MASS SPECTROMETRY

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INTRODUCTION

Mass spectrometry is an analytical technique which allows to determine the mass to charge ratio of gas-phase ions. It is widely used in both organic and inorganic chemistry research and has a broad application range in chemical, pharmaceutical and biomedical research. These days, mass spectrometers are everywhere, even in space.

This course will cover following aspects related to mass spectrometry:

- what is mass
- what information do we get from a mass spectrum
- what types of instruments exist
- how do the instruments work
- hyphenation to other analytical techniques
- what are the applications of mass spectrometry

At the end of the course students should be acquainted with terminology used in mass spectrometry and understand scientific papers containing this technique.

1

THE MASS OF ATOMS AND MOLECULES

1.1 mass of atoms

1.1.1 constituents of atoms

For a single atom, atomic mass is the sum of the protons, neutrons and electrons. The protons and neutrons (nucleons) are found in the nucleus of atoms. The nucleus of an atom is surrounded by empty space in which there are electrons.

Protons and neutrons have nearly the same mass while electrons are much lighter. If we assume that a neutron has a mass of 1, then the relative masses are:

- neutron = 1
- proton = 0.998 623 49
- electron = 0.000 543 867 34

Relative masses, however, don't give the actual masses of these particles. In kilograms, the masses are:

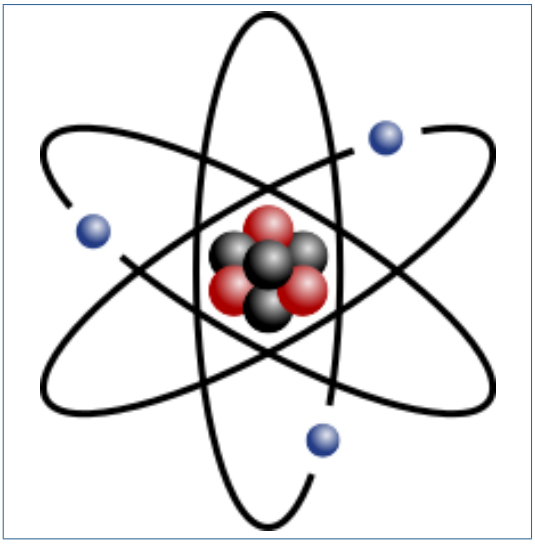

Figure 1.1: representation of the structure of an atom

- neutron = 1.674 928 6 $\times$ 10^{-27} kg
- proton = 1.672 623 1 $\times$ 10^{-27} kg
- electron = 9.109 389 7 $\times$ 10^{-31} kg

There is another unit, called an electron volt (eV), that scientists use when talking about small things like protons, neutrons and electrons. An electron volt is actually a measurement of energy, but scientists can get away with using it to measure mass since mass and energy are related by Einstein's famous equation:

$$E = m\,c^2$$

So, in terms of MeV (Megaelectron volts, 1 MeV = 1 000 000 eV), the masses are:

- neutron = 939.56 MeV
- proton = 938.27 MeV
- electron = 0.511 MeV

Atoms are described by their proton number (Z), which is the number of protons they contain, and their nucleon number (A), which is the number

of nucleons their nucleus contains (nucleon number = number of protons + number of neutrons).[1] For example the element 'X' below has a proton number of 'Z' and a nucleon number of 'A'.

$$^{A}_{Z}X$$

All the elements have a unique proton number that determines the position of the element in the periodic table. For example the element 'C' (carbon) below has a proton number of '6' and a nucleon number of '12'.

$$^{12}_{6}C$$

1.1.2 isotopes

Isotopes are atoms which have the same number of protons but different numbers of neutrons. Isotopes are all atoms of the same element. For example the element 'C' exist mainly with a proton number of '6' and a nucleon number of '12'. About 1.1% is present with a proton number of '6' and a nucleon number of '13' (carbon-13).

$$^{12}_{6}C \qquad\qquad ^{13}_{6}C$$

Often, isotopes are written with only the nucleon number: ^{12}C, ^{13}C, ^{32}P.

The majority of the elements are present in nature under different isotopic forms (e.g., ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn). Some elements are present as one isotope only (e.g.,^{23}Na, ^{19}F).[2]

There are stable and unstable (radioactive) isotopes. Stable isotopes maintain constant concentrations on earth over time. Unstable isotopes are atoms that disintegrate at predictable and measureble rates to form other isotopes by emitting a nuclear electron or a helium nucleus and radiation. These isotopes continue to decay until they reach stability. As a rule, the heavier an isotope is than the most common isotope of a particular element, the more unstable it is and the faster it will decay.

[1] the nucleon number is also called *the mass number*
[2] see appendix for a table of naturally occurring isotopes

Unstable atomic nuclei can undergo a variety of radioactive decays:

alpha decay

$$ {}^{A}_{Z}\mathrm{X} \longrightarrow {}^{A-4}_{Z-2}\mathrm{Y} + {}^{4}_{2}\mathrm{He} $$

beta decay

$$ {}^{A}_{Z}\mathrm{X} \longrightarrow {}^{A}_{Z+1}\mathrm{Y} + e^{-} + \overline{v}_{\mathrm{e}} $$

positron emission

$$ {}^{A}_{Z}\mathrm{X} \longrightarrow {}^{A}_{Z-1}\mathrm{Y} + e^{+} + v_{\mathrm{e}} $$

electron capture

$$ {}^{A}_{Z}\mathrm{X} + e^{-} \longrightarrow {}^{A}_{Z-1}\mathrm{Y} + \overline{v}_{\mathrm{e}} $$

gamma decay

$$ {}^{A}_{Z}\mathrm{X}^{*} \longrightarrow {}^{A}_{Z}\mathrm{X} + \gamma $$

1.1.3 definition of mass unit

The symbol of the mass unit[3] (u) represents 1/12th of the mass of the most abundant naturally occurring stable isotope of carbon (^{12}C).[4]

$$ 1u = m_u = \frac{1}{12} m(^{12}\mathrm{C}) = 1.661 \times 10^{-27}\,\mathrm{kg} $$

$$ 1u\,c^2 = 931.19\,\mathrm{MeV} $$

All masses expressed in mass units can be considered as relative masses.

When protons, neutrons and and electrons are combined to an atom, the mass of that atom will not correspond to the sum of the masses of the constituents. This difference corresponds to the **binding energy** and can be written as:

$$ E_B = c^2(m_{{}^{A}_{Z}\mathrm{X}} - (A-Z)m_n - Zm_p - Zm_e) $$

[3] also called *unified atomic mass unit*

[4] **amu** was also used as the symbol for mass unit but the *current IUPAC recommentdations* no longer recommends to use it. The term **dalton (Da)** is also sometimes used, mainly in biochemistry.

The binding energy of nucleons is extremely large compared to other atomic energy scales.

1.1.4 average atomic mass

The average atomic mass or atomic weight of an element (M_{av}) can be calculated from the masses (M) and abundances (A) of the isotopes of that element.[5]

$$M_{av} = \sum_{i=1}^{n} M_i \times A_i$$

1.2 mass of molecules

The mass of molecules can be calculated from the mass of atoms. Depending on which atomic mass is used, we can distinguish:

- monoisotopic molecular mass
- average molecular mass
- nominal mass

The **monoisotopic mass** of a molecule is the sum of the masses of the atoms in that molecule using the mass of the principal (most abundant) isotope for each element.

The **average mass** of a molecule is the sum of the average masses of the atoms in that molecule. This value takes the presence of isotopes into account and can be found on the periodic (Mendeljeev's) table.

The **nominal mass** of a molecule is the sum of the nominal masses of the elements. The nominal mass of an element corresponds to the atom number of the the most abundant isotope.

Which mass one should use depends on the situation. A chemist weighing a substance wants to know how many molecules are on the balance. He/she uses the average mass because the molecules contain a mixture of isotopes present in ratios as found in nature.

[5] see appendix for a list of atomic masses

1.3 nitrogen rule

> *The nominal mass of a non-charged molecule with paired electrons is even when the number of nitrogens is even*

The nitrogen rule is not really a rule, but the consequence of the fact that all atoms with an even nominal mass are bound to an even number of other atoms (e.g., carbon: 4) while atoms with an odd nominal mass have an odd number of bonds (e.g. phosphorous: 3 or 5). The only exception is nitrogen: nominal mass equals to 14 while having 3 bonds.

For non-radical ions the rule can be reversed: an even mass means an odd number of nitrogens. Similarly, we can state that the nitrogen rule can be applied to radical ions which are charged.

It is important to know that the nitrogen rule is only valid for nominal masses. For molecules containing a lot of atoms, the monoisotopic mass can deviate more than 1 mass unit and will lead to wrong interpretations.

Examples:

- CH_4: M = 18, even number of nitrogens, even nominal mass
- NH_3: M = 17, odd number of nitrogens, odd nominal mass
- NH_4^+: M = 18, ammonium ion with odd number of nitrogens and even nominal mass
- $C_6H_6^{\cdot+}$ M = 78, benzene radical with even number of nitrogens and even nominal mass

1.4 degree of unsaturation

The elemental composition of a neutral molecule allows the determination of the **degree of unsaturation** (DU), this means the sum of the number of rings and double bonds.[6]

[6] also called the **ring and double bond equivalent** (RDBE) or **index of hydrogen deficiency** (IHD)

$$DU = 1 + \frac{1}{2}\sum n_i \times (v_i - 2)$$

with n_i the number of atoms with valence v_i

For example: benzene (C_6H_6):

DU = 1 + 1/2 (6 $\times$ (4-2) + 6 $\times$ (1-2)) = 4 (rings + double bonds)

1.5 calculation of isotopic abundances

Mass spectrometers are able to measure the abundance of each isotope individually. On the spectra we 'see' the isotope patterns. These patterns are very informative regarding the presence (or absence) of certain elements in the molecules.

There exist tools for the calculations of isotope abundances and these can be useful for the calculation of complicated cases. For simple molecules, is it very easy to perform the calculations by hand.

As an example, the isotope distribution for a compound containing one Cl and one Br atom is calculated.[7]

	Cl	Br
M	0.7577	0.5069
M+2	0.2423	0.4931

First, for each of the expected masses (M, M+1, M+2, ...) the probability is calculated. In this example, we only have to consider M, M+2 and M+4.

