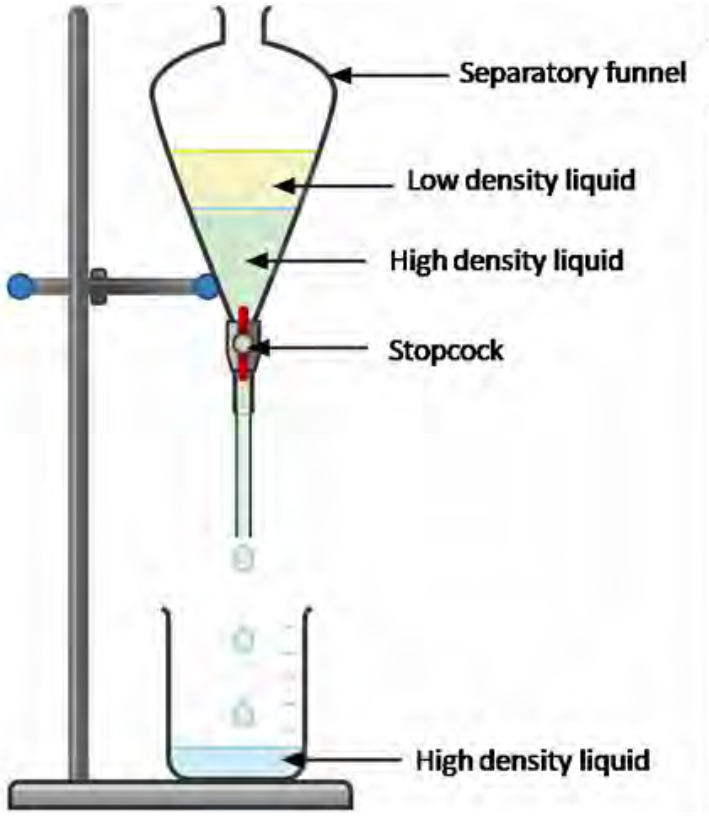


Figure 5.6. Separatory funnel for separation of immiscible liquids.

After extraction, the organic solvent can be evaporated to concentrate the sample. Evaporation of the sample to the dryness allows changing the solvent to another solvent (mixture), which fits for example more to the instrumental determination. As the solvent consumption is high in this procedure, this can be lowered by using less solvent, but keeping the solvent to sample ratios, the sample volumes are considerably reduced as well.

This is more cost-effective and less harmful to the environment, and the same enrichment factor values can be achieved. In some cases, a polar organic solvent (e.g. acetonitrile or acetone) is added to the two immiscible phases (water and e.g. hexane) to facilitate the mixing of two phases and the phase transfer of the analytes. If the separation of phases is not complete, centrifugation is applied.

The solvent consumption is even lower in various liquid phase microextraction methods, for example in single-drop microextraction (SDME), when only a single drop of solvent is used to isolate and preconcentrate analytes from a sample matrix. In a mode of SDME the organic solvent drop held at the tip of a syringe needle, is directly immersed into the water sample. The drop is withdrawn into the syringe after extraction and then injected to the compatible system for analysis.

Another frequently used and powerful sample preparation method is the solid phase extraction (SPE, see also Unit 6.2.). The solid phase (sorbent) is filled into a cartridge and after conditioning of the solid phase, the water sample is loaded to the sorbent dropwise (Figure 5.7.). The solid phase retains the components. The goal of conditioning to remove impurities and to wet the phase. To accelerate the process, vacuum is applied. Then removal of matrix components (washing step) is followed by the elution of target components by an organic solvent. The collected eluate is then evaporated to the dryness (in some cases it is not recommended) and resolved or the volume is set to a known value. Many other varieties of the SPE procedures is known in the literature, depending on the analytical problem. In all cases these procedures should be optimized, including the composition and the volumes of the solvents and also the sorbent selectivity and its capacity should be considered. There are a lot of different sorbent materials on the market, but the most commonly used sorbents are the modified silica or carbon based sorbents. Depending on the chemical modification of the silica surfaces, its polarity and selectivity is tailored. Thus, selection of the appropriate solid phase is based on the polarity target compounds. For special target compounds (e.g. glyphosate and AMPA) molecularly imprinted polymers are also commercially available. Regarding the format of sorbents, there are also more

possibilities, sorbents can be filled into the pipet tips or disks as well. The main drawback of SPE that it is time consuming and clogging often occurs, therefore samples are usually filtered by paper prior to loading. In this way three orders of magnitude or even higher enrichment of the analytes of interest can be achieved, for example from 1 L water sample a 1 mL final extract is measured. There are also commercially available automatic systems to perform SPE sample preparation procedures, which are useful in optimization of procedures and the results are more reproducible as well.

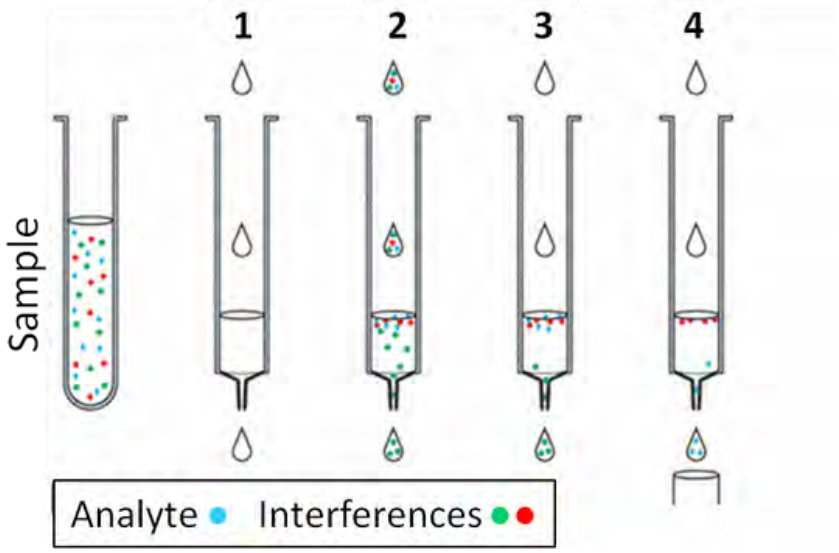


Figure 5.7. Usual steps of the solid phase extraction (SPE): 1. Condition the solid phase, 2. loading the sample, 3. removal of interferences (washing), 4. elution of target components.

5.2.3 Preparation of soil samples prior to determination of pesticide residues

Extraction of a solid (e.g. soil or food) sample can be carried out by manual or machine shaking with an organic solvent or solvent mixture. Ultrasound agitation is also often used to facilitate the removal of compounds of interest from the surface of soil particles. Then it is generally necessary to separate the two phases (solid and organic solvent) by centrifugation. Polarity and solubility of target compounds in the extraction solvent is a crucial point, therefore efficiency of this step is examined by determination of the recovery from the matrix. Pre-concentration of the extract is inevitable in the trace analysis, if the concentration is out of the dynamic range of the instrumental analytical technique. Many other technical solutions are also commercially available to carry out liquid extraction (e.g. microwave assisted), where the extraction of analytes is accelerated by high temperature and/or pressure. Using these systems allow faster extraction and less solvent consumption.

The Soxhlet extraction was frequently used for the extraction of solid samples, but due to its high solvent consumptions, its use has been reduced. Here the sample is filled into a porous thimble and the solvent is boiled (Figure 5.8). The condensed vapor drops to the sample and solves the compounds. If the level of the solvent exceed the

appropriate height in the extraction chamber, the liquid flows down through the siphon arm and the cycle starts again. More cycles are required for the exhausted extraction, but here the fine solid particles do not contaminate the extract.

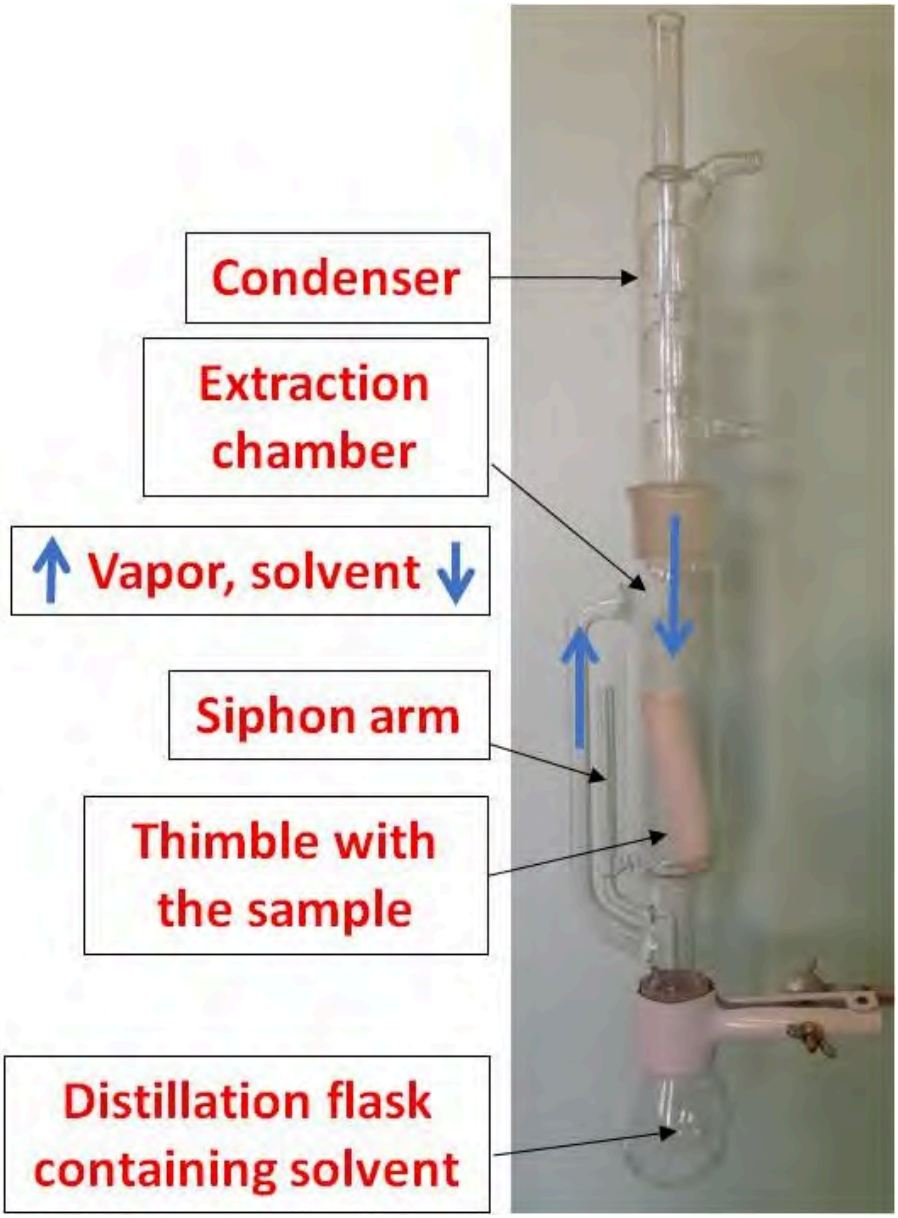


Figure 5.8. Soxhlet extractor. The vapors of the heated extraction solvent condense, drop into the thimble containing the sample. It solves the compounds and when the level collected extraction solvent exceeds the top of the siphon arm, the solvent is returned into the flask.

appropriate height in the extraction chamber, the liquid flows down through the siphon arm and the cycle starts again. More cycles are required for the exhausted extraction, but here the fine solid particles do not contaminate the extract.

5.2.4 The QuEChERS method

Recently the so called QuEChERS method (quick, easy, cheap, effective, rugged, safe) became very popular for multi-residue analysis in food, but it was applied for soil samples as well. Advantage of the method that sample preparation for the determination of pesticide residues in food products was simplified and less solvent is used. Extraction with methanol known as the Alder method was used earlier, but acetonitrile proved to be better for determination of multiple pesticide residues.