M	0.7577 $\times$ 0.5069 =	0.3840
M+2	(0.2423 $\times$ 0.5069) + (0.7577 $\times$ 0.4931) =	0.4964
M+4	0.2423 $\times$ 0.4931 =	0.1195

Finally, the values are normalized to 100%.

[7] other elements are omitted in this example as their contribution is not significant

M	0.3840 / 0.4964 × 100 =	77.36
M+2	0.4964 / 0.4964 × 100 =	100.0
M+4	0.1195 / 0.4964 × 100 =	24.07

2

INSTRUMENTATION

Mass spectrometers consist mainly of three parts: an ion source, the analyzer(s) and a detector. For each of the parts several different types exist which will be discussed in this chapter.

In the ionization source ions are generated from the analytes. These ions are subsequently separated by their mass over charge ratio (m/z) and detected by the detector.

When ions travel through the mass spectrometer every obstacle can cause the loss of signal intensity or errors in the measurements. Any presence of particles (molecules) should be avoided inside the mass spectrometers and therefore the instruments are kept under high vacuum (low pressure).

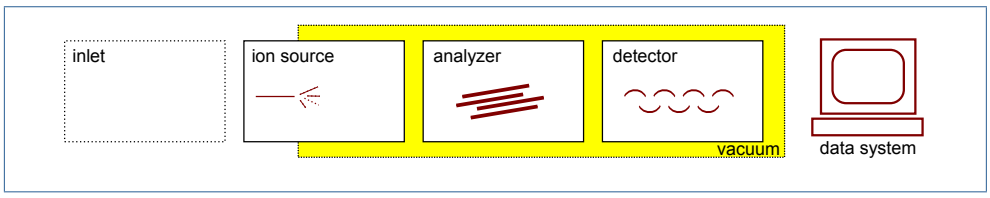

Figure 2.1: components of a mass spectrometer

2.1 ion sources

In ion sources the ionization of the analyte takes place prior to analysis in the mass spectrometer. A variety of ionization methods are available for mass spectrometry. In each method energy is transferred to the analyte. Some ionization techniques are very energetic and cause extensive fragmentation. Other techniques are softer and ionize molecules keeping them intact.

Ion sources can also be divided in two groups: methods where ionization takes place under vacuum and methods where ionization takes place under atmospheric pressure.

The result of ionization are ions and can be protonated or deprotonated molecules, adducts or charged radicals.

2.1.1 electron ionization

Electron ionization (EI), also called *electron impact* was widely used in organic mass spectrometry in the 20th century. This ionization technique works well for many gas-phase molecules but induces extensive framentation. Often the molecular ions are not observed.

The source consists of a heated filament giving off electrons. These are accelerated towards an anode and on their way interaction takes place with the gaseaous molecules of the analyte.

Electrons are associated with a wave whose wavelength λ is given by

$$\lambda = \frac{h}{m\,v}$$

where m its mass, v its velocity and h Planck's constant. This wavelength corresponds to 1.4 Å for a kinetic energy of 70 eV. When the frequency corresponds to a transition in the molecule, energy transfer can occur leading to various electronic excitations. When there is enough energy, an electron can be expelled.[1] The optimum wavelength for organic molecules

[1] The electrons do not 'impact' molecules and hence the term *electron impact* should be avoided

corresponds to 70 eV. For ionization 15 eV is enough in most cases. The excess of energy leads to extensive fragmentation. This fragmentation is not necessarily a drawback because it can give useful structural information and can be used for structure elucidation of unknown compounds.

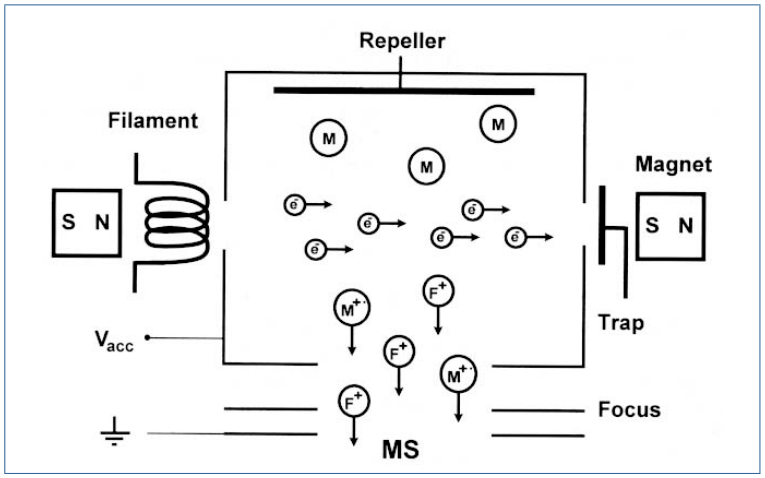

Figure 2.2: electron ionization

The source consists of a small box (usually a few centimeters) and has holes for sample introduction, an inlet and outlet for the electrons and a slit where the formed ions can leave and be injected into the analyzer.

In order to increase the ion production a magnetic field causes the electrons to follow a circular path and allowing a better interaction with the analyte.

Samples have to be introduced into the vacuum because the ionization takes place *in vacuo*. Solid samples and liquids have to be heated in order to bring the analyte molecules into the gas phase. Gases can be introduced directly, which makes this ionization method very suitable to hyphenate with gas chromatography.

Negative ions can be produced by electron capture and are obtained by reversing the repeller and ion optics voltages.

2.1.2 chemical ionization

The source for chemical ionization (CE) is almost identical to the one used in EI. The sample is introduced together with a reagent gas. Methane (CH_4), isobutane (C_4H_{10}) and ammonia (NH_3) are common reagents. In a first step the reagent gas is ionized followed by a proton transfer to the analyte:

$$CH_4 + e^- \longrightarrow {CH_4}^{\cdot +} + 2e^-$$
$${CH_4}^{\cdot +} + CH_4 \longrightarrow {CH_5}^+ + {CH_3}^{\cdot}$$

The analyte is then protonated

$$M + {CH_5}^+ \longrightarrow MH^+ + CH_4$$

The ionization process is less harsh than with EI (less than 5eV energy is transferred) and in addition to protonated molecules, sometimes reagent gas adducts can be observed.

$$M + {CH_5}^+ \longrightarrow [M + H + CH_4]^+$$

2.1.3 plasma desorption

A breakthrough in the analysis of biomolecules came in 1974 with the introduction of plasma desorption mass spectrometry (PDMS). This technique uses ^{252}Cf fission fragments to desorb large molecules from a target. The target is made of a thin aluminum foil, often covered with a layer of nitrocellulose, to which a droplet of the sample solution is applied. The adsorption of proteins to nitrocellulose is believed to be based on hydrophobic interactions and allows salts to be washed off and chemical reactions to be carried out on the target. Alternatively, the sample can be electrosprayed directly onto Ni or Al foil, a technique which is more effective for smaller peptides.

Two atomic particles are produced by the ^{252}Cf fission reaction, one causing desorption of the analyte and the other providing the start signal for the time-of-flight measurement. A time-of-flight mass analyzer,

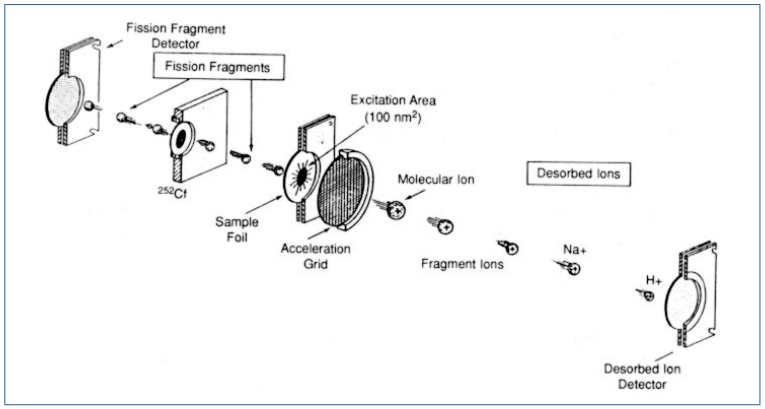

Figure 2.3: plasma desorption mass spectrometry

described below, is generally used for ion separation. Similar to other desorption techniques, cluster formation and multiple charging is observed. Suppression effects, i.e. the inability to ionize some molecules due to the presence of other compounds present, are also sometimes observed.

2.1.4 FAB/LSIMS

Fast atom bombardment (FAB) and secondary ion mass spectrometry (LSIMS) are ionization methods developed in the 1980s. They consist of focusing a high energy beam of neutral atoms of ions onto the sample. Essential for both methods is the fact that the sample should be dissolved in a (low volatile) liquid matrix. Typical matrices are m-nitrobenzylic alcohol and (thio)glycerol. Although these matrices work in both positive and negative ion mode, bases such as triethanolamine are often added in negative mode.

The difference between the two method is the origin of the primary ion beam. In FAB inert gases such as argon or xenon are first ionized and accelerated to about 5 keV. At the exit, the radicals are neutralized but they conserve momentum as neutral species. In LSIMS the primary ion beam consists of cesium ions (Cs^+) which are generated by heating a cesium salt pellet by a filament. Thes ions are accelerated and focused by lenses onto the target containing the sample.

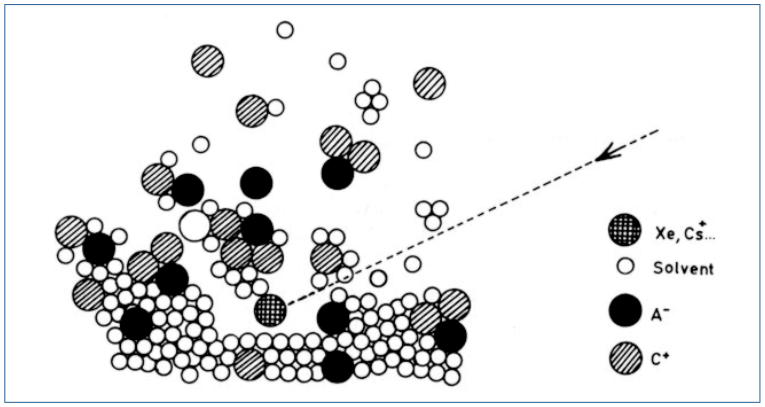

Figure 2.4: FAB and LSIMS

Ions are formed by proton transfer ($[M + H]^+$) from the matrix to the analyte upon collision of the primary ion beam with the sample dissolved in the matrix. FAB and LSIMS are soft ionization techniques, leaving the molecules mostly intact. Often also adducts of matrix (M + H^+ + glycerol) or cations (Na^+, K^+) are observed as well as dimers (such as $[2\,M + H]^+$).

2.1.5 MALDI

The use of lasers in mass spectrometry goes back to the early 1960's. A wide range of wavelengths, from UV to IR, have been used with many different types of mass spectrometers for isotope measurements, elemental composition analysis, and for pyrolysis of small organic and inorganic molecules.

A major breakthrough came in 1988 with the introduction of matrix assisted laser desorption ionization (MALDI), a technique which now is able to detect biomolecules over 300,000 Da in size.[2] The technique involves embedding the analyte in a solid matrix which absorbs energy at the wavelength of the laser.[3] The actual mechanism of MALDI, a combination of desorption and ionization, is still being investigated. One hypothesis proposes a hydrodynamic model

[2] In 2002 part of the Nobel Prize Chemistry was given to Koichi Tanaka for his contribution to the development of MALDI
[3] for a list of common matrices, see appendix

for the desorption of matrix and embedded biomolecules. The laser energy absorbed by the matrix, typically on the order of 106 watts/cm^2, leads to intense heating and generation of a plume of ejected material that rapidly expands and undergoes cooling. The phase transition (evaporation and sublimation) is probably the rate determining step in ion formation. Generation of ions is believed to arise through ion/molecule reactions in the gas phase. However, depending on the matrix used, enough energy can be transferred to the molecule to break weak bonds. Generally, the $[M + H]^+$ ion, or $[M + Na]^+$, $[M + K]^+$, etc., are preferentially formed in the positive ion mode, and $[M - H]^-$ ions in the negative ion mode. However, the technique also generates singly- and multiply-charged clusters of the analyte of low intensities, an undesirable situation in that these tend to complicate the spectrum. Mass resolution obtainable with MALDI is highest when using laser power close to the threshold level required to produce ions from the solid sample, i.e., at low signal strength.

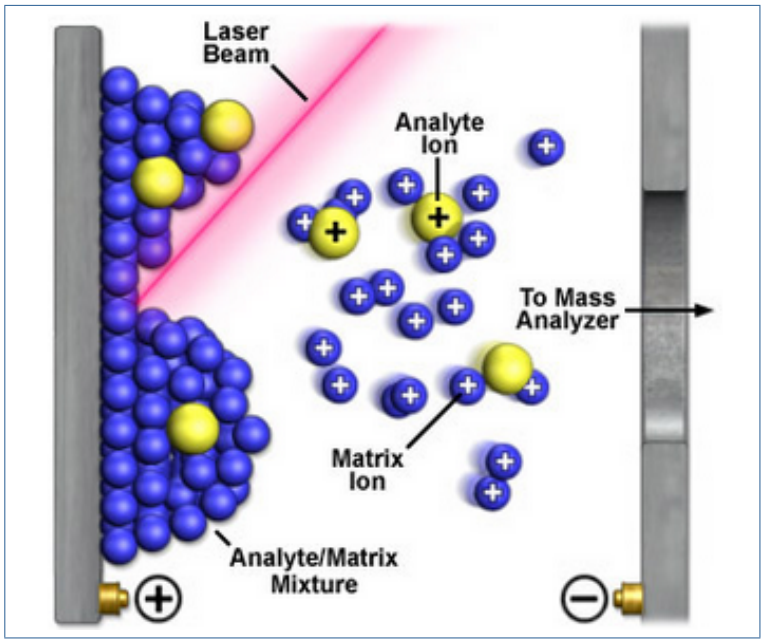

Figure 2.5: matrix assisted laser desorption ionization

MALDI produces a relatively intense matrix background, generally below m/z 1000. This chemical background depends on the matrix and laser wavelength

chosen. MALDI is a very fast and sensitive technique, implemented on small, relatively inexpensive instruments that do not require extensive expertise in mass spectrometry. Such instruments are ideally suited for biological scientists that need molecular mass information more quickly and more accurately than can be obtained by gel electrophoresis.

This ionization technique is mainly used together with time-of-flight analyzers. A shot is fired with the laser beam triggering the time measurement of the flight time for the ions to reach the detector.

2.1.6 thermospray

With thermospray (TSP) a sample containing the analyte is pumped into a steel capillary which is heated to high temperature. The heated liquid is converted to a supersonic beam when entering a vacuum chamber. A fine droplet spray occurs, containing ions, solvent and sample molecules. The droplets can be charged by corona discharge and are finally extracted to the mass analyzer.

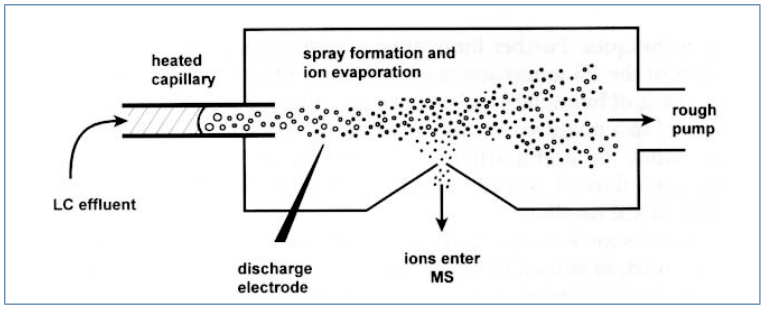

Figure 2.6: thermospray

Thermospray generates protonated ions and was mainly used for coupling of HPLC to mass spectrometers. Nowadays other techniques such as electrospray and atmospheric pressure ionization are used instead.

2.1.7 electrospray

Electrospray ionization (ESI) is an ionization method where ionization takes place under atmospheric pressure.[4] Compared to CI, the ionization efficiency is much higher. The problem with atmospheric pressure ionization methods is that the ions have to be transferred into the mass spectrometer which is under reduced pressure (vacuum).

An electrospray is formed when a strong electric field is applied under atmospheric pressure to a liquid passing a very narrow capillary. The electric field is obtained by applying a potential difference of 2.5 - 6 kV between the capillary tip and a counter electrode at a distance of 0.2 - 2 cm. This field induces a charge accumulation at the liquid surface at the tip of the capillary which will break to form highly charged droplets. Upon evaporation, droplets shrink to a point where repelling coulombic forces cause the droplets to divide. Each rupture generates smaller droplets finally leading to desorption of ions from the droplet surface. Evaporation of the droplets is promoted by applying heat, usually in the form of a heated nitrogen gas counterflow.

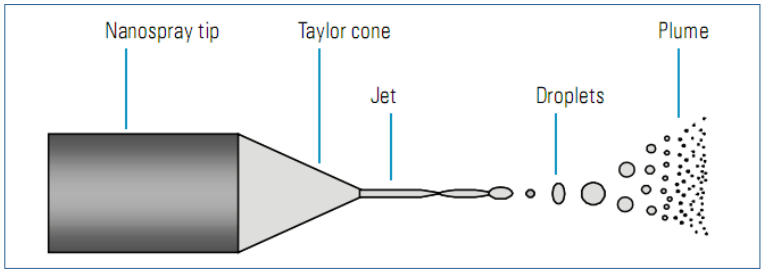

Figure 2.7: nanospray

When the capillary diameter is 10 - 20 μm the droplets that are formed are small enough to be used directly. The spray causes the remaining liquid to flow at a flow rate of about 10 - 1 000 nL/min. This version is therefore called **nanospray**.

[4] In 2002 John Fenn received the Nobel Prize Chemistry for his work on ESI

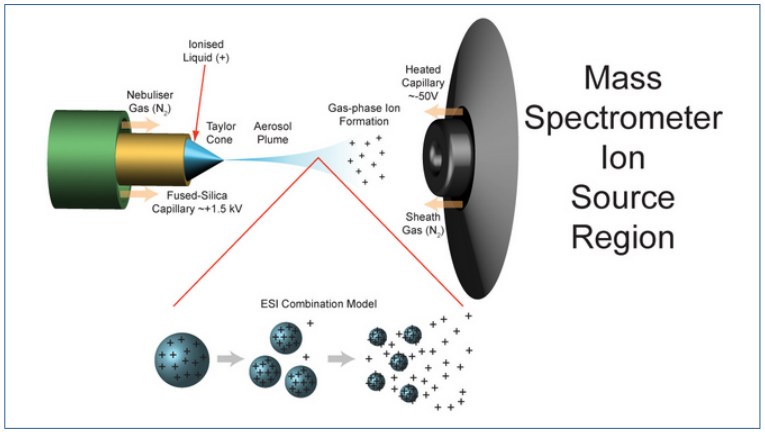

Figure 2.8: pneumatically assisted electrospray

Nanospray can be coupled with liquid chromatography, but special low flowrate pumps are needed. There exist columns for nanospray where the tip is integrated with the column.

For higher flow rates and larger capillary diameter, coaxially gas is applied in order to generate an aerosol with small droplets. This is sometimes called **pneumatically assisted electrospray** or often simply **electrospray**.

The solubility and lipophilicity of the analytes play an important role in the ionization efficiency of the molecules. With electrospray often matrix effects and signal suppression are observed which should be taken into account when performing quantitative work.

Electrospray ionization generates mainly (de)protonated molecules. Sometimes cation adducts and dimers are observed. Electrospray allows the ionization of polar and lare molecules and is widely used for the analysis of biomacromolecules like proteins and oligonucleotides. These large molecules carry multiple charges and their electrosprayed ions are therefore often multiply charged.

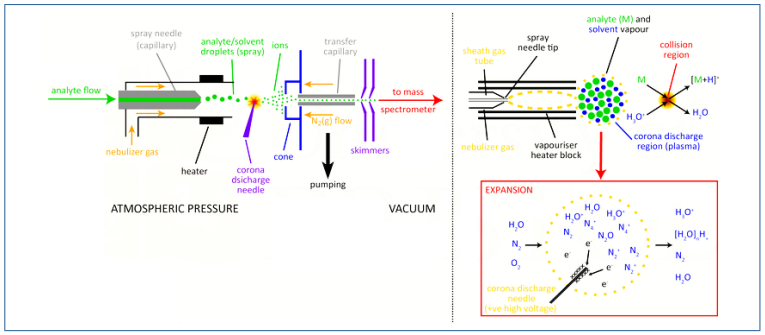

Figure 2.9: atmospheric pressure chemical ionization

2.1.8 atmospheric pressure chemical ionization

Atmospheric pressure chemical ionization (APCI) is an ionization technique that uses gas-phase ion-molecule reactions at atmospheric pressure. Primary ions are formed by corona discharge which is obtained by applying a high voltage on a stainless needle. Often, not the voltage is controlled, but the current is kept constant in the range 10 - 30 μA.