The original QuEChERS procedure consists of extracting the homogenized sample by hand-shake or Vortex with the same amount of acetonitrile in order to have a final extract, which is concentrated enough without the need of a solvent evaporation step. High recoveries are achieved even for polar pesticides by including a salting out step, when a proper combination of salts (anhydrous magnesium sulphate and sodium chloride) are added to the extract. Anhydrous MgSO4 is an effective drying agent and also enhances the extraction efficiency. After centrifugation, clean-up and removal of residual water is performed simultaneously. In contrast to SPE procedure, the sorbent is directly added to the extract (dispersive solid-phase extraction, dSPE), and partial removal of matrix components is easily carried out by shaking and centrifugation.

A primary secondary amine (PSA) sorbent and more anhydrous MgSO4 are mixed with the sample extract in dSPE used for the clean-up. PSA is a weak anion exchanger which removes fatty acids, sugars and some other hydrogen bonding matrix co-extractives. The original QuEChERS method (See also Unit 6.4.) has been modified and buffered (citrate or acetate), and the method was validated for the base sensitive pesticides. For fatty matrices silica C18 sorbent is also included in the dSPE to reduce interferences. In some cases graphitized carbon black sorbent is also employed to remove pigments and polyphenols from the extract. Many other varieties were developed depending on the matrix. The procedure has to be optimized (e.g. sorbent types, amount of sorbents) according on the matrix characteristics. QuEChERS based methods are validated as official procedures for the extraction of more than 400 pesticides.

The main disadvantage of QuEChERS versus other common methods is that the 1 g sample/mL final extract concentration is lower than the concentrated extracts obtained with most of the traditional procedures. After the dSPE and centrifugation, the removed supernatant has to be concentrated to achieve the desired limits of quantification (LOQ) in modern analytical systems. If this concentration can lead to analyte losses, injection of large volumes or spitless injection (GC) is needed.

Unit 5.3 Analytical measurement

Mária Mörtl, Borbála Gémes,
Eszter Takács

When we have the homogeneous extract from the studied matrix, we have to analyze the sample for pesticide residues generally by an instrumental analytical technique. Chromatography is the most common method used for separation of sample components, which is based mostly on the partition of compounds between the phases. Chromatographic systems consist of a mobile phase, which flows through the system and the stationary phase. Based on the state of the mobile phase, there are liquid (LC) and gas chromatographic (GC), or less commonly supercritical fluid chromatographic (SFC) systems. Some compounds interact stronger with the stationary phase, and these differences in the retention lead to the different mobility rates and separation of components. Solubility of analytes in the mobile phase (eluent) also influence the partition of the target compounds between the mobile and stationary phase in the liquid chromatography, whereas the mobile phase (carrier gas) has no special interaction with the analytes in the gas chromatography. After the separation compounds enter the detector at different retention times. There are several detection modes, but most important are based on mass spectrometry in the field of pesticide residue analysis. Qualification in the chromatography is carried out by the retention times and some additional information (e.g. mass spectra), whereas quantitation is based on detector signal intensities (peak areas). Calibration of the instrumental analytical method is performed either by external or by internal calibration.

5.3.1 Gas chromatography

One of the most convenient routine analytical techniques is the gas chromatography (GC), which is suitable for determination of thermally stable and volatile organic materials. Typically non-polar and middle polar pesticides are amenable to GC. After extraction and separation of pesticide residues with GC, their selective detection and identification are commonly achieved by a mass spectrometer (MS) coupled to GC. The major components of the gas chromatograph are shown in Figure 5.9. The mixture of pollutants enter the column through the heated inlet, where they are partly or entirely evaporated. The injected volume is usually 1 µL from a liquid, but programmable temperature vaporization (PTV) system, allowing the injection of big volumes of sample (20–100 µl), is also often applied in trace analysis (e.g. pesticide residues). The components are transported by the pressurized carrier gas (mobile phase) through a column containing commonly a liquid phase (stationary phase) coated on the capillary wall (Figure 5.10). Columns with different dimensions are available on the market, but the typical capillary column used for determination of pesticide residues have a length of 30 m, 0.25 mm inner diameter and film thickness of 0.25 µm. Highest resolution (separation of compounds) is afforded by the narrow columns, which provide short retention times and

faster analysis as well. However, the sample capacity for thin-film columns is low and they require high-sensitivity detectors.

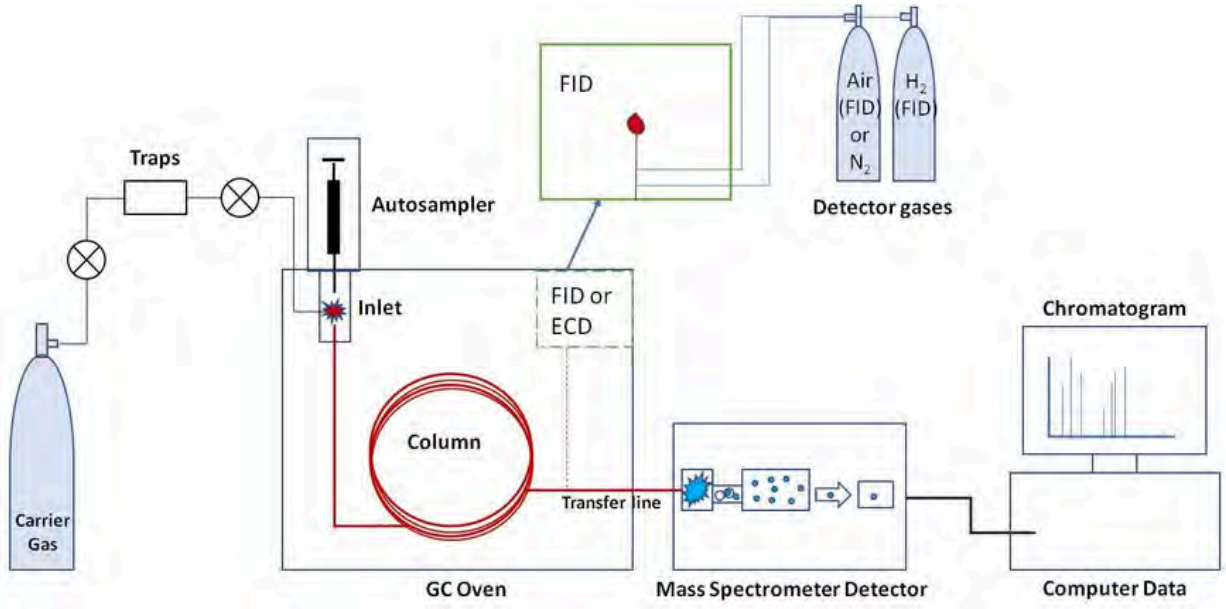
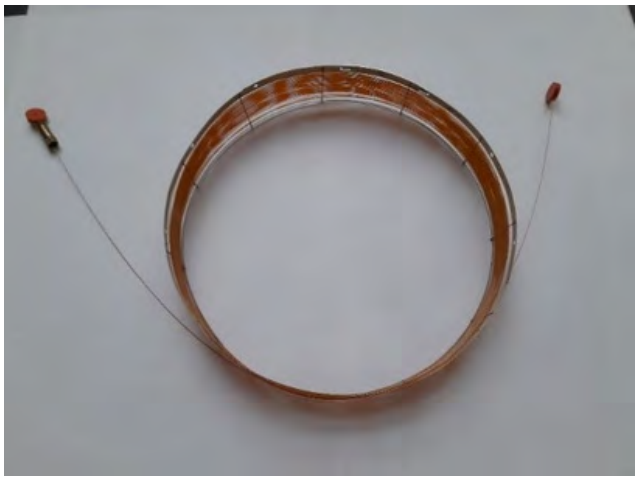


Figure 5.9. The major components of the gas chromatograph.



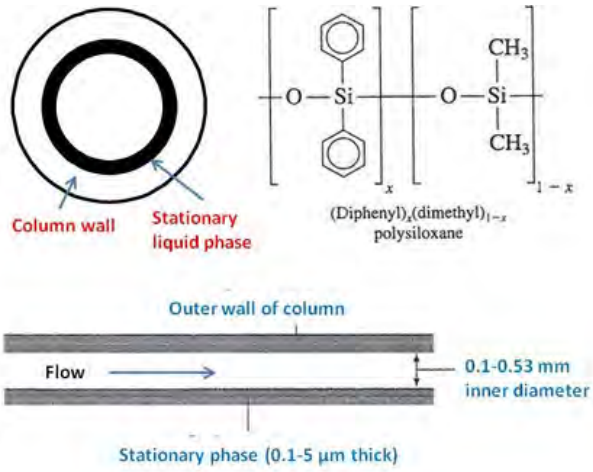


Figure 5.10. Wall coated open tube (capillary column) and the structure of the most common stationary phase.

Each component will be partitioned between the carrier gas and the solid (Figure 5.11). As the carrier gas moves, the components leave the stationary phase, then they move and solve again in the gas. Partition coefficients depend on the characteristics of analyte and the stationary phase, and strongly influenced by temperature as well.

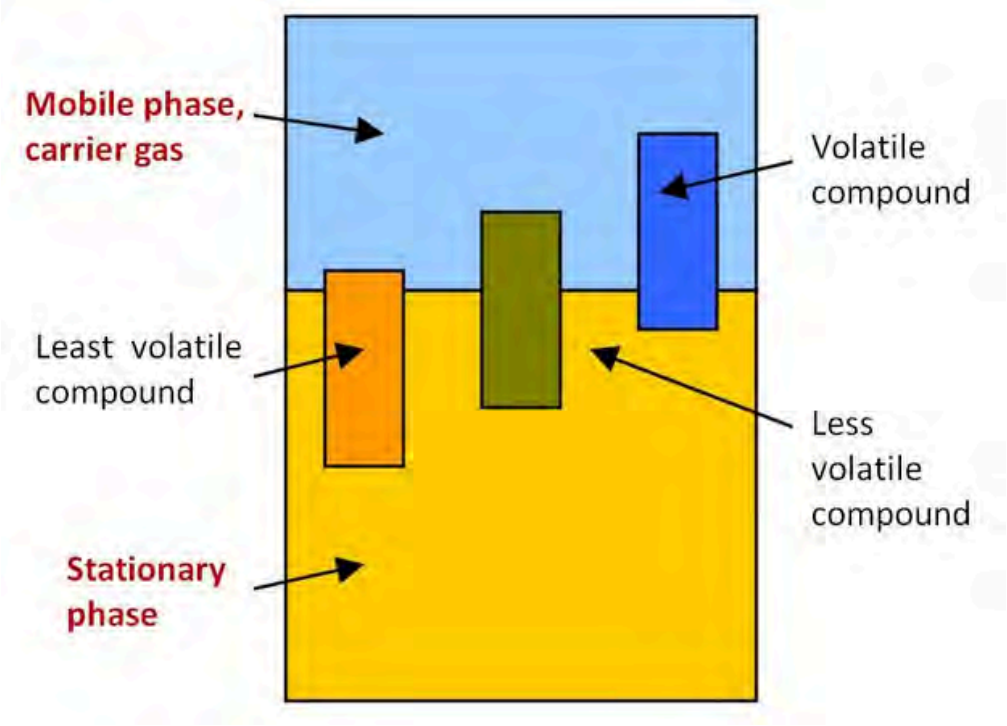


Figure 5.11. Partition of volatile components between the mobile and stationary phases.

Isothermal measurements, when the oven containing the column is maintained at a constant temperature throughout the analysis are rarely performed. For the determination of pesticide residues, the temperature programming is performed due to the wide polarity and volatility (boiling point) range of pesticide active ingredients, and to prevent

excessive broadening of later eluting peaks. Application of temperature gradient including sometimes multiple ramps in oven operation reduces analysis time and produces sharper peaks. According to the rule of thumb “Like dissolves like”, the interaction of the polar analyte with polar stationary phase is stronger, thus it is more retained. Although equilibrium is not achieved as the mobile phase moves, but differences in the partition results in separation of components.