Compared to electrospray ionization, APCI can generate ions for less polar compounds. While electrospray works at very low flowrates, APCI needs higher solvent flows (0.2 - 2 mL/min) and hence more heat for dissolvation. Both methods are these days the main ionization techniques used when HPLC is coupled to a mass spectrometer.

2.1.9 other ionization methods

Many ionization methods derived from the previously mentioned techniques have been published the past decennium.

Desorption electrospray ionization (DESI) is a method where an electrospray beam is used as a primary ion source for desorption of ions from a solid surface. This allows sample introduction without the need of sampe preparation.

Atmospheric solids analysis probe (ASAP) is also a method where a liquid spray is aimed on a solid sample. The desorbed molecules are then ionized

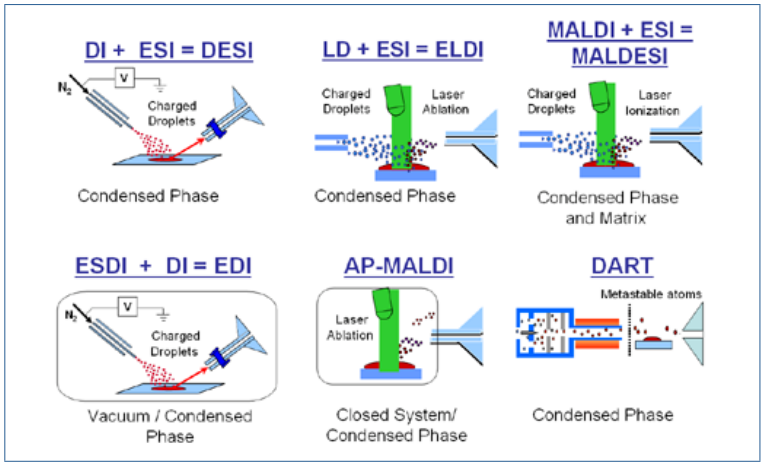

Figure 2.10: ambient ionization techniques

by corona discharge.

2.2 mass analyzers

Once ions are produced in the source, their m/z has to be measured. Different approaches are possible and several analyzer types have been developed.

An ideal mass analyzer has following properties:

- has a high resolving power
- has a high ion transmission (sensitivity)
- has a high upper mass limit
- low cost
- is compact

2.2.1 magnetic sector

As moving charges enter a magnetic field, the charge is deflected to a circular motion of a unique radius in a direction perpendicular to the applied magnetic field. Ions in the magnetic field experience two equal forces: force due to the magnetic field and centripetal force.

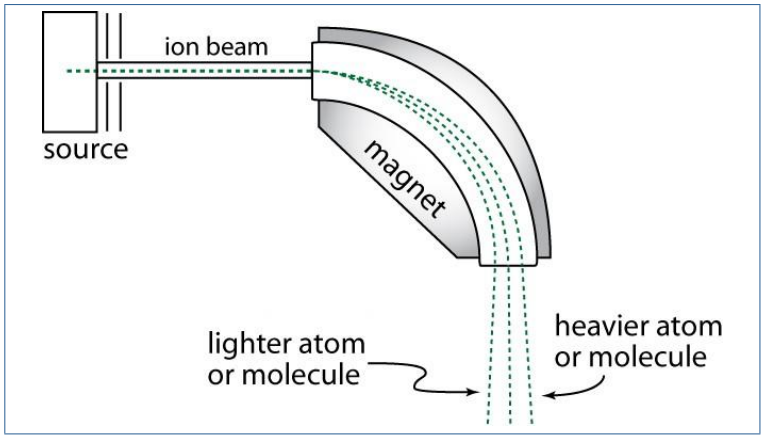

Figure 2.11: electrostatic analyzer

$$F_B = zvB = F_c = \frac{mv^2}{r}$$

where z = charge, v = velocity, B = magnetic field, r = radius and m = mass.

The above equation can then be rearranged to give:

$$v = \frac{Bzr}{m}$$

If this equation is substituted into the kinetic energy equation:

$$E_k = zV = \frac{mv^2}{2}$$

where V = acceleration potential

$$\frac{m}{z} = \frac{B^2r^2}{2V}$$

The radius that the charges (ions) follow, depends on their kinetic energy. If ions with the same *m/z* ratio are dispersed the mass resolution will decrease. In order to avoid this dispersion, the kinetic energy must be controlled. This can be achieved with an electrostatic analyzer.

2.2.2 electrostatic analyzer

An electrostatic sector analyzer consists of two curved plates of equal and opposite potential. As the ion travels through the electric field, it is deflected

and the force on the ion due to the electric field is equal to the centripetal force on the ion. Here the ions of the same kinetic energy are focused, and ions of different kinetic energies are dispersed.

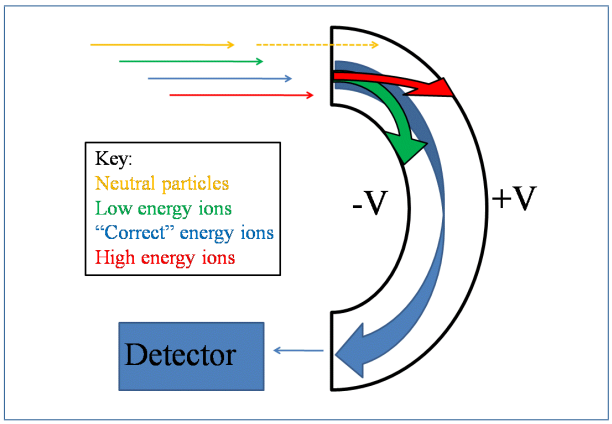

Figure 2.12: electrostatic analyzer

$$E_k = zV = \frac{mv^2}{2}$$

$$F_E = zE = F_c = \frac{mv^2}{r}$$

with E being the electric field.

Electrostatic sector analyzers are energy focusers, where an ion beam is focused for energy.

Electrostatic and magnetic sector analyzers when employed individually are single focusing instruments. However when both techniques are used together, it is called a double focusing instrument because in this instrument both the energies and the angular dispersions are focused. Double focusing instruments are usually high resolution instruments with resolving power around 10 000 - 20 000. The upper mass limit can be as high as 10 000 u but these magnets and the necessary electronics are expensive.

2.2.3 quadrupole

A quadrupole is a device that uses the stability of the trajectories in oscillating electric fields to separate ions according to their m/z ratio. Quadrupole analyzers are made up of four parallel rods with circular or, ideally, hyperbolic section.

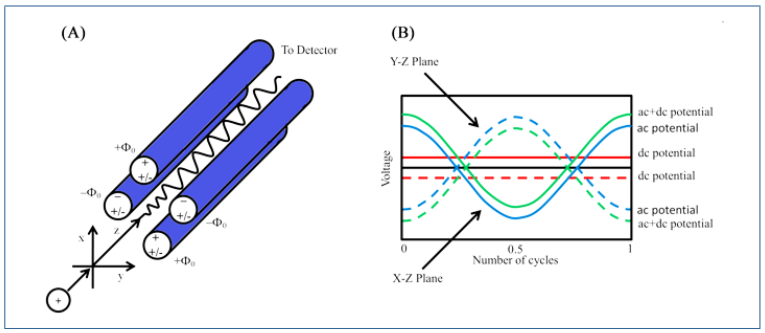

Figure 2.13: quadrupole analyzer

The resolution of quadrupole instruments depends on the scan rate and is typically in the range of 1 000 - 3 000. The upper mass limit can be as high as 8 000 u.

When no DC voltage is applied, a quadrupole is a device that can guide ions along its length. Often these RF-only devices are made up of more parallel rods like in the case of hexapoles or octapoles.

2.2.4 ion trap

A quadrupole ion trap or quistor is made up of a circular electrode with two ellipsoid caps on the top and the bottom. Conceptually, an ion trap can be imagined as a quadrupole bent on itself in order to form a closed loop.

In quadrupole instruments, the potentials are adjusted so that only ions with a selected m/z value go through center of the the rods. In an ion trap, ions with different masses are present together inside the trap and are expelled according to their masses so as to obtain the spectrum.

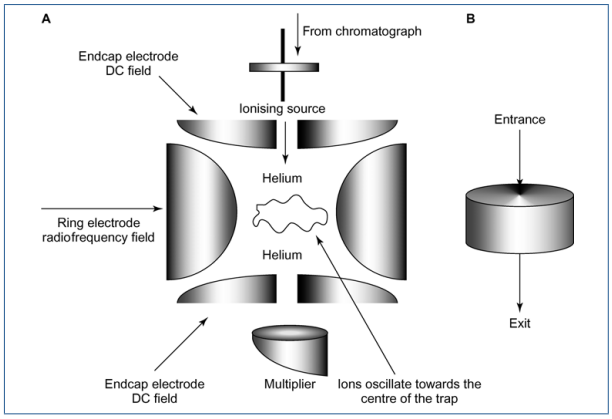

Figure 2.14: quadrupole ion trap

The linear ion trap can be seen as a quadrupole mass analyzer where the entrance and exit sides of the rods have additional electrodes on which potentials can be applied in order to keep the ions from leaving the trap.

Ions of the same polarity repell each other in the trap and their trajectories expand as a function of time. To avoid ion losses by this expansion the trap is filled with helium gas (10^{-3} Torr or 0.13 Pa) that removes excess energy from the ions.

The properties in terms of resolution and upper mass limits are comparable with quadrupole mass analyzers.

2.2.5 FT-ICR

Ion cyclotron resonance (ICR) is an ion trap that uses a magnetic field in order to trap ions into an orbit inside of it. In this analyzer no separation of ions with different m/z occurs. Instead all the ions of a particular range are trapped inside, and an applied external electric field helps to generate a signal for all ions simultaneously. As mentioned earlier, when a moving charge enters a magnetic field, it experiences a centripetal force making the ion orbit. Again the force on the ion due to the magnetic field is equal to the

centripetal force on the ion.

$$zvB = \frac{mv^2}{r}$$

Angular velocity of the ion perpendicular to the magnetic field can be substituted here

$$\omega_c = \frac{v}{r}$$
$$zB = m\omega_c$$
$$\omega_c = \frac{z}{m}B$$

Frequency of the orbit depends on the charge and mass of the ions, not the velocity. If the magnetic field is held constant, the charge to mass ratio of each ion can be determined by measuring the angular velocity ω_c. The relationship is that, at high ω_c, there is low *m/z* value, and at low ω_c, there is a high *m/z* value. Charges of opposite signs have the same angular velocity, the only difference is that they orbit in the opposite direction.