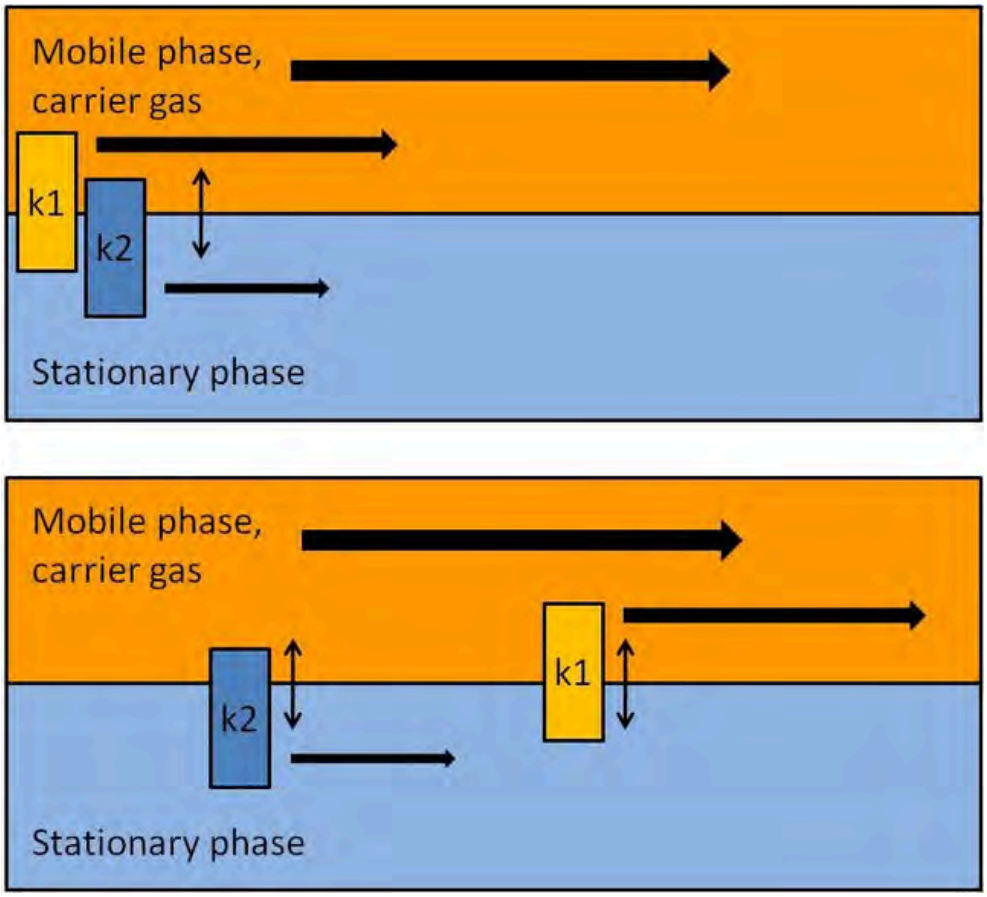


Figure 5.12. Differences in partition of components between the mobile and stationary phases results in differences in their mobility rates.

The less volatile compounds spend more time in the stationary phase (Figure 5.12), whereas all compounds spend the same time (dead time) in the mobile phase. Based on their different retention by the stationary phase, they will emerge from the end of the column at different retention times, which in an important parameter to identify a sample component.

Chromatogram is a graph showing the detector response as a function of time (Figure 5.13). The retention time for each component is the necessary time for the pesticides to reach the detector after the injection of the mixture. There is a minimal time, which is required for the unretained component to travel through the system, which is called dead time. Retention times are often corrected (adjusted) with it in further calculations. As the exact retention time depends on chromatographic conditions (temperature program, flow rate, column dimensions, etc.), therefore to compare the results obtained in different chromatographic systems, to facilitate method transfer or identification of compound, retention indices were defined.

These are valid to the component separated on the same stationary phase, but almost independent from other parameters. The most frequently used is the Kovats

retention index relates the retention time of a solute to the retention times of linear (normal) alkanes measured under the same conditions. The peak area is the parameter used to measure the quantity of the sample component, as it is proportional with the amount of the analyte.

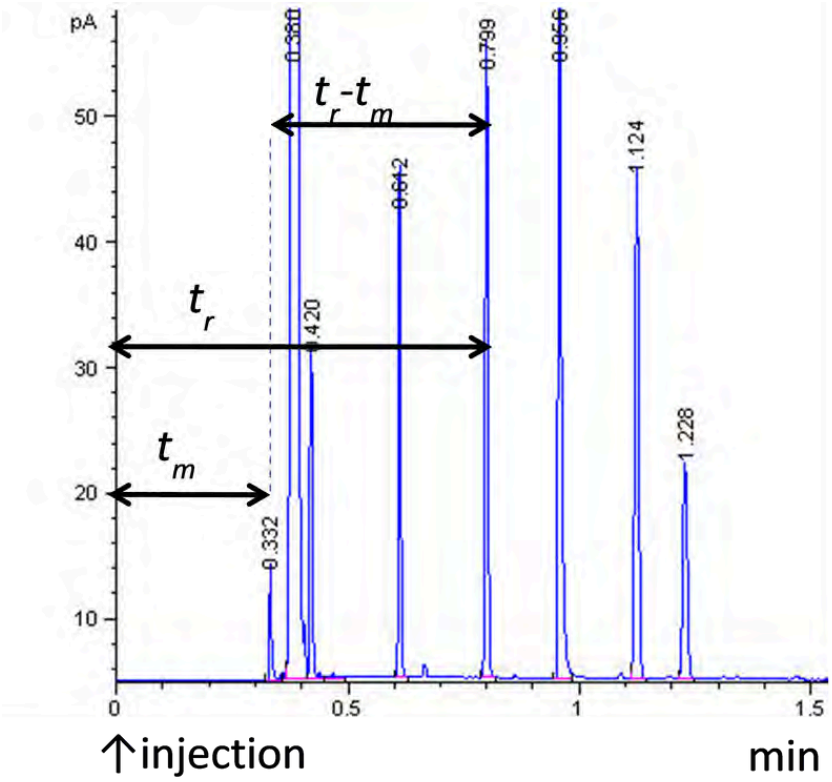


Figure 5.13. Typical chromatogram. t_m is the retention time of an unretained component, t_r =retention time, $t_r - t_m$= adjusted retention time.

There are a lot of commercially available stationary phase, which have different polarity and selectivity. Polydimethylsiloxane is a non-polar stationary phase, which separates the components according to their volatility. Polarity and retention of polysiloxanes is increased

especially for aromatic compounds by substitution of methyl groups with phenyl moiety, but other substitutions for special purposes (e.g. chiral separation) are also put into practice. The most polar phases are polyethylene glycol based (WAX) phases. The most commonly applied stationary phase for the separation of pesticide residues is the non-polar dimethylpolysiloxane contaning 5% of phenyl groups. When the separated compounds elute from the column, they enter the detector, which responds to sample components. Different detectors (e.g. FID=flame ionization detector, ECD=electron capture detector) are applied, but for the recognition (identification) of component mass spectrometry is the most convenient method (see later). ECD has a special role in the field of pesticide analysis. Among the pesticide active ingredients, especially the first period in the 1960s, there were many, which contained halogens, mainly chlorine, but also bromine. Before the mass spectrometers became commonly used routine equipment, detection of these residues was performed by ECD. In this device, the electrons are emitted from a β-radiating 63Ni foil, which ensures a stable electrical current and signal in the detector cell (Figure 5.14). Electrons produced by the radioactive source ionize the nitrogen molecules (makeup gas) flowing through the

detector cell and migrate to the anode. If the entering molecule contains electron withdrawing groups (halogens, nitro, carboxyl etc. groups) and it is able to capture electron, then the absorption results in reduction in the number of electrons and thus the current (detector signal). ECD is more sensitive, when an aromatic system is bound to the electron withdrawing group making possible the delocalization and stabilization of the negative charge. There is no response for other molecules, but ECD offers selectivity for halogen containing pesticides (e.g. DDT, lindane, deltamethrin, etc.) that can be determined at very low levels, occasionally even at lower levels compared to MS (Figure 5.15).

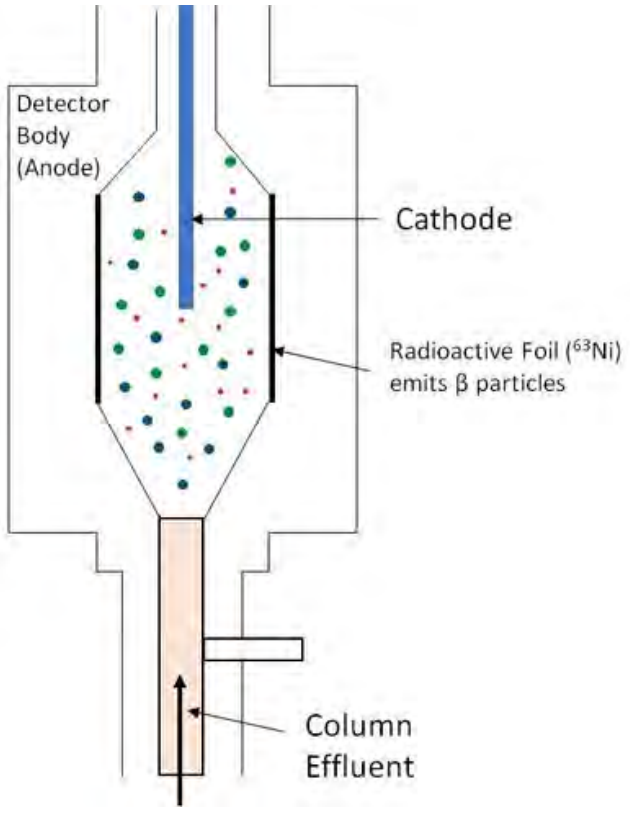


Figure 5.14. ECD detector.

Therefore it can be used for confirmation of those components, for which ECD is selective and more sensitive than MS and it presents a useful complement. This tool was also used to demonstrate the presence of chlorofluorocarbons (CFCs) in the atmosphere over the Atlantic Ocean between England and Antarctica, which pollutants were proven to react with stratospheric ozone in a chain reaction causing the depletion of ozone (ozone hole).

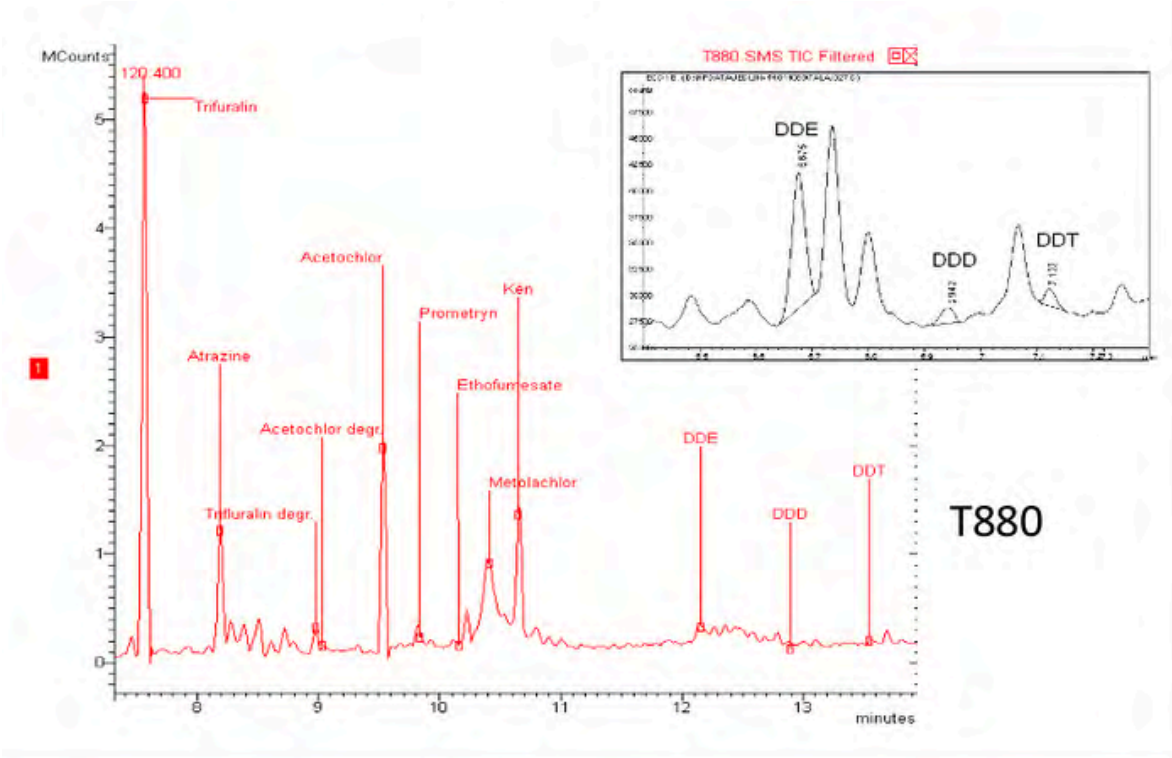


Figure 5.15. The total ion chromatogram (red) and the chromatogram of the same sample recorded by ECD (black). DDTs (DDE and DDD are DDT metabolites) have low signal intensity at the levels present in the soil extract recorded by MS, but they are confirmed by paralel measurement using ECD.

5.3.2. Mass spectrometry coupled to gas chromatography

Selective detection and identification (qualification) of pesticide active ingredients are generally performed by mass spectrometry. Although qualification is based on retention times, but some additional information is also required to reveal the structure. Earlier either the measurement of retention times on two different stationary phases and/or application of selective detectors (e.g. electron capture detector for halogen containing compounds) were the proofs to identification of a pesticide active ingredient. These are useful and in some cases convenient tools, but nowadays the mass spectrometric detection mode is the most commonly used technique in the field of pesticide residues. Single quadrupole (SQ) is more frequently used, than the more expensive triple quadrupole (QQQ) instruments, which provides selective and sensitive detection only for those compounds, which are included in the analytical method. When the molecules enter the mass spectrometer, they are ionized by high energy electrons in the high vacuum (Figure 5.16).

This electron impact is followed by so called fragmentation, when different parts of the molecule split/cleavage upon the collision and then the produced ions are separated by their mass-to-charge ratio (m/z). Generally single charged ions are formed in GC-MS, and the ion intensities as a function of mass gives the mass spectrum (Figure 5.17).

The detector response, which is proportional with the total number of the ion as a function of retention times is the total ion chromatogram (Figure 5.18). To all peaks belongs a mass spectrum. As fragmentation occurs at weak bonds, the mass spectrum is characteristic to the structure of the molecule.

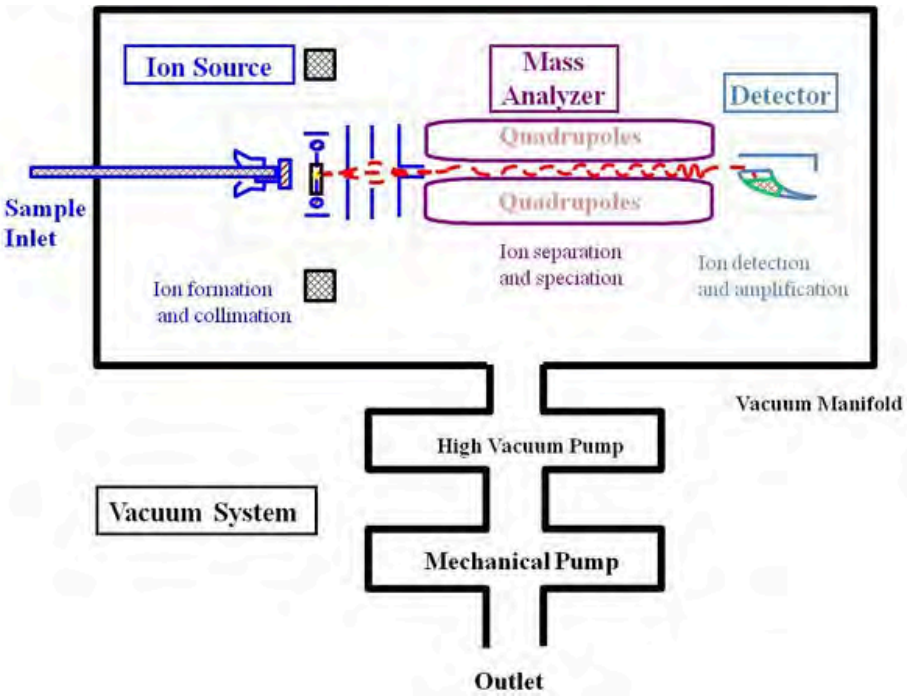


Figure 5.16. Main parts of a single quadropole mass spectrometer.

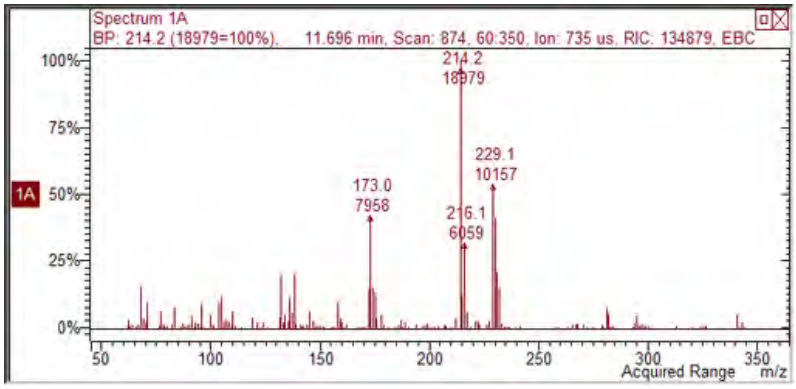


Figure 5.17. Mass spectrum of terbuthylazine measured in water sample extract (TIC is shown in Figure 5.18) at 11.7 min. Molecular ion M+=229, base peak (100%) m/z=214, qualifier ions m/z=173 and 229.

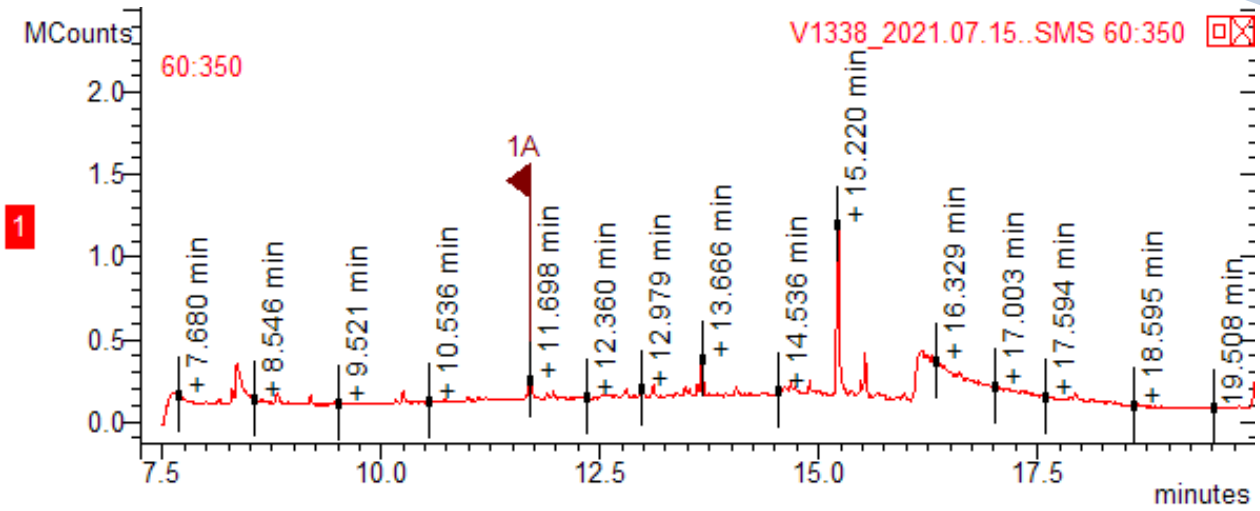


Figure 5.18. The total ion chromatogram (TIC) of a water sample, which contained terbuthylazine (retention time 11.7 min).

Mass spectra depend on the energy of the impacting electrons in a certain region, but they became unchanged above a limit. Mass spectra recorded at 70 eV are suitable for identification of unknown compounds, which is based on comparing the measured mass spectrum with those stored in database (Figure 5.19) or with that of recorded for the standard solution of the corresponding analyte under the same conditions (Figure 5.20). The most intensive ion is the base peak and the intensity of other fragment ions are given as the ratio to the intensity of the base peak. Molecular ion has often low intensity, but the other characteristic fragment ions and their intensity ratios serve as a fingerprint in the qualification (see Section 5.4.2.). It is not necessary to identify all the fragment ions, but if the fragmentation pattern of an unknown analyte and known pollutants match, then the probability is high that the two compounds are the same. Of course retention time for the compound, or more precisely retention index measured on the

same stationary phase should be also in accordance with the literature data. These are also stored in different data bases (e.g. NIST, https://webbook.nist.gov/chemistry/).

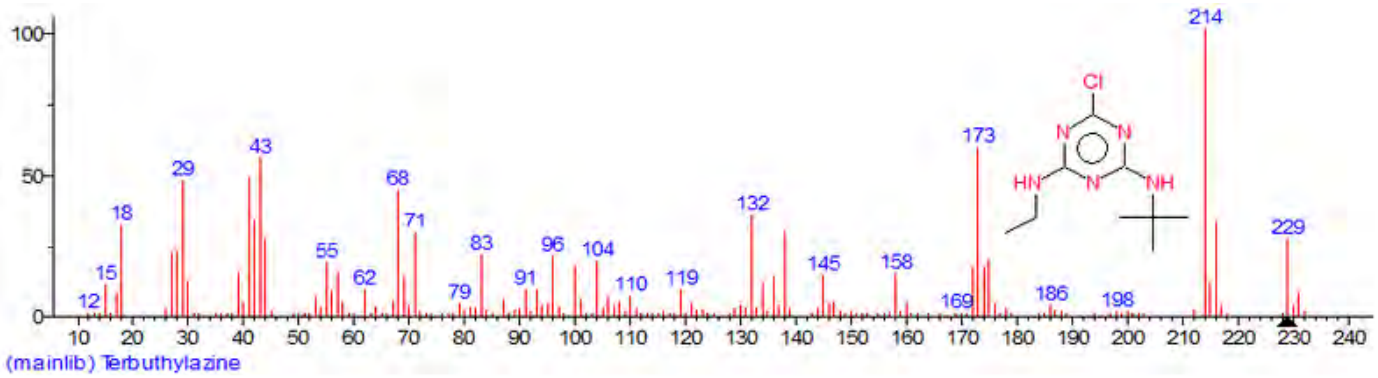


Figure 5.19. Mass spectrum of terbuthylazine, stored in NIST data base.

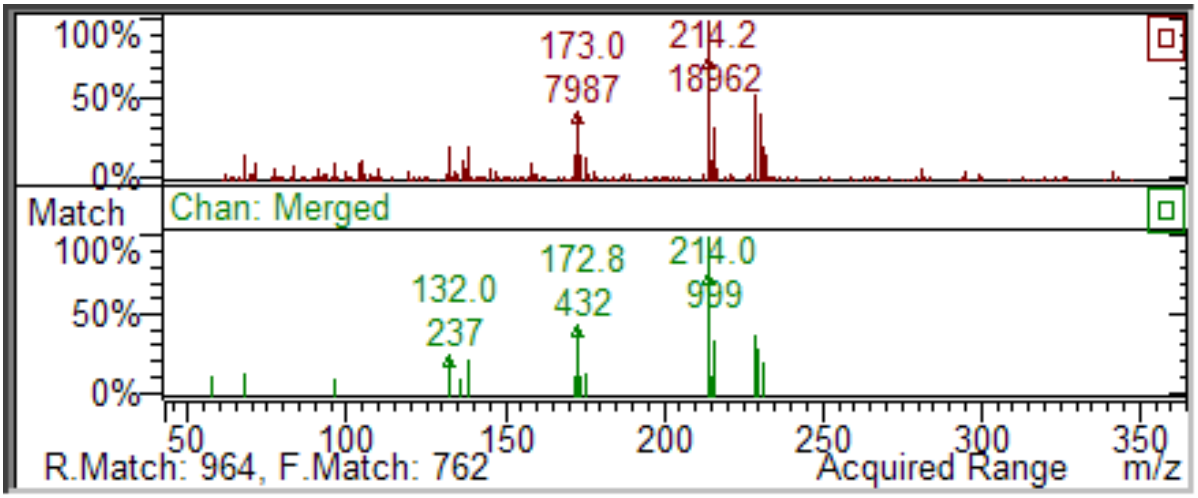


Figure 5.20. Mass spectrum of terbuthylazine (red) measured in water sample extract (Fig. 5.18.) at 11.7 min (upper) and that of recorded earlier (green) by using the same method for terbuthylazine standard solution (stored simplified in the method).