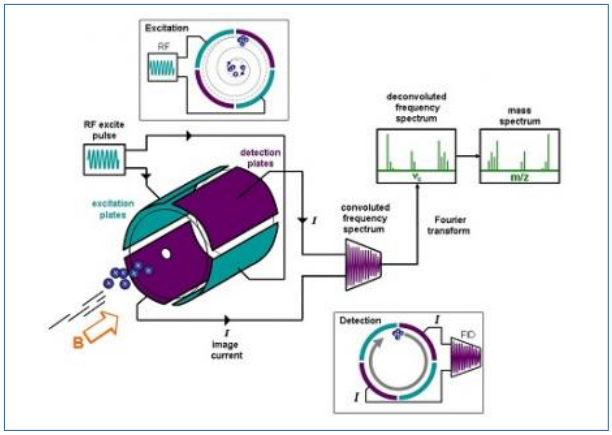

Figure 2.15: FT-ICR

To generate an electric signal from the trapped ions, a variable electric field is applied to the ion trap

$$E = E_o cos(\omega_c t).$$

When the ω_c in the electric field matches the ω_c of a certain ion, the ion absorbs energy making the velocity and orbiting radius of the ion increase.

In this high energy orbit, as the ion oscillates between two plates, electrons accumulate at one of the plates over the other inducing an oscillating current, or current image. The current is directly proportional to the number of ions in the cell at a certain frequency.

In a FT-ICR, all of the ions within the cell are excited simultaneously so that the current image is coupled with the image of all of the individual ion frequencies. A Fourier transform is used to differential the summed signals to produce the desired results.

The resolution of FT-ICR instruments is very high (10^6) and depends on the Fourier transform parameters and the acquisition time used.

2.2.6 orbitrap

The orbitrap mass analyzer was invented by Alexander Makarov and published in 2005. The orbitrap consist of a central spindle shaped and a barrel like outer electrode. The working principle of the orbital mass analyzer is based on the orbital trapping of ions. Injected ions cycle around the central electrode and at the same time oscillate along the horizontal axis.

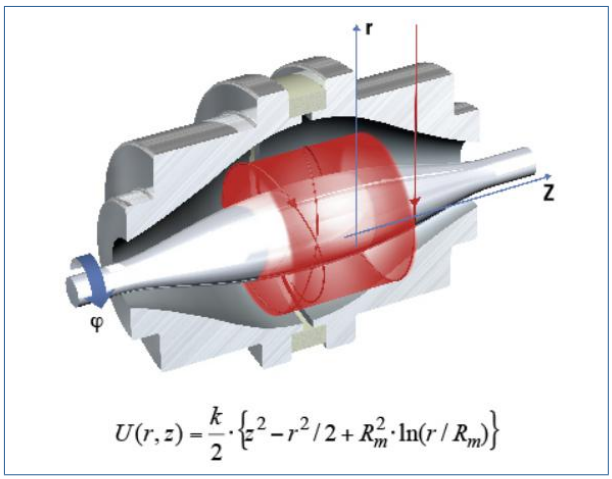

Figure 2.16: orbitrap

The frequency of the oscillations in the axial direction depends on the individual *m/z* values of the ions and can be processed by Fourier transform.

Although orbitrap mass analyzers operate in a pulsed fashion, they can be coupled to continuous sources. Ion storage devices (like quadrupole ion traps) had made this coupling possible.

Orbitrap mass analyzers operate with good sensitivity, high mass resolving power (up to 150 000), mass accuracies in the order of ppm and mass range up to *m/z* 2000.

2.2.7 time-of-flight

Time-of-flight (TOF) analyzers separate ions by time without the use of an electric or magnetic field. With TOF the separation is based on the kinetic energy and velocity of the ions.

Ions of the same charges have equal kinetic energies; kinetic energy of the ion in the flight tube is equal to the kinetic energy of the ion as it leaves the ion source:

$$E_k = \frac{mv^2}{2} = zV$$

The time of flight, or time it takes for the ion to travel the length of the flight tube is:

$$T_f = \frac{L}{v}$$

with L is the length of tube and v is the velocity of the ion.

Substituting the equation for kinetic energy in equation for time of flight:

$$T_f = L\sqrt{\frac{m}{z}}\sqrt{\frac{1}{2V}} \propto \sqrt{\frac{m}{z}}$$

During the analysis, L (length of tube) and V (the voltage from the ion source) are held constant, which can be used to say that time of flight is directly proportional to the root of the mass to charge ratio.

Unfortunately, at higher masses, resolution is difficult because flight time is longer. Also at high masses, not all of the ions of the same *m/z* values reach their ideal TOF velocities. To fix this problem, often a reflectron is added to the analyzer. The reflectron consists of a series of ring electrodes of very

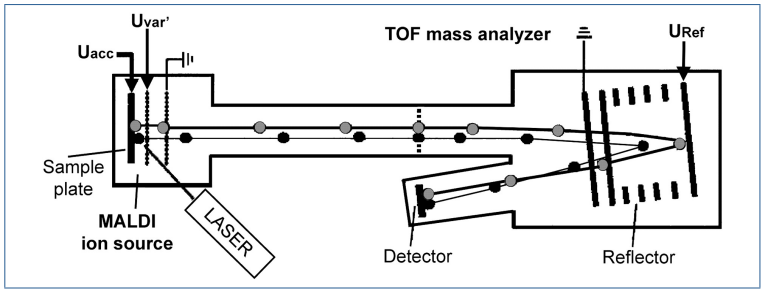

Figure 2.17: time-of-flight instrument with reflectron

high voltage placed at the end of the flight tube. When an ion travels into the reflectron, it is reflected in the opposite direction due to the high voltage.

The reflectron increases resolution by narrowing the broadband range of flight times for a single m/z value. Faster ions travel further into the reflectrons, and slower ions travel less into the reflector. This way both slow and fast ions, of the same m/z value, reach the detector at the same time rather than at different times, narrowing the bandwidth for the output signal.

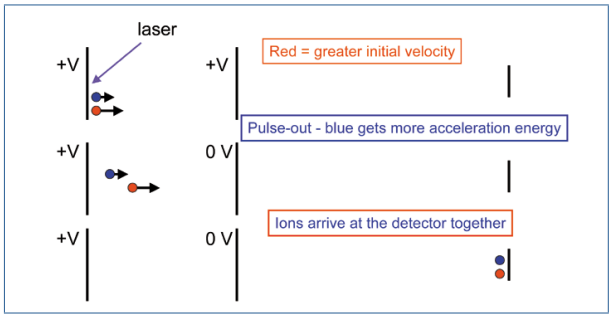

Figure 2.18: delayed extraction

2.2.8 traveling wave

The traveling wave device is in fact not really a mass analyzer but acts as an ion guide similarly to RF-only multipoles. Traveling wave devices consist of a large set of parallel plates with a hole in the center. On each of the plates a potential can be applied. Typically, a sinusoidal potential is applied

on consecutive plates and this wave shifts along the longitudinal axis as a function of time, pushing the ions to the exit of the device.

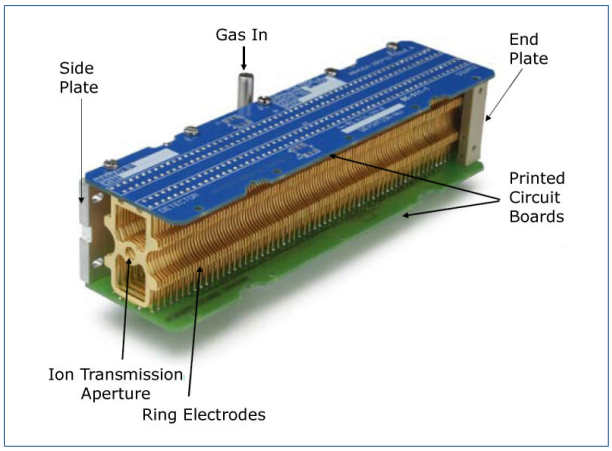

Figure 2.19: traveling wave

2.2.9 ion mobility

Ion mobility spectometers have the ability to separate ions not according to their *m/z* values, but to their size (cross-section). The timescale of the separation is in the order of milliseconds.

There are different types of ion mobility spectrometers and there are different types of mass spectrometers. In principle it is possible to combine every type of the former with any type of the latter.

Drift tube ion mobility does not employ RF voltage which may heat ions, and it can preserve the structure of the ions. The rotationally averaged collision cross section (CCS) which is a physical property of ions reflecting the shape of the ions can be measured accurately on drift tube ion mobility. The resolving power is high (CCS resolution can be higher than 100). Drift tube ion mobility is widely used for structure analysis. It is usually coupled with time-of-flight (TOF) mass spectrometer. Ion mobility (μ) is determined from the drift velocity (v_d) attained by ions in a weak electric field (E) in the drift tube, according to the simple equation

$$v_d = \mu E$$

High-field asymmetric-waveform ion-mobility spectrometry (FAIMS or RF-DC ion-mobility spectrometry) is a mass spectrometry technique in which ions at atmospheric pressure are separated by the application of a high-voltage asymmetric waveform at radio frequency (RF) combined with a static (DC) waveform applied between two electrodes.

Triwave technology which enables ion mobility separations comprises three traveling wave devices which are used to manipulate (trap, accumulate, release, separate and fragment) ions in a very precise, rapid and efficient manner. As ions are driven by wave forms in the traveling wave device, they interact/collide with the neutral buffer gas which slows them down, causing ions of different size, shape, charge and mass to transit at different rates. Species with high mobility (more compact) surf more on the wave front and are overtaken by the wave less often than those species of low mobility (more extended), hence mobility-based separation occurs.

2.3 detectors

When the ion beam has passed through the mass analyzer, it has to be detected and the signal has to be transformed into a usable electrical signal. There exist two types of ion detectors: *direct detectors* that allow a direct measurement of the charges that reach the detector and *multiplier detectors* that increase the intensity of the signal.