Quantification is carried out by using the calibration curve, as detector signal intensity is proportional with the concentration of analyte in the dynamic range of the detector. The used calibration is often an external

calibration, when the detector response is recorded for different levels of the analyte. Although the total ion intensity is suitable for calibration, but quantification is usually performed by using the intensity of the most intensive characteristic ion to improve selectivity and eliminate some of the possible co-eluting compounds (Figure 5.21).

Sometime solutions of the standard materials (pesticide active substance) are diluted by analyte protectant or the whole sample preparation is performed with the spiked samples (matrix-matched calibration). During the spiking known amounts of the analyte are added to the matrix and these are prepared as the sample itself and these extracts are used as calibrating solutions. This method is recommended in those cases, when the presence of the matrix significantly alters the detector signal intensity (matrix effect). The effect is less common in the GC measurements and related usually to the adsorption of the analytes in the sample inlet. Calibration curve is based either on the total ion or on most intensive characteristic ion intensity, but there are some other possibilities as well.

For example, the selected ions can be also recorded in the retention time window of the target component (SIM-selective ion monitoring). This allows more selectivity and lower detection limits. Even more detection modes are available by

using a triple quadrupole (QQQ or GC-MS/MS) instrument. Higher selectivity is achieved, if we choose a characteristic fragment ion (parent ion), then it is further fragmented and the intensities of both ions (parent and daughter ions) are recorded.

In this way interfering matrix components are eliminated, and only the fragments characteristic to the target molecules are monitored and detected in the timeframe set in the method. Worthy of note that other fragments, which are not selected in advance are not detected. Due to its high selectivity and low LODs, GC-MS/MS is commonly used for the determination of pesticide residues from food samples, but also frequently used in the field of environmental analysis.

There are other even more expensive mass spectrometric techniques, where retrospective analysis of the measured sample is possible and a new evaluation may result in discovery of a new pollutant. For example time of flight (TOF) mass spectrometers based on their exact mass measurement capacity are suitable to detect pollutions, which were not considered earlier (e.g. doping crime). All details related to the analytical tools provided by mass spectrometry cannot be given here, but the most often applied analytical techniques are the GC-MS and GC-MS/MS for determination of pesticide residues in food and in environmental samples.

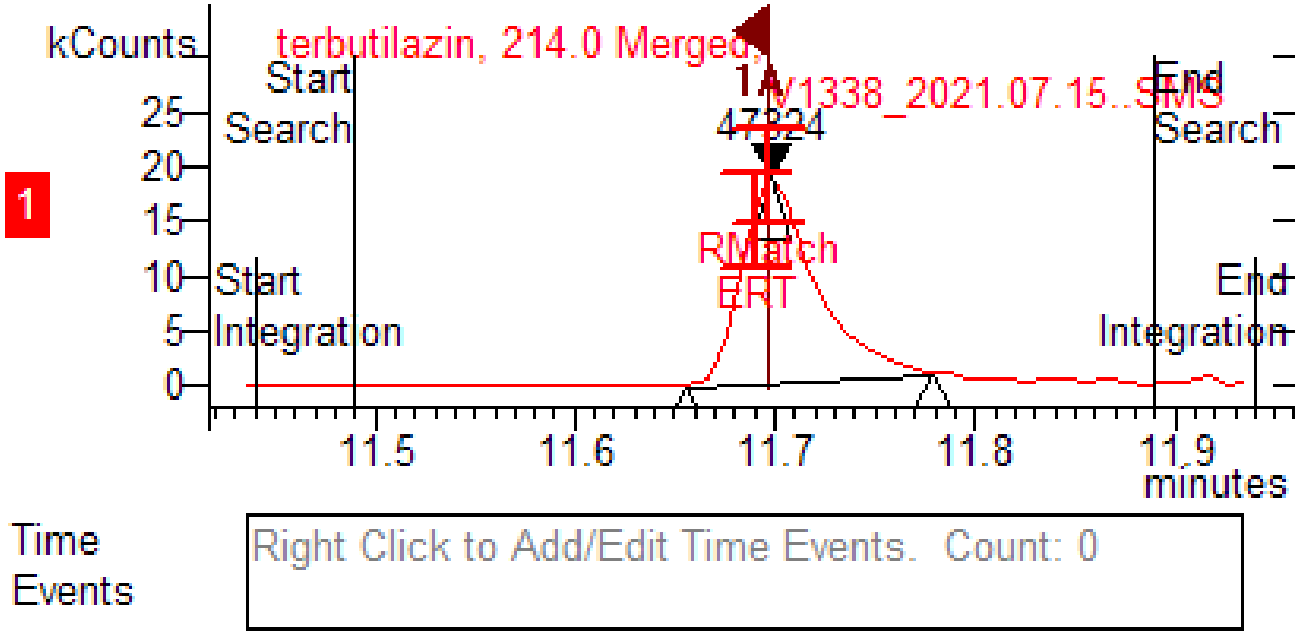


Figure 5.21. Intensity of the m/z=214 ion in the region of terbuthylazine retention time. The peak appeared close to the retention time recorded earlier by using the same method for terbuthylazine standard solution (ERT) and the time, when the measured mass spectrum is similar to that of the standard (RMatch). All these confirm the presence of terbuthylazine in the sample.

5.3.3. Liquid chromatography

More polar and in some cases thermally labile pesticide active ingredients have been emerged in the decades on the market. The new ingredients are often systemic and therefore they spread in the whole plant to protect it not only locally against the pest. In contrast, contact ingredients are less polar and if they are thermally stable, then GC-MS is appropriate instrumental analytical technique to determine them. There was a crucial need for the development of new instrumental analytical methods for the determination of polar or thermally labile compounds in many fields, including in biological samples. The separation and detection of polar pesticides is generally achieved with liquid chromatography (LC) coupled to a mass spectrometer. In contrast to GC, volatility is not required in the LC, but sample must be soluble in the mobile phase. Analysis in LC is carried out close to room temperature, whereas the target molecule must be able to survive high temperature in the injection port and column in the GC. There is no upper theoretical limit regarding the molecular mass and the most polar compounds are also amenable in LC (Fig. 5.2.). Comparing the GC and LC, both stationary phase and mobile phase take part in the separation by LC, while mobile phase is only an inert sample carrier in GC. Although GC-MS is still a convenient technique for determination of (semi)volatile, non-polar pesticides (e.g. organochlorine, triazines or

pyrethroids), most of the currently used pesticides are of medium or high polarity, which fit better with LC-based methods. Nowadays for large-scope screening/determination of pesticides with wide polarity and volatility range, the combined use of both methods (GC-MS/MS and LC-MS/MS) appears as the most efficient approach in the field of multi-residue analysis.

After extraction of pesticide active ingredients and concentration of the extract, the final solution is usually supplied with internal standard solution and injected to an LC instrument (Fig. 5.22). There are a plenty of varieties belonging to the high performance liquid chromatographic (HPLC) techniques including the different stationary and mobile phases and detection modes as well. In the field of pesticide residue analysis the reversed phase chromatography is the most commonly applied when the mobile phase (liquid) is more polar than the stationary phase. Modified silica containing octadecyldimethylsilyl (C18) groups on the surface is dominant for the separation of components. Target compounds are adsorbed on the surface of the particles, but their partition between the mobile and stationary phase is usually different and based on this they can be separated.

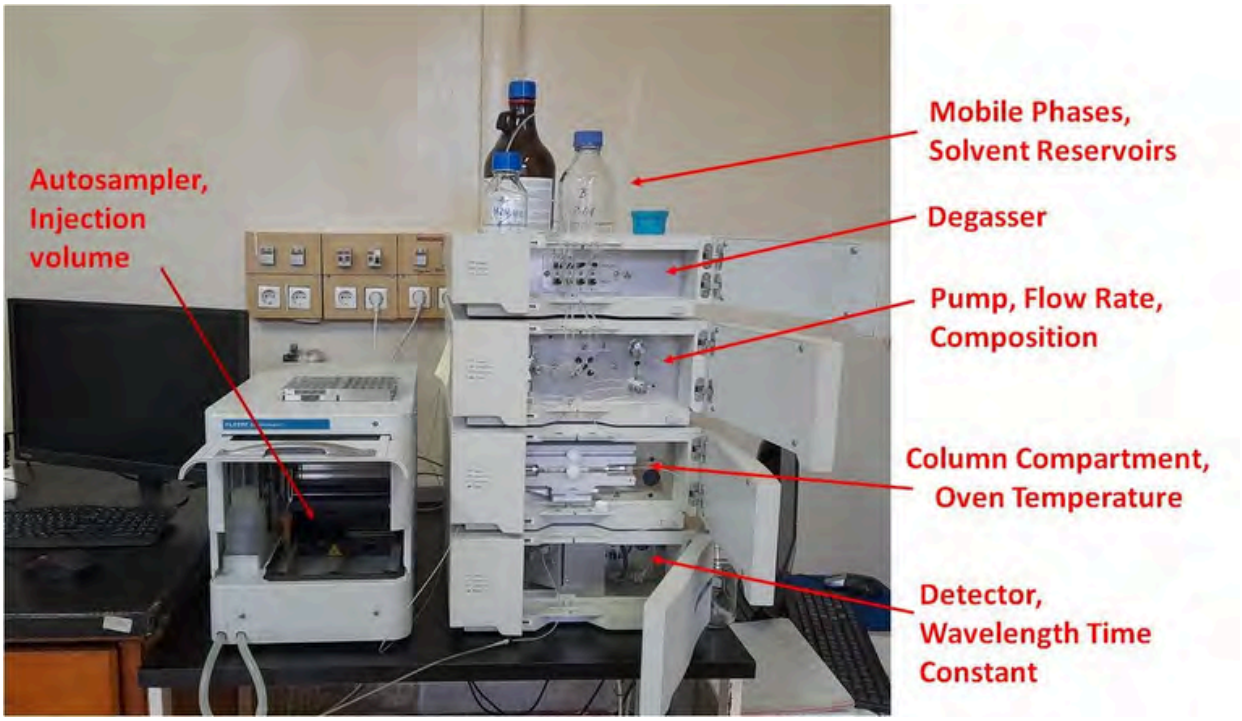


Figure 5.22. The main parts of an HPLC-UV system.

Stationary phase is filled into the columns which lengths are typically 5-30 cm, diameter is 0.4-0.46 cm and the particle size is 3-5 µm in the conventional HPLC (Fig. 5.23.). As relatively broad peaks are obtained with these columns, they can be applied for the separation of simple mixtures. Separation of complex mixtures and identification of many components (e.g. multi-residue methods) requires more effective separation resulting narrow peaks in short time. Nowadays smaller particles, around or less than 2 µm, and smaller inner diameter columns (e.g. 0.2 cm) are used. Higher pressure is required to maintain the optimal flow rate in this system, therefore these are often called UPLC instruments.

The mobile phase (eluent) in the reversed phase chromatography consists of water and the organic solvents

Figure 5.23. HPLC columns of different sizes.

are typically acetonitrile or methanol. The solvent delivery system has three basic functions providing accurate and constant flow rate, accurate mobile phase compositions and the force necessary to push the mobile phase through the tightly packed column.

The composition of the eluent determines the solubility of the analytes as well, which is one of the most important parameter in LC. Elution strength in the reversed-phase LC increases from water to methanol and further to acetonitrile. Isocratic elution, when the mobile phase composition remains constant throughout the time of the chromatographic run, are rarely applied due to the general elution problem, occurring with the mixture of wide range in polarity (e.g. pesticides). These include poor resolution of early eluting peaks, increase in peak width and decrease in peak height for later eluting peaks, long analysis times and risk of column contamination with strongly retained components. Therefore gradient elution is applied, when the mobile phase composition is changed during the separation. Increasing the ratio of organic modifier (methanol or acetonitrile) in the eluent results in shifting of partition to mobile phase and this increases simultaneously the velocity of less polar compound.

In addition to the ability to separate complex samples within a shorter analysis time, the further advantages of gradient elution are the improved resolution and detection due to sharper peaks, and it decreases also the column deterioration due to strongly retained components. Unfortunately the column re-equilibration with the initial eluent compositions takes some time, which lengthen the whole chromatographic cycle.