2.3.1 photographic plate and Faraday cylinders

The first mass spectrometers used photographic plates to detect ions. Ions with the same m/z exit the analyzer and reach the plate at the same place. The darkness of the spots gives a value of the beam intensity.

Faraday cylinders or Faraday cups are devices that also can be used to detect ions. When ions reach the cylinder, they are neutralized and the discharge current can be amplified and measured. The sensitivity of such detectors is limited but they have the property to generate signal intensities that correspond to the charge of the ions (and not to the energy or mass).

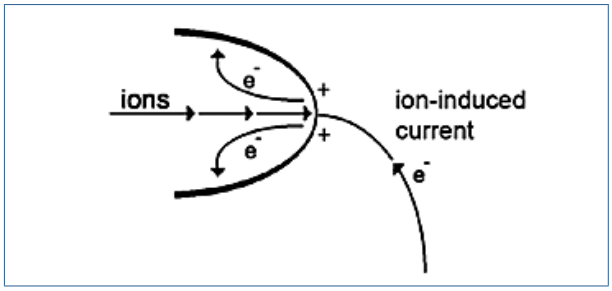

Figure 2.20: Faraday cup

2.3.2 electron multipliers

When charged particles hit a high voltage electrode, electrons are dislodged. These electrons pass further to the next charged plate causing more electrons to be emitted. This is repeated several times and the result is a measurable electric current.

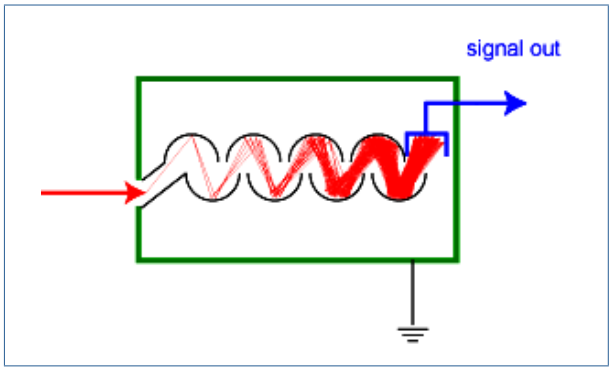

Figure 2.21: electron multiplier

The cascade multiplier is preceded by a conversion dynode that converts the ion beam coming from the analyzer into secondary particles. These secondary particles can include positive ions, negative ions, electrons and neutrals.

The multiplying dynodes can consist of individual electrodes (discrete dynodes) or it can be a continuous dynode. Their amplifying power is typically 10^7.

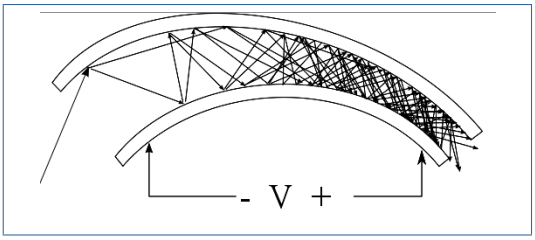

Figure 2.22: cascade multiplier

2.3.3 array detectors

Array detectors (multichannel plate detectors) work based on the sample principle as the continuous dynode electron multipliers. They consist of a plate where parallel cylindrical channels have been drilled. The channels have diameters in the range from 4 to 25 nm with a center-to-center discance from 6 to 32 nm. A voltage difference of 1 kV is kept over the input and output side of the plate.

In order to improve the collision efficiency of the electrons with the channel surface, curved channels can be used or two plates can be sandwiched together in a Z-shape (chevron assembly).

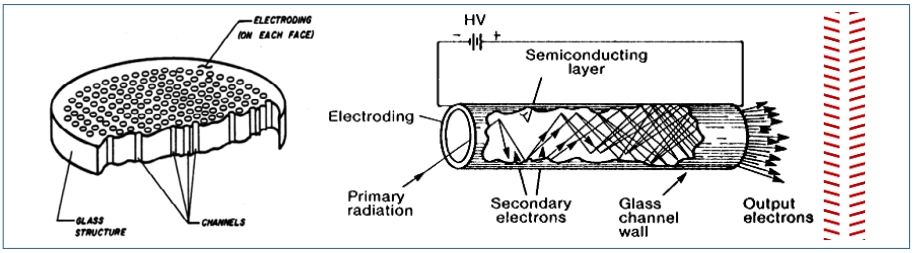

Figure 2.23: array detector

2.3.4 photon multipliers

This type of detector consists of a phosphorescent screen and a multiplier. Ions are converted to electrons and accelerated towards a phosphorescent screen where they are converted into photons. These photons enter a vaccuum tube containing a photocathode and an electromultiplier.

The amplification range is usually 10^4 to 10^5. Their lifetime is longer than electron multipliers.

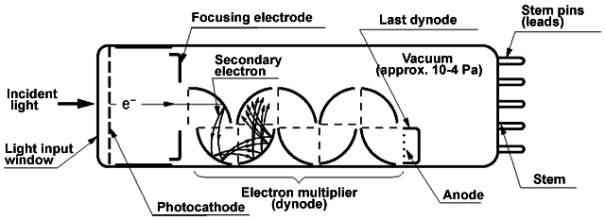

Figure 2.24: photon multiplier

2.3.5 electric signal measurement

The output of electromultipliers (and photon multipliers) is an electric current. This is converted into a voltage which is fed into an analog-to-digital converter (ADC). The digital values are then processed and stored on a datasystem.

2.4 vacuum system

Mass analyzers require high levels of vacuum (very low pressure) in order to operate in a predictable and efficient way. Ions need to be guided on a specific pathway only influenced by electric, magnetic and radiofrequency fields and all collisions with residual air molecules have to be eliminated. The mean free path (distance ions can travel without coliisions) is given by the formula

$$L = \frac{0.661}{P}$$

with L = distance (in cm) and P = pressure (in Pa)[5]

Modern mass spectrometers have a vacuum system consisting of a dual differential pumping system. The first step is a rough vacuum pump, usually

[5] 1 Pa = 0.007 5 Torr. 1 bar = 100 000 Pa ≈ 750 Torr

a vane rotary pump. The second step is a high vacuum tubomolecular or diffusion pump. The vacuum needs depend on the analyzer type.

2.4.1 oil diffusion pump

In diffusion pumps oil is heated and rises up the chimney. It escapes through circular openings at various levels and condenses in contact with the cool walls. Gases are trapped in the vapours and directed downwards to the exhaust of the pump connected to a forline pump.

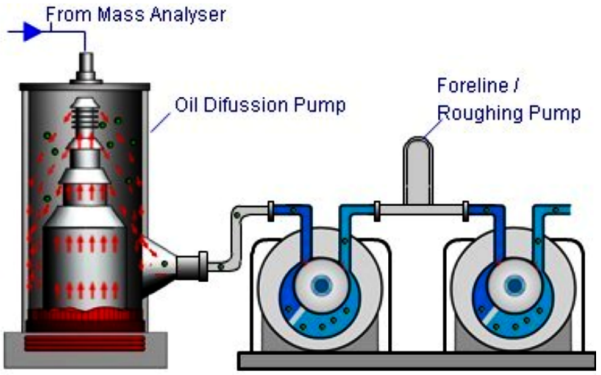

Figure 2.25: diffusion pump with foreline pump

The boiler operates between 280 and 300°C generating oil vapour of around 100°C. The pump capacity is similar as for turbomolecular pumps (200-500 L/s). Water cooled diffusion pumps have coils through which cooling water circulates during operation.

2.4.2 turbomolecular pump

These pumps consist of a system of rotating foils or blades that are angled so that air molecules are compressed and progressively drawn to the vent port. The speed of rotation is up to 60 000 rpm and their capacity is 150-200 L/s. The exhaust is connected to a foreline pump.

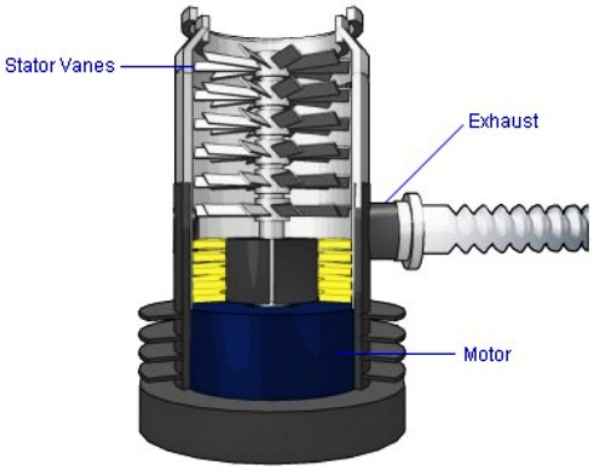

Figure 2.26: turbomolecular pump

2.4.3 rotary pump

Rotary pumps are pumps that move fluid using the principles of rotation. The vacuum created by the rotation of the pump captures air into the liquid. A rotary pump consists of vanes mounted to a rotor that rotates inside a cavity. The eccentric drive causes air to be moved from the inlet to the exhaust. Depending upon the design of the mass spectrometer, foreline pumps (also called roughing pumps) are employed to assist the high vacuum pumps directly attached to the instrument. Rotary vane pumps can reach pressures to 0.1 Pa (10^{-3} Torr) mainly due to the vapour pressure of the sealing oil. The air evacuation capacity depends on the rotary speed and is typically between 50 and 150 L/min at 1 400 rpm.

The function of the foreline pumps is to reduce the pressure within a perticular region of the spectrometer to approximately 1 Pa (10^{-2} Torr) prior to the high vacuum pump establishing the required analyzer pressure.

2.5 tandem mass spectrometers

Tandem mass spectrometry (MS/MS) employs at least two stages of mass analysis in series. The first mass analyzer can be followed by a collision

device where ions are fragmented and subsequently analyzed by the second mass analyzer. This process can be repeated by selecting one of the obtained fragments and fragment again. This is then labeled as MS/MS/MS or MS^3 experiments. The number of steps can further be increased to yield MS^n experiments where n refers to the number of generations of ions that are analyzed.

Tandem mass spectrometry can be performed in space or in time. In space means that the selection of ions and analysis is performed in two physically distinct instruments (QqQ configuration). In time means that the analysis is performed in one mass analyzer, but at a different time (ion traps). Scanning mass analyzers (sector instruments, quadrupoles and ion traps) can be used as both first and second stage analyzers while other analyzers are mainly used in the second stage.

Fragmentation of ions is a valuable method for determination of the structure of compounds. It allows also to obtain sequence information for biopolymers such as peptides and oligonucleotides.

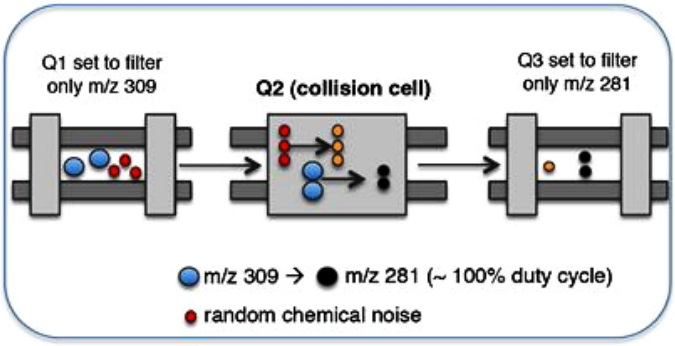

Figure 2.27: triple quadrupole tandem mass spectrometer

Scanning mass analyzers can be used in scanning or in static mode: scanning mode means that a spectrum is recorded by changing the potentials for transmission of *m/z* in such a way that all *m/z* values in a range will be transmitted one by one in function of time. In static mode only one *m/z* value is transmitted all the time. For a single quadrupole operating in static mode

this mode is called *selected ion monitoring* (SIM). In this method both a high sensitivity and selectivity is obtained.

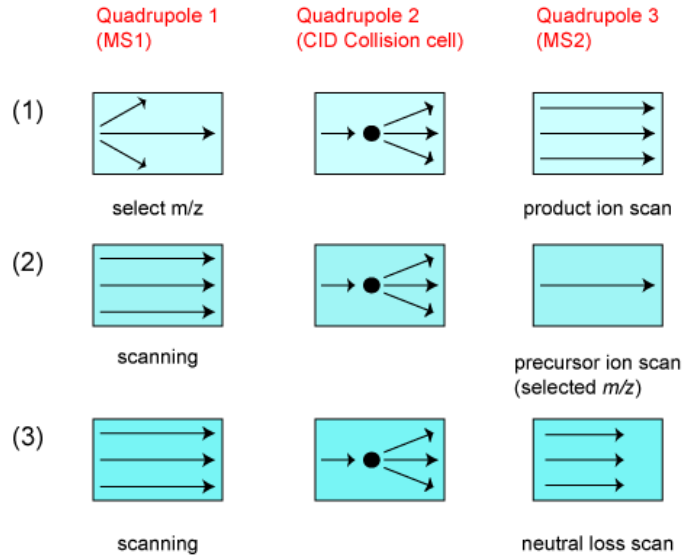

Figure 2.28: scan modes in a QqQ configuration tandem mass spectrometer

As an example we will discuss the possibilities of a triple quadrupole instrument.[6]

product ion scan

In product ion scan mode the first mass analyzer is set to static mode in such a way that the *m/z* of the ion that one wishes to fragment (the precursor ion) is transmitted. In the collision cell, filled with an inert gas (argon, helium), the selected ions collide and fragment. The generated fragments are then analyzed by the second quadrupole by scanning a mass range. Fragments have by definition a lower mass, but fragments from a multiple charged precursor ion can have higher *m/z* values.

The resolution setting of the first quadrupole determines whether isotopes are also transmitted through the first quadrupole. This will influence the spectrum obtained after fragmentation. If the precursor ion corresponds to a

[6] although it is called a *triple quadrupole*, these instruments contain only two quadrupoles

monoisotopic peak, then the product ion spectra will not contain any isotope peaks.

precursor ion scan

In this mode, the first quadrupole is scanning a m/z range while the second quadrupole is set to the m/z value of an expected fragment. The obtained spectrum will contain all precursor ion m/z values that generate the fragment set on the second mass analyzer. This technique is useful for characterizing compounds which are similar and generate the same fragment.

neutral loss scan

A neutral loss scan is obtained by scanning both the first and the second mass analyzer, but at each time point the m/z are set in such a way that an offset is present between the two analyzers. This technique allows to scan for ions that lose a neutral fragment.

multiple reaction monitoring

Comparable to single ion monitoring on a single quadrupole instrument, on a triple quadrupole instrument one can set both mass analyzers to a fixed m/z value.

This mode allows for a highly selective and sensitive detection of compound of which the fragmentation is known. If only one transition is measured during an LC/MS run, we call it *selected reaction monitoring*. When multiple transitions are followed (by alternating the settings every few milliseconds), we call this *multiple reaction monitoring*.

3

DATA ACQUISITION AND PROCESSING

3.1 the mass spectrum

3.1.1 X-axis: *m/z*

The X-axis in a mass spectrum is given in *m/z* units. This represents the data as they were recorded. When the spectrum contains multiply charged ions (for example with electrospray ionization), it is often more clear to perform a deconvolution of the spectrum in order to show the real mass of the components. Deconvolution is a software tool and yields a spectrum with mass units on the X-axis instead of *m/z*.

3.1.2 Y-axis: abundance

The Y-axis gives the intensity of the detected ions. Nowadays, the scale is normalized: the largest peak in a spectrum is (also called *base peak*) set to 100% and the intensities of all the peaks are given relative to the intensity of the largest peak. Earlier, absolute intensities were given, but this has no practical meaning since it depends on the sample concentration, instrument type and tuning.

If needed, parts of the spectrum can be magnified in order to visualize peaks with small intensities. The magnification factor and range are usually indicated at the top of the spectrum.

3.1.3 isotope distribution

In the spectra not only the *m/z* values for the ions can be found, but also other useful information is available. The isotope peaks give information about the presence of elements (such as Cl, Br), the amount of carbon atoms in the ion and the charge state of the ions.

3.1.4 raw data

MS data collected with an instrument are presented as either profile or centroid mode.

In raw data mode (also called profile mode), a peak is represented by a collection of signals. The advantage of profile data is that it is easier to classify a signal as a true peak from noise of the instrument. It also contains and shows the information about the resolution of the mass analyzer used.

3.1.5 centroid data

In centroid mode, the signals are displayed as discrete *m/z* values with zero line widths. The advantage of centroid data is the file size is significantly smaller as there is less information describing a signal. At the same time, information about the resolution of the instrument can not be deduced from the spectra. This information can sometimes be extracted from the instrument settings.

3.1.6 deconvolution

Multiply charged ions give peaks at different *m/z* values for different charge states. By processing the data they can be deconvoluted to a peak giving the mass of the measured compound. There are several software algorithms

available for this deconvolution, each instrument vendor uses his own method.

3.2 spectrum recording

3.2.1 full scan

In full scan mode, a spectrum is recorded. Depending on the analyzer type, this is done by a one-shot procedure (time-of-flight) or by setting electronic parameters to allow to measure a range of m/z values one by one (magnetic sector, ion trap, quadrupole).

3.2.2 selected ion recording

Selected ion recording is mainly used in LC/MS experiments. The mass analyzer is set to a fixed m/z value and records only the intensities for this m/z as a funtion of time. The advantage over recording spectra is the increased sensitivity, but as a consequence, information is lost as only one m/z is monitored.

It is possible to record multiple ions by alternated switching between different m/z values for a short time.

3.2.3 selected reaction monitoring

Tandem mass spectrometers consist of more than one analyzer. In a triple quadrupole instrument, the first and second quadrupole can be set to a precursor and fragment respectively, allowing to monitor a reaction. Similarly as with selected ion monitoring, this technique is mainly used in LC/MS experiments. The fragmentation and monitoring of a fragment strongly increases the specificity of the detection: only ions with a certain m/z which generate a fragment set in the second quadrupole will be detected.

Also, as with selected ion monitoring, multiple reactions can be measured by alternating the settings.

3.2.4 MSn

Mass analyzers such as ion traps allow not only the selection of an ion, followed by fragmentation and detection of the fragments. In addition, one of the fragments can be selected and subsequently fragmented generating MS/MS/MS spectra (also called MS^3 spectra).

3.3 hyphenated techniques

Coupling separation techniques (HPLC, capillary electrophoresis, gas chromatography) to mass spectrometers allows the analysis of complex mixtures. The separation of components prior to the mass spectrometric analysis generates less complicated spectra which is beneficial for interpretation.

In general, during a separation experiment the mass spectrometer records data (full scan, ion monitoring or reaction monitoring mode). It is important that at least ten datapoints are collected over a chromatographic peak. For a conventional HPLC experiment, chromatographic peak widths are around 20 seconds, meaning that the mass spectometer should record datapoints at least every 2 seconds.

If full scan spectra were recorded, post-processing allows the extraction of different chromatograms. *Total ion chromatograms* (TIC) are generated by plotting for each recorded spectrum the sum of al the ion intensities recorded in a spectrum. The disadvantage is the fact that spectra with a lot of noise (lot of low intensity peaks) will also generate a substantial intensity in the total ion chromatograms.

Base peak chromatograms (BPC) are chromatograms obtained by plotting the peak with the highest intensity (base peak) from each spectrum. The advantage over the total ion chromatogram is that peaks usually will be better visible because of the reduction of the noise.

Once full spectra are recorded, it is also possible to extract the intensities of a certain *m/z* value of interest. This is called an *extracted ion chromatogram*

(XIC).

Software usually allows to detect peaks, just as with HPLC instruments. It is also possible to subtract background spectra or to sum spectra from a region of the chromatogram. This is useful for identifying compounds in a chromatographic peak.

When chromatography is coupled to mass spectrometry, this adds a third dimension to the data. It is obvious that such data can be represented in a 3D plot or contour plot.

4

APPLICATIONS

4.1 small molecules

4.1.1 accurate mass measurements

From accurate mass measurements (less than 2 ppm error) the elemental composition can be calculated. This can be used for structural identification. In addition, fragmentation and H/D exchange can provide additional information about the structure of compounds.

For accurate mass measurements, the analyzer should be capable of recording data with a high resolving power. The presence of peaks in close vicinity of the peaks for the compound under investigation can introduce errors in mass measurements.

In order to reduce the error, the instrument has to be well calibrated. In general, reference compounds are used to calibrated mass analyzers. These compounds (or mixtures of compounds) ideally generate peaks spread over the entire mass range of interest. By recording the mass spectra of these calibrants, the relation between the physical properties (current through a magnet, DC and RF settings on a quadrupole, time-of-flight etc.) can be established by applying a multipoint calibration function. Often a polynomial

function is applied. Once this relation is known, the instrument is ready to measure.