The other main factor is the interaction of the analyte with the stationary phase, which is influenced by the polarity of substances. Less polar compounds are more retained by the non-polar stationary phases (e.g. C18) in the reversed phase chromatography, whereas the more polar target compounds move rapidly, as their affinity to the mobile phase is higher. Separation of components is based on the differences in the partition similarly to GC (See Fig. 5.11. and 5.12.), but in contrast to GC, the mobile phase is not inert in LC, it takes part also in the interaction with the analyte resulting in differential migration rates. Instead of volatility, the solubility of the analyte in the mobile phase and its interaction with the stationary phase have crucial role in LC. There are hundreds of different stationary phases to be purchased, but the most often applied are the C18 modified silica phases. For special purposes (e.g. chiral separation) many other possibilities are commercially available.

Column dimensions have significant role in the solvent consumption, as the optimal flow rate depends on these parameters. Shorter column length allows shorter retention times, but generally separation of a complex mixture is required in pesticide analysis. Changing of other parameters decreases more effectively the solvent consumption. For example using of columns with smaller internal diameters is advantageous in trace analysis, especially if sample amounts are limited. Disadvantage is that the column frits prone to clogging. Another way to shorten the analysis time and decrease the solvent consumption as well is to reduce the particle size. In addition we may decrease column length as well, if the baseline separation of peaks is ensured. Changing of column is effective only in those cases, if the instrumentation have low extra-column volumes (detector, tubes, etc.), otherwise the desired improvement in separation (resolution of peaks) will not be achieved.

The most common detector coupled to LC is based on absorption of the UV light (HPLC-UV). This is a simple and convenient detection mode, but it gives satisfactory structural information for the identification of compound only in rare cases. However, absorption of the UV light at different wavelengths or even the whole UV spectrum can be recorded by the appropriate device, but this is not enough to confirm the pollutant, only false positive results can be excluded in some cases or undesired matrix interferences caused by a co-eluting compound can be observed.

5.3.4. Mass spectrometry coupled to liquid chromatography

As for the GC, mass spectrometric detection modes together with the retention time provide the information required to the identification of pesticide. The main difference between the MS coupled to LC compared to that of coupled to GC is the interface, which connects the chromatographic instrument to the mass analyzer. Mass spectrometers operate in high vacuum, therefore the coupling (interface) have to solve the evaporation of the solvent and somehow the analyte has to be ionized as well. For this purposes two main strategies (ESI and APCI) are applied, and many compounds will work well with both, however, analyte characteristics may determine the ionization source chosen. Electrospray Ionization (ESI) is predominant among the Atmospheric Pressure Ionization (API) techniques. Nebulization and ionization takes place simultaneously, eluent is removed and ions enter into the MS (See Fig. 5.24.). First, the HPLC effluent with ions in the solution emerges from the tip of the nebulizing needle, and the strong electric field (potential difference) between the nebulizer and the counter-electrode charges the surface of the emerging liquid and forms a fine spray of charged droplets. These droplets migrate through a counter flow of hot nitrogen drying gas, which drains the droplets and the uncharged material flows out of the system by gravity to the drainage port.

Evaporation results in shrinking of the charged droplets, and if the repulsive electrostatic (Coulombic) forces exceed the droplet cohesive forces, then the droplets repeatedly explode (See Fig. 5.25.). Successive explosions lead to solvent-ion clusters and finally to the ions in the gas phase. The emerging ions are then passed through the capillary sampling orifice into the low pressure region of the ion source. As ions form in solution and ionization takes place under mild conditions, therefore thermally instable compounds are also suitable for ESI. Regarding the analyte, no volatility is necessary, but some polarity is required. During the ESI process multiple charged ions may form as well. Relatively higher flow rates up to 1 mL/min can be applied.

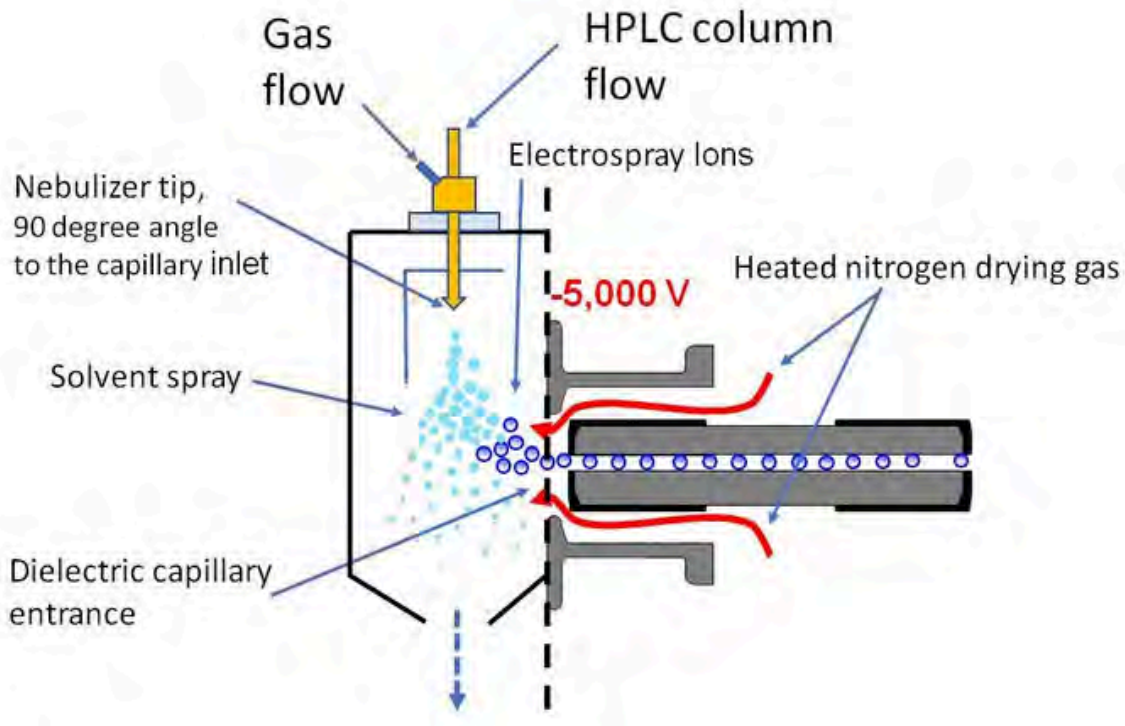


Figure 5.24. ESI LC-MS interface.

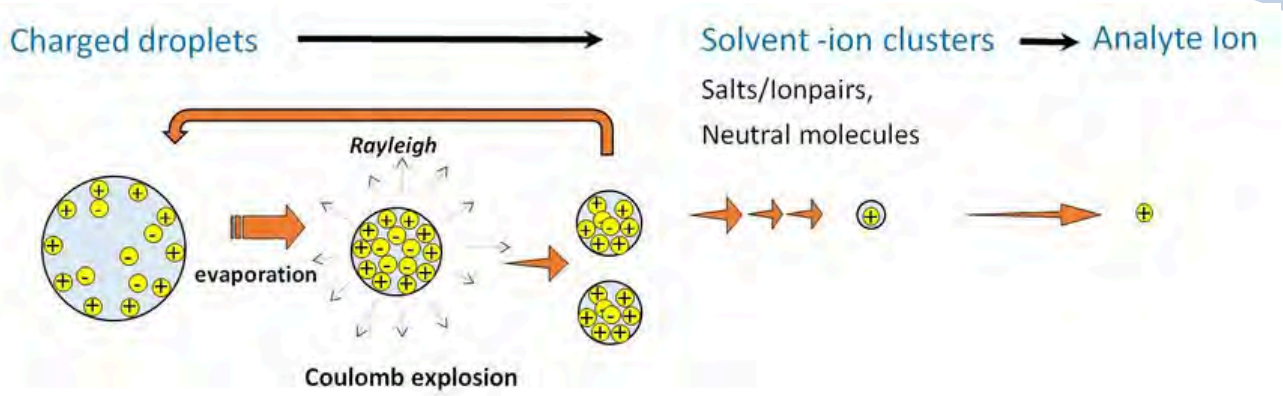


Figure 5.25. ESI process: ionization followed by evaporation.

Another way of atmospheric pressure ionization is the chemical ionization of the analyte in gas phase (Atmospheric Pressure Chemical Ionization, APCI). First the liquid analyte is nebulized into small droplets, followed by evaporation of droplets to produce gas phase analyte molecules (Fig. 5.26.). Heating also contributes to the removal of solvent and the rapid vaporization preserves the molecular identity of the sample, the thermal decomposition is minimal. The corona discharge needle serves as a charge source, providing electrons for the formation of reactant positive ions. These primary ions ionize the mobile phase solvent molecules by collision with the reagent ions induced by a corona discharge needle. Typically, the mobile phase becomes the source of reagent ions, then the gas phase analyte is ionized by collision with the reagent gas formed from the eluent (Fig. 5.27.).

Proton addition $(M+H)^+$, proton abstraction $(M-H)^-$, or electron capture $(M)^-$ are the common processes in this chemical ionization. For positive mode, the solvent must be capable of donating a proton and the analyte must have higher proton affinity than the reagent gas.

For example, acetonitrile is not a protic solvent, because it does not have a proton to donate. In addition, water in the gas phase is rather a strong base, because heterolytic cleavage of a bound is not favored in the nonpolar gaseous phase. Therefore, acetonitrile/water is not a good solvent choice for ACPI positive mode, despite the fact that it is a commonly used as a mobile phase. In negative mode APCI, the reagent gas must be able to abstract a proton or capture an electron. Similarly to ESI process, heated drying gas is still used, but at a lower flow rates. As the ions are generated in gas phase in the APCI process, therefore thermal stability and some volatility of the compounds is required, but no polarity is necessary.

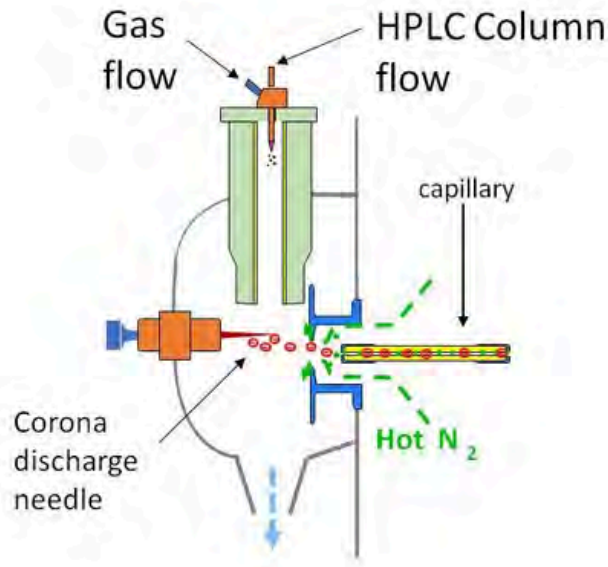


Figure 5.26. APCI LC-MS interface.

The APCI interface is used for low to medium polarity analytes, where evaporation is followed by chemical ionization resulting in usually only single charged ions. In special and rare cases the

Atmospheric Pressure Photoionization (APPI) is applied, when a photoionization takes place in gas phase directly, or indirectly, when the analyte is ionized by dopant ions formed upon irradiation.

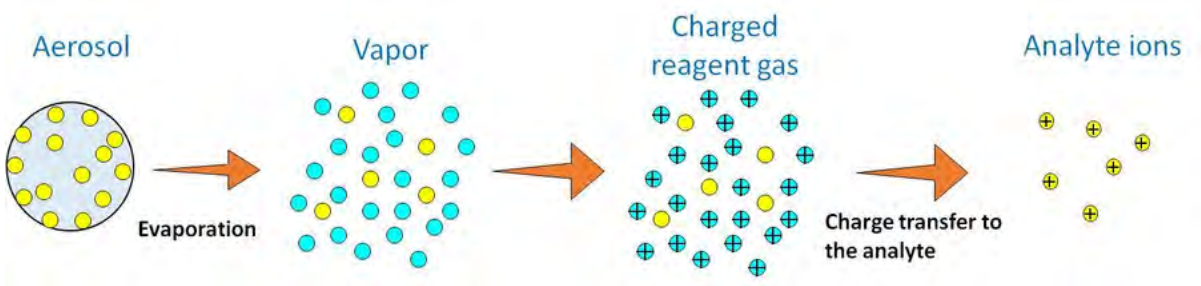


Figure 5.27. APCI process: evaporation is followed by chemical ionization.