For accurate mass measurements some instruments use a *lockmass*. This is the simultaneous introduction of a reference compound allowing the recalibration of spectra (often on-the-fly) resulting in better mass accuracy.

4.1.2 use of stable isotopes

functional group determination

For the characterization of functional groups, hydrogen atoms can be exchanged with deuterium. This is accomplished by dissolving the compound in D_2O. Mainly hydrogens on heteroatoms (amines, thiols, alcohols) are exchanged. The increased mass gives an indication of the presence of these functional groups.

Similarly, $H_2{}^{18}O$ can be incorporated in carboxylic groups, phosphates and ketones allowing to identify them.

structural analysis

The synthesis of analogs containing stable isotopes is useful for the study of fragmentation patterns of molecules. By comparison of the labeled and non-labeled compounds it is often possible to find out what is going on during the fragmentation in a mass spectrometer. It allows to detect and follow the participation of hydrogens in the fragmentation pathway.

quantitation

Quantitative analysis can be performed by creating calibration curves by LC/MS using increasing concentrations. The ionization efficiency is not guanranteed to be constant over time, and therefore an internal calibrant is often needed in order to achieve acceptable results. Any compound can be used as internal reference compound on condition that it does not interfere with the compound(s) to be analyzed. The best reference compound, however, is the one with the same retention time as the compound under

investigation and having the same chemical and physical properties. The best match for such a reference compound is a derivative with stable isotopes incorporated (such as ^{13}C, ^{2}H, ^{18}O or ^{15}N).

From the ratio of the intensities of the unknown to the reference compound it is possible to determine the amount. The selection of the reference substance should carefully be performed: there should be enough heavy isotopes incorporated in order to avoid interference of the isotopes of the compound under investigation with the monoisotopic peak of the reference compound. In most cases the reference compound should have a mass at least three mass units higher, often five mass units are preferable.

protein conformation by hydrogen/deuterium exchange

In a folded protein, the solvent can access only parts of the sequence. By exchanging hydrogens in these parts of the molecule and measuring the increase of mass it is possible to assess the surface of proteins and obtain a (rough) picture of the folding of proteins. This techniques can also be used to study the folding/unfolding of proteins.

The location and relative amount of deuterium exchange along the peptide backbone can be determined roughly by subjecting the protein to proteolysis after the exchange reaction has been quenched at pH 2.6. Individual peptides are then analyzed for overall deuteration of each peptide fragment. Pepsin, an acid protease, is commonly used for proteolysis, as the quench pH must be maintained during the proteolytic reaction. To minimize the back-exchange, proteolysis and subsequent mass spectrometry analysis must be done as quickly as possible. HPLC separation of the peptic digest is often carried out at low temperature just prior to electrospray mass spectrometry to minimize back-exchange. More recently, UPLC has been used due to its superior separation capabilities.

4.1.3 metabolomics

Cells produce metabolites and the study of the contents of the pool of metabolites related to the life, growth and environment of the cells is called

metabolomics.

Because the huge amount of different metabolites and the complex composition of cells, protocols exist to prepurify the samples and run them by LC/MS. The goal of identification as many metabolites as possible can only be achieved by instruments capable of measuring accurate masses (high resolution instruments) and is usually performed in an unsupervised manner. Software procedures such as principal component analysis allow the identification of metabolites that have changed from one growth condition to another.

4.1.4 ADME

When a drug is taken by a patient, the chemical substance is absorbed, distributed in the organism, metabolized and excreted (ADME). The study of this process can be approached qualitatively (identification of metabolites formed by the liver enzymes) or quantitatively (plasma concentrations of a drug). For quantitative work often isotopically labeled derivatives are used.

4.2 peptides and proteins

4.2.1 sequencing

When subjected to fragmentation, covalent bonds along the backbone are cleaved in peptides. The site of fragmentation can be described using the symbols a, b and c for the amino terminal and x, y and z for the carboxy terminal fragments. The index (for example b_1, b_2 etc.) represents the number of amino acids in the fragment.

The ions that are formed depend on the fragmentation method applied. From the fragment ion spectra it is possible to reconstruct the peptide sequence using software. The larger the peptides are, the more difficult it will be to obtain the correct sequence. Also, for large peptides, the whole sequence will not be covered from all the fragments found in a fragment ion spectrum.

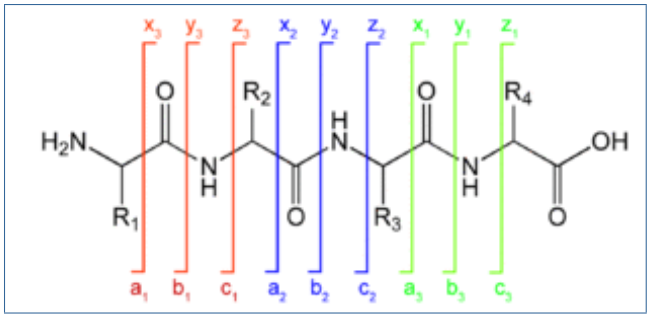

Figure 4.1: peptide fragmentation in a mass spectrometer

4.2.2 digestion

Large proteins (mass larger than 10 kDa) are generally too large to be fully sequenced by mass spectrometry. Often, is is advisable to digest the protein to smaller pepetides and then to sequence the obtained pieces. Mostly enzymes like trypsin and chemotrypsin are applied. They cleave adjacent to a basic amino acid generating peptides containing at least one basic residue which is beneficial for the ionization of the peptides in the mass spectrometer.

One should be careful with disulfide bridges. If they interfere with the analysis they should be reduced to thiol groups using dithiothreitol (DTT). In order to prevent reoxidation, the thiol groups are fot that purpose immediately alkylated using iodoacetamide (IAA).

4.2.3 proteomics

Proteomics is the large-scale study of proteomes. A proteome is a set of proteins produced in an organism, system, or biological context. We may refer to, for instance, the proteome of a species (for example, *Homo sapiens*) or an organ (for example, the liver). The proteome is not constant: it differs from cell to cell and changes over time. To some degree, the proteome reflects the underlying transcriptome. However, protein activity (often assessed by the reaction rate of the processes in which the protein is involved) is also modulated by many factors in addition to the expression

level of the relevant gene.

Proteomics is used to investigate:

- when and where proteins are expressed
- rates of protein production, degradation, and steady-state abundance
- how proteins are modified (for example, post-translational modifications (PTMs) such as phosphorylationor glycosylation)
- the movement of proteins between subcellular compartments
- the involvement of proteins in metabolic pathways
- how proteins interact with one another

Proteomics can provide significant biological information for many biological problems, such as:

- Which proteins interact with a particular protein of interest?
- Which proteins are localized to a subcellular compartment?
- Which proteins are involved in a biological process?

Several high-throughput technologies have been developed to investigate proteomes in depth. The most commonly applied are mass spectrometry based techniques such as tandem-MS and gel-based techniques such as differential in-gel electrophoresis (DIGE). These high-throughput technologies generate huge amounts of data. Databases are critical for recording and carefully storing this data, allowing the researcher to make connections between their results and existing knowledge.

The identification of proteins in a sample is a crucial step. If possible, the sample should be fractionated or purified before analysis. The analysis can consist of a digestion followed by recording a mass spectrum on a MADI-TOF instrument or subjecting the sample to a LC/MS experiment where not only the masses of the peptides, but also the fragment ion spectra are recorded.

After the data collection is finished, the spectra are compared with theoretical masses of peptides and their fragments obtained by calculation from sequences from (genome) databases.

Once proteins are identified, further analyses can be performed in order to assess the quantity of that protein as a function of cell growth, environment, stress factors and so on.

4.3 nucleic acids

4.3.1 nucleic acids sequencing

The existence of good DNA sequencing methods (Sanger) causes mass spectrometry mainly to be applied for the characterization of post-transcriptionally modified nucleosides in RNA and in the research of DNA and RNA modifications by drugs or environmental factors.

The tools and protocols are similar to those described for peptides. Oligonucleotides are usually measured in negative ion mode as the phosphate backbone is negatively charged. Fragment ion spectra can be generated and from the fragments the sequence can be derived. Compared to peptides, the number of residues that can be sequenced is usually lower because the mass per residue is larger (about 300 u compared to 100 u for peptides). Obviously, the nomenclature is different too and the most important ions for sequencing are generally the a-B and w-ions.

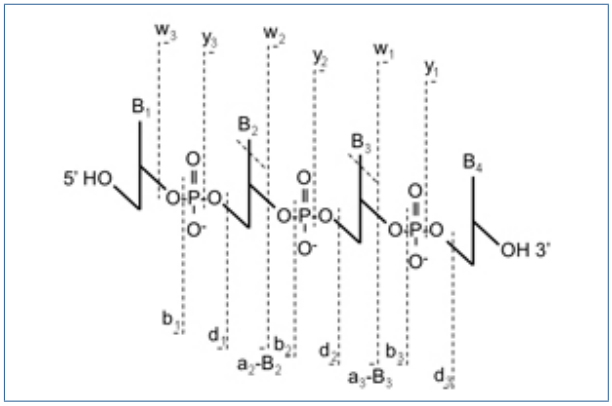

Figure 4.2: oligonucleotide fragmentation in a mass spectrometer

4.3.2 nucleic acids digestion

For the characterization of modifications in DNA and RNA, the nucleic acids can be digested to nucleotides using exo- and endonucleases. The choice of the buffer is important as it may not interfere with the ionization. In particular, sodium and potassium ions have a negative effect on the formation of negative ions as they tend to form adducts. As nucleotides are too polar to be separated on reversed phase columns, the phosphate group is removed using phosphatases first. The nucleosides are then analyzed using LC/MS methods. The presence of a (weak) glycosidic bond enhances the fragmentation of the nucleosides into a base and sugar part. This is not a drawback, but gives important information about the site of any modification.

In order to obtain the sequence location of the modifications, nucleic acids are digested into oligonucleotides. For RNA a typical enzyme is T_1 endonuclease which cleaves at the 3' end of a guanosine generating oligonucleotides ending on -Gp. The obtained oligonucleotides can then be analyzed using LS/MS. The chromatographic system has to be adapted as oligonucleotides are very polar due to the presence of the phosphate groups. In order to obtain retention on a C18 reversed phase column, ion pairing reagents are used in the buffer (triethylamine with 1,1,1,3,3,3-hexafluoro-2-propanol).