Using the above ionization methods and then separation of the ions produced according to their mass-to-charge ratios (scanning mode) gives us the single quadrupole mass spectrum. As ionization in LC occurs under mild conditions, therefore fragmentation is generally not so extended, mass spectra are more simple compared to those typically recorded in GC-MS. Protonated molecular ion [M+H]+ is commonly the most abundant ion in positive mode, but formation of other adducts with the co-eluting components (e.g. sodium, ammonium, etc.) is also often observed. In addition, ionization efficiency and fragmentation are influenced by the applied parameters and the instrumental design as well. Parameters include among others the composition of the eluent, the temperature and flow rate of drying gas, ionization mode, which are depending on the applied method and instrument as well.

These parameters can be adjusted to obtain optimal performance of the analytical method. The best choice is to record the mass spectrum under the same conditions and built an own data base for identification of compounds. In some cases more or less expensive databases developed for a dedicated application, are provided by the instrument manufacturers.

In addition to the above mentioned detectors, other MS based devices and detection modes are also applied. Other mass selective detector types like time-of-flight (TOF) are more expensive, but they are very fast and based on their exact mass measurement capacity identification of target molecules can be easily done even more years after the measurement. The softness of API results in molecular ions of analytes, but for confirmation of compound identity detailed structural information is desired. By using a triple quadrupole MS (LC-MS/MS), the selected ion (parent ion) is further fragmented by collision gas (e.g. nitrogen), and intensity of daughter ion is determined (Fig 5.28.). The degree of fragmentation is compound-dependent and higher ion energies lead to more extended fragmentation. The other compounds are not detected here, only the target compounds are measured in the timeframe specified in the method. Qualification is based on the measured retention times and ions completed with their intensity ratios.

These parameters can be adjusted to obtain optimal performance of the analytical method. The best choice is to record the mass spectrum under the same conditions and built an own data base for identification of compounds. In some cases more or less expensive databases developed for a dedicated application, are provided by the instrument manufacturers.

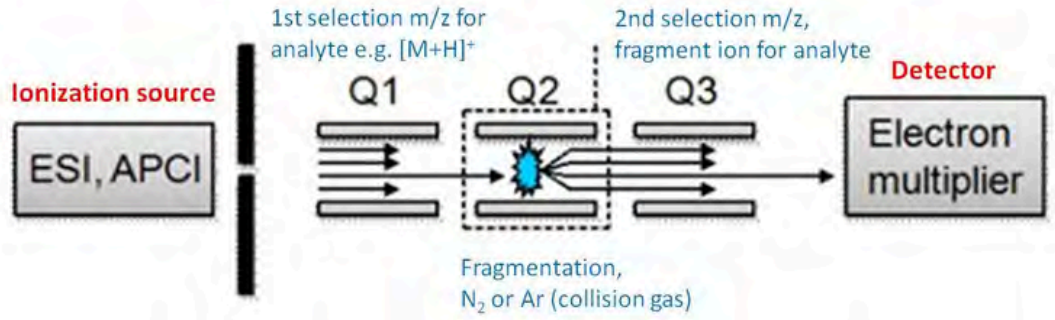


Figure 5.28. Main parts of a triple quadrupole mass spectrometer. Precursor or parent ion is selected by Q1, which is fragmented in the collision cell (Q2) and the product or daughter ions are analyzed by Q3.

Similarly to GC-MS, selective ion monitoring (SIM) or MRM (multiple reaction monitoring) modes are in practice to improve selectivity and avoid false positive results. Ion trap (IT) mass detectors are also used as their operation in MSn mode (MS/MS/….) allows confirmation and identification pollutants, set into the analytical method. When using different MS detectors and different modes, then their selectivity, sensitivity and/or linearity range has to be also considered. Of course, in the frame of this learning material all advantages, disadvantages, limitations and other details regarding the different MS techniques cannot be presented.

In the LC-MS measurements, rather internal than external calibration is used. The matrix effect is more significant in LC due to co-eluting components, which significantly influences the efficiency of the ionization the target analyte. Therefore deuterated or 13C-labelled pesticides or another compound, which is originally not in the sample are used as internal standards to eliminate these effect. Of course, not all the pesticide active ingredient are added to the sample in isotopically labeled form, because of the high cost of these standard materials. Detector responses are determined in a solution containing the target analytes and internal standard in a specified time region. Composition of the solution is often similar to the matrix and it is also close to the initial eluent composition of the applied HPLC method.

5.3.5 Immunoanalysis

Immunoassays are suitable tools for quantitative, semiquantitative, or qualitative detection of analytes. The base of all immunoassay type is the specific interaction between the antibody and the target substance (antigen). Target substances can be micro- and also macromolecules, like e. g. pesticides, toxins, or viruses, but in human healthcare some of the body's own proteins can be the targets. Antibodies act as labels in the immune system of vertebrates and are proteins that can be produced in „virtually unlimited variety" against diverse foreign compounds. There are synthetic antibodies on the market, but immunization (induced antibody production in laboratory animals) is also a possibility to get the needed antibody after purification. The decades of consecutive development resulted several techniques based on the specific antibody-antigen interaction, including e. g. microfluidic immunoassays, lab-on-a-chip technologies or surface plasmon resonance-based instruments for label-free technologies.

As mentioned above, antibodies are labels of their antigens via their specific binding sites. Because of the diversity of the targets, their recognized parts (the epitopes) are also diverse, and that is why the part of an antibody that binds to the

epitope (and is called a paratope) has to be also variable and takes place in the variable region at the N terminus of the protein (Fig. 5.29.). The other part of the antibody at the C terminus is called constant region and is far less variable and is the part that interacts with effector cells and molecules. There are five known classes of antibodies present in the human body, but for immunoassays immunoglobulin G (IgG) is the most frequently used. The IgG has the widely known typical Y-shape of the antibodies, and consists of four polypeptide chains. Two of them are identical heavy chains in the inner side, and the other two are identical light chains at the outside.

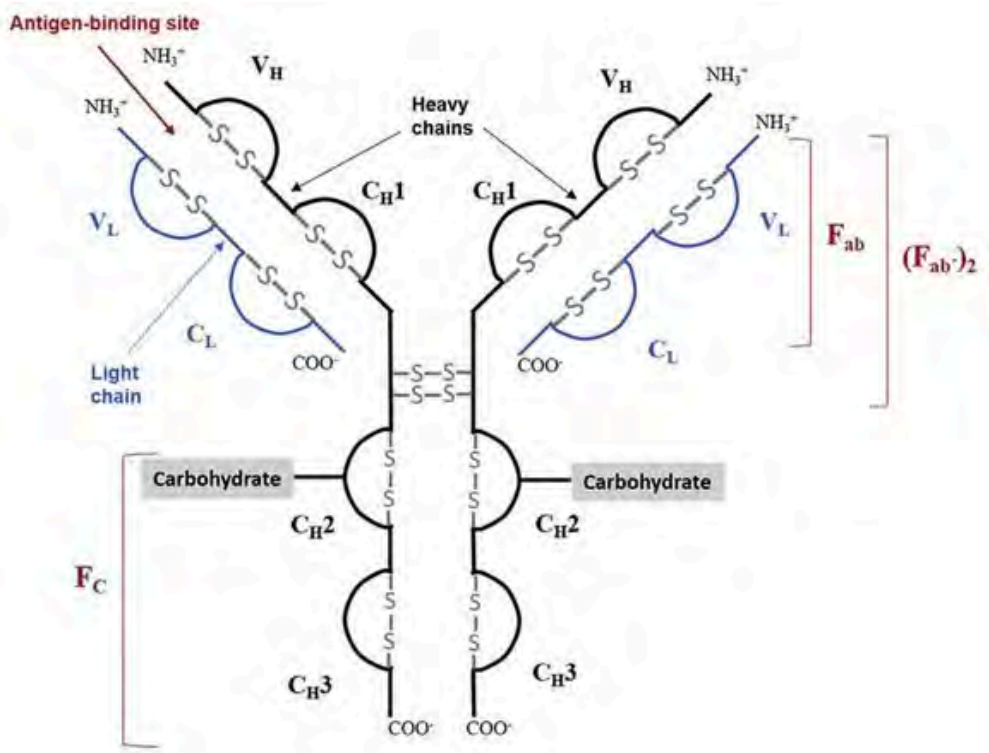


Figure 5.29. Schematic structure of an IgG (Vashist, S. K., & Luong, J. H. 2018. Immunoassays: an overview. In: Handbook of Immunoassay Technologies (Vashist, S. K., & Luong, J. H. Eds.), Academic Press, Elsevier Inc., 1-18. (doi: 10.1016/B978-0-12-811762-0.00001-3)

Light chains are containing a variable (VL) and a constant (CL) domain. Heavy chains are also containing a variable (VH) domain, but they have three constant domains (CH1, CH2 and CH3). Both antigen-binding sites are formed by the V regions of light and heavy chains on each arms of the Y-shape, which means that IgG-s are bivalent, and the antigen-binding parts of the heavy and light chains are attached to each other by noncovalent interactions and covalent disulfide bonds.

The total molecular weight of an IgG is around 150 kDa, and contains ~440 amino acids. Each heavy chain is around 50 kDa and contains two times more amino acids than a light chain (molecular weight ~25 kDa). Beside the four subclasses of IgG-s in humans, namely IgG1, IgG2, IgG3, and IgG4 there are other classes with different heavy chains. IgM has m-chains, IgA has a-chains, IgE has ε-chains and IgD has d-chains. However, there are only two main types of light chains, i.e., kappa (k) and lambda (l). IgD, IgE and IgG are monomers, whereas IgA is typically in dimer form having 4 antigen-binding sites but also can be a monomer, and IgM is a pentamer with 10 antigen-binding sites.

The different antibodies have different functions in different types of immune responses (Table 5.1.).

Table 5.1. Properties of the five types of human antibodies.

Name	Molecular weight	Number of antigen binding sites	Functions
IgA	385 kDa	4	Found in mucous, saliva, tears and breast milk.Protection against pathogens.
IgD	180 kDa	2	Is a part of the B-cell receptor. Activates basophils and mast cells.
IgE	200 kDa	2	Protects against parasitic worms. Involved in allergic reactions.
IgG	150 kDa	2	Secreted by plasma cells in the blood. Able to cross the placenta.
IgM	900 kDa	10	Can be attached to the B-cells surface or secreted into the blood. Responsible for early stages of the immunity.

A question to decide during planning an immunoassay is to use monoclonal or polyclonal antibodies. Both of them are against a specific target, but monoclonal antibodies have the same recognizing site to the identical epitope of the antigen, while polyclonal antibodies may recognize different epitopes of the same substance. Both antibody types have advantages and disadvantages to consider (Table 5.2.). As monoclonal antibodies are produced by the clones of one single B-cell and have only the same recognizing site, they are mostly synthetized by cell cultures, whereas polyclonal antibodies are produced by multiple B-cells of immunized laboratory animals. As polyclonal antibodies have more types of paratopes, there is

higher risk of cross-reactivity, which affects the specificity. Cross-reactivity means that the antibody interacts with some other substance containing the same epitope like the targeted one.

Table 5.2. Properties of monoclonal and polyclonal antibodies.

Property	Monoclonal antibodies	Polyclonal antibodies
Produced by	the same clone of plasma B cells	different clones of plasma B cells
require hybridoma cell lines	yes	no
antibody population	homogenous	heterogeneous
interact with	a particular epitope of the antigen	different epitopes of the antigen
production price	expensive	not expensive
required skills to handle the technique	more	fewer
production time needed	more	less
cross-reactivity	less	comparatively higher
used	as therapeutic drugs	in general research
advantages	high specificity, high reproducibility	high affinity, tolerance of minor changes, more robust

Beside buying on the market there is a possibility to produce antibodies in laboratory animals. Immunization means introducing

the antigen to the immunosystem. After the required time the blood will contain the sufficient amount of the specific antibody and can be purified to get the antiserum, an applicable form in immunoassays. Numerous vertebrate species are suitable for antibody production including horse, rabbit, goat, or smaller species like mice, frogs or fishes. The selected one depends on the needed quantity of antiserum, the phylogenetic relation between the antigen protein donor and the recipient and the character of the antibodies made by the recipient species.

Immunoassay types

As result of the years long development there are a lot of methods based on the antigen-antibody reaction. All of them are useable to evaluate an unknown concentration of the analyte within a sample by compare it to a calibration curve. In some cases sample preparation may be necessary. Although enzyme-linked immunosorbent assays (ELISAs) are the most widely used immunoassays, other possibilities include e.g. immunostrip, immunohistochemistry or surface-plasmon resonance-based immunoassays (a label-free technology).

Immunoassays can be classified in various ways, and the first one is using label or not using label (label-free) technologies. Label using assays are divided to homogeneous and heterogeneous assays, where both homogeneous and

heterogeneous assays can be competitive or noncompetitive. Label-free immunoassays are mostly immunosensors, and electrochemical, optical, piezoelectric immunosensors and lateral flow assays are the four most important types. In electrochemical immunosensor technique the biological reaction turns into electrochemical signal. These sensors have high selectivity, high sensitivity and low cost. Electrochemical impedance spectroscopy (EIS) is an example of the group and is a suitable tool to detect e. g. the construction of an immunocomplex composed of an antibody and its antigen.

Optical immunosensors are monitoring the optical variation during biomolecule absorption. Advantages are the portability, the compatibility with other computerized instruments and the low limit of detection. An example is the noncompetitive SPR immunosensor developed to detect triazophos, an organophosphate insecticide.

Piezoelectric immunosensors are developed to measure the proportional decrease in oscillating frequency of a piezoelectric quartz crystal in an oscillator circuit, resulted by the subsequent absorption of material. Quartz crystal microbalance (QCM) is an example that detect resonance frequency variation associated with mass change on the chip surface and is able to measure nanogram sized mass changes.

Lateral flow assays (LFA) are simple, rapid and do not require expensive instrumentation or additional reagents. Its most known form is the pregnancy test. LFA is based on antigen-antibody reaction with aggregation on a nitrocellulose membrane which provides a visually detectable signal. To improve the efficiency of these methods often used tools are nanoparticles, metal dots, quartz crystals, etc.

Enzyme-linked immunosorbent assays (ELISAs)

ELISAs are the most widely used types of immunoassays. Between all immunoassays, where antibodies are used as reagents, ELISAs are using enzymes attached to one of the reactants to allow quantification. The basis of all ELISA forms belongs to one of the three main types, namely: direct, indirect or sandwich ELISA (Fig 5.30.).

Direct ELISA is the simplest form, where the antigen is bound to the solid phase (microplate, membrane, etc.) and its own antibody is labelled with the enzyme allowing the quantification upon giving its substrate.

In indirect ELISAs antigen is bound to the solid phase again, but instead of antigen detecting antibodies additional antibodies against the detecting ones are labelled. This step improves the specificity of the method.

In sandwich ELISA the detecting („capture”) antibody is bound

to the surface to recognize the antigen. Another antibody against the antigen is labelled with an enzyme and is given to the system to allow the enzymatic reaction leading to quantification. All of these three types are able to be the basis of competitive or inhibition ELISAs.

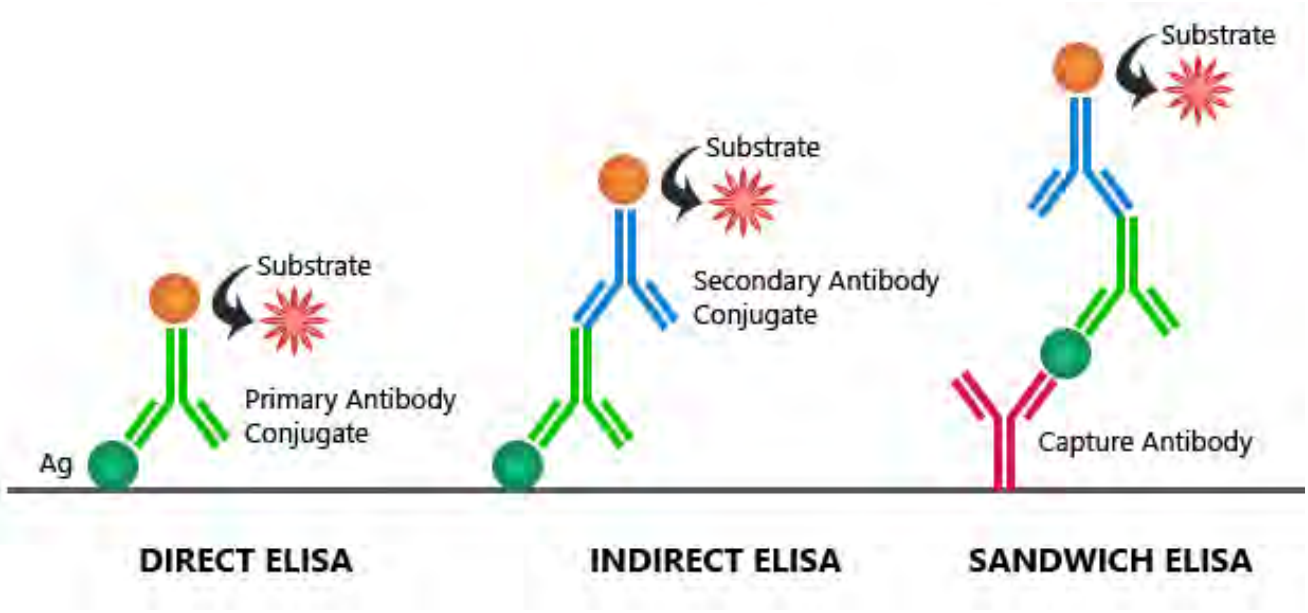


Figure 5.30. Schematic figure of direct, indirect and sandwich ELISAs.

Labelling

In case of ELISA the label is an enzyme. Usually horseradish-peroxidase (HRP) with TMB (3, 3', 5, 5'-tetramethylbenzidine), o-Phenylenediamine (OPD) or other chromophores with hydrogen peroxide as oxidizing agent. The forming color is the simplest possibility to detect with colorimetric equipment, but e. g. 10-Acetyl-3,7-dihydroxyphenoxazine (ADHP) is another substrate of the enzyme forming resorufin as product with color and also detectable fluorescent sign.

Other common enzyme immunoassay is the enzyme multiplied immunoassay technique (EMIT) used basically for determination of therapeutic and recreational drugs in human samples. The analyte in the sample competes with a labelled one to bind the antibody.

Other labelling possibilities include radioactive isotopes in radioimmunoassays (RIAs) where the bound antigen-antibody complexes are emitting detectable radioactivity. Its advantage is the very high sensitivity and specificity, but because of the possible danger of radioactivity this method is relatively rarely used.

Fluoroimmunoassay (FLIA) and chemiluminescence immunoassay (CLIA) are similar to RIA, with emitted fluorescent or chemiluminescent signal.

4 parameter logistic model

To quantify the unknown concentration of the measured analyte, the signal of the sample is compared to a calibration curve (Fig 5.31.). Parameters of the curve based on the Rodbard equation are the followings: upper plateau, lower plateau, inflection point (here the IC50) and the slope factor at the inflexion.

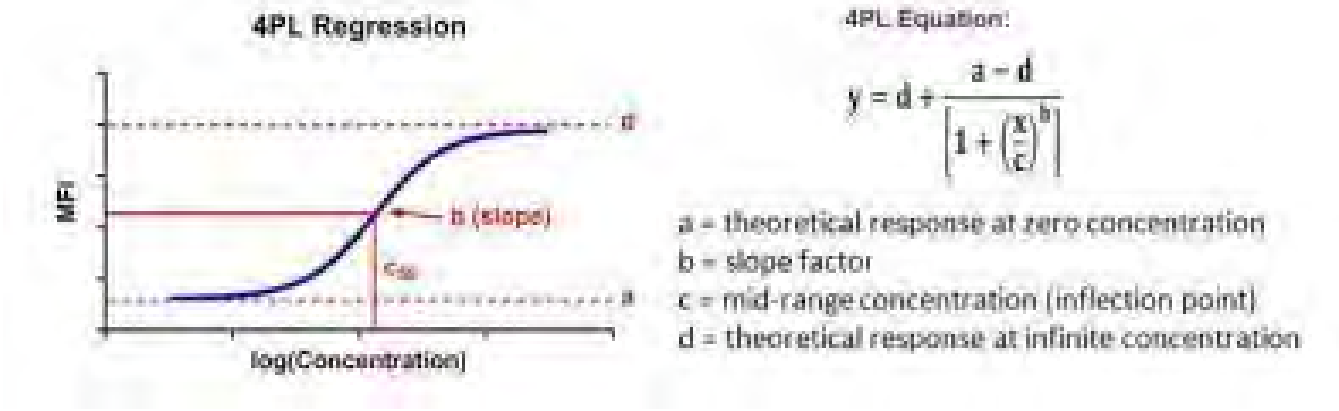


Figure 5.31. General form (left) and parameters (right) of the 4 parameters logistic model (Johnson, K. Curve Fitting for Immunoassays: ELISA and Multiplex Bead Based Assays (LEGENDplex™). Available online. URL: https://www.biolegend.com/en-us/blog/curve-fitting-for-immunoassays-legendplex (accessed on 26 January 2023)

Fields of use

As possible analytes of immunoassays have a wide range including e. g. proteins, toxins, pathogen bacteria and other micro- and macromolecules, fields of use are ranging from diagnostic medicine and food safety to environmental research and agricultural biotechnology. EMIT is one of the main tools in monitoring of drugs in human samples, ELISAs are suitable to detect several pesticides in eggs, food samples or even groundwater. Different types of biosensors are used in e. g. food industry, metabolic engineering, medical science or plant biology.

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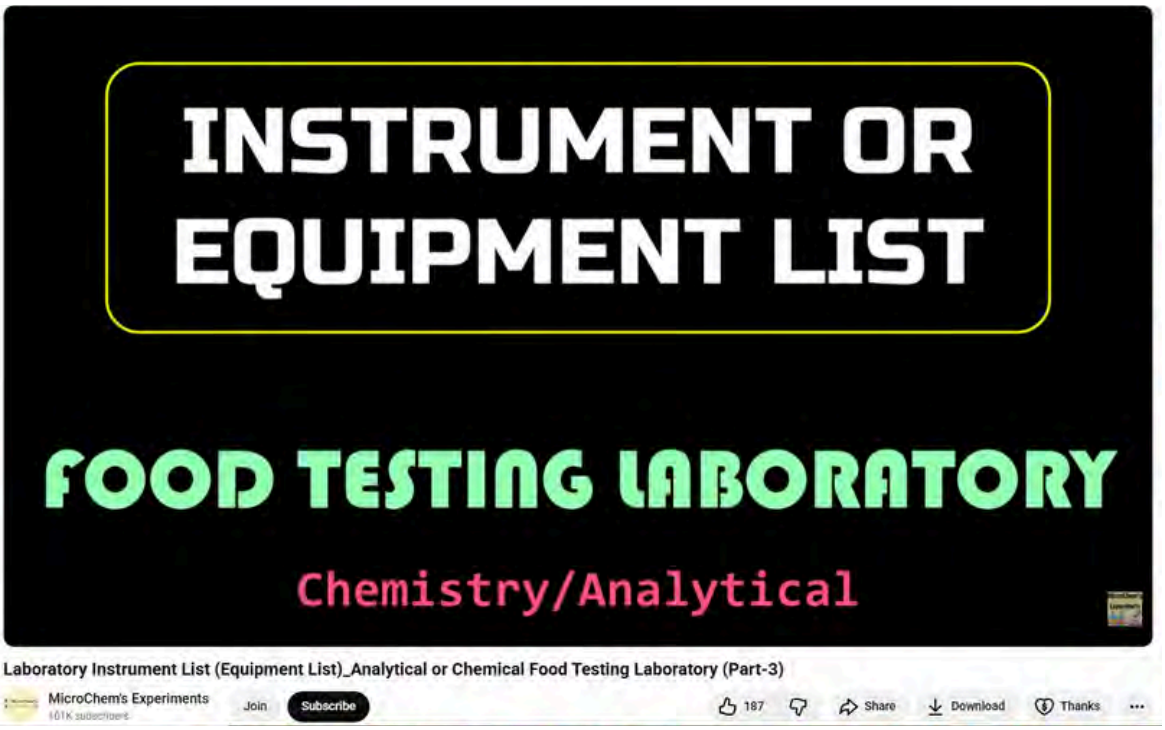
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