

Chapter 4

MASS SPECTROMETRY

In this chapter, you will learn about . . .

- ◆ . . . the basic principle and instrumentation of mass spectrometry (MS).
 - ◆ . . . the two most important techniques for the analysis of biomolecules: MALDI-TOF/MS and ESI-MS.
 - ◆ . . . how mass spectrometry is used to determine the molecular weight of even large biomolecules like DNA and proteins.
 - ◆ . . . how mass spectrometry is used as a separation method.
 - ◆ . . . and how mass spectrometry can be used to obtain structural information about peptides and polynucleotides.
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Mass spectrometry is among the most powerful tools in protein and DNA analysis. It can determine molecular weights of biomolecules as large as 500,000 Da with high accuracy. Structural information like the amino acid sequence in a peptide or the sugar sequence in an oligosaccharide can be obtained. Some mass spectrometers can be coupled directly to a separation method such as LC or CE to combine the strengths of both techniques.

4.1 The Principle of Mass Spectrometry

A mass spectrometer determines the molecular weight of ions in vacuum. The sample molecules are first ionised in what is known as the *ion source* (Fig. 4.1). The gaseous ions are then introduced into a *mass analyser* and separated according to their *mass-to-charge* (m/z) ratio. A *detector* registers the signals and passes information to a computer for analysis and spectrum recording. To avoid collisions between ions and air molecules, a high vacuum of about 10^{-5} Pa is required.

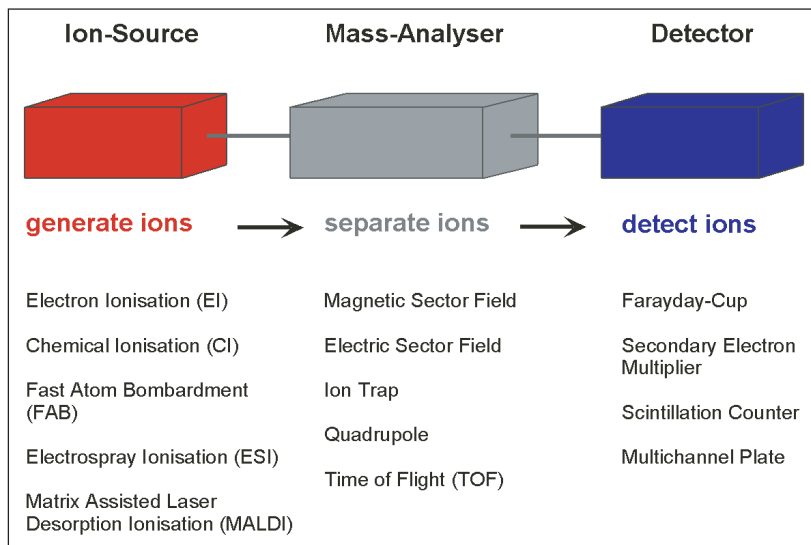


Fig. 4.1. The principal components of a mass spectrometer.

4.1.1 Ionisation

The sample molecules can be ionised by one of several techniques. In *electron impact ionisation*, (EI), electrons are fired at the sample molecules, whereas in *chemical ionisation*, (CI), the sample molecules are collided with a reactive gas. The sample can also be bombarded with argon atoms (*fast atom bombardment*, FAB) or the dissolved sample can be sprayed into an electric field (*electrospray ionisation*, ESI). Furthermore, the sample can be co-crystallised with a matrix and then ions can be generated by exposure to photons (*matrix assisted laser desorption ionisation*, MALDI).

All these techniques result in positively and/or negatively charged ions in the gaseous phase. *Hard ionisation methods* like EI and FAB lead to the breakdown of the sample molecules into smaller fragments. These fragments give a “fingerprint” of the sample and thus valuable information. *Soft ionisation methods* like ESI and MALDI lead to molecular ions $[M]^+$, and quasi molecular ions such as $[M + H]^+$ which can be used for molecular weight determination.

4.1.2 Mass Analyser

The mass analyser separates the ionised species according to their mass-to-charge (m/z) ratio. This can be achieved by magnetic or electric sector fields, an ion trap,

a quadrupolar magnetic field with high frequency, or in a time-of-flight (TOF) analyser.

4.1.3 *Detector*

A Faraday-cup, a secondary electron multiplier, a scintillation counter or a multichannel plate are used for ion detection.

In bioanalytical chemistry, soft ionisation methods such as ESI and MALDI are preferred as they allow analysis of whole protein or DNA molecules. MALDI is usually combined with TOF analysers, whereas ESI is combined with quadrupole analysers. These two methods are explained in detail in the following sections.

4.2 Matrix Assisted Laser Desorption Ionisation – Time Of Flight Mass Spectrometry (MALDI-TOF/MS)

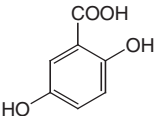
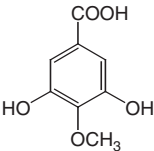
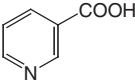
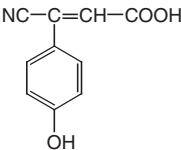
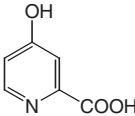
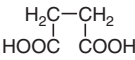
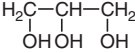
Koichi Tanaka presented experiments for *soft laser desorption ionisation (SLD)* of proteins in 1987. However, the predominant and most widely used version of SLD, *matrix assisted laser desorption ionisation, MALDI*, was introduced shortly afterwards by Michael Karas and Franz Hillenkamp. Tanaka was awarded the Nobel Prize for his cornerstone invention in 2002. Prior to that, no method was available to transfer large biomolecules with molecular weights of more than 1,000 Da into the vacuum without fragmenting them.

With MALDI-TOF, molecular weights above 500,000 Da can be determined with sensitivities as low as fmol and mass accuracies as high as 0.1–0.01 %. Furthermore, small amounts of contaminants are tolerated, sample preparation is fairly straightforward and the information obtained can be submitted automatically for a database search.

4.2.1 *Ionisation Principle*

The ionisation principle is based on the soft desorption of the solid sample molecules into the vacuum and subsequent ionisation. First, the sample is co-crystallised with a 1,000–10,000 excess of a suitable *matrix* on a metallic plate. Small, organic, UV-absorbing molecules like sinapinic acid are used as matrix materials (Table 4.1). An electric field is applied between the sample plate and the entrance to the time-of-flight analyser (Fig. 4.2). A pulsed laser beam is then

Table 4.1. Typical matrix substances used for MALDI in biochemical analysis.

| Compound | Wavelength | Used for |
|--|--|--|
| 2,5-Dihydroxy benzoic acid (DHBA)  | 337 nm 355 nm | peptides, proteins, oligosaccharides |
| Sinapinic acid (SA)  | 337 nm 355 nm | proteins, peptides, glycoproteins |
| Nicotinic acid  | 266 nm | proteins, peptides, oligonucleotides |
| α -Cyano-4-hydroxy-cinnamic acid (α -CHCA)  | 337 nm 355 nm | peptides, proteins, oligosaccharides |
| 4-Hydroxy-picolinic acid  | 337 nm 355 nm | oligonucleotides |
| Succinic acid  | 2.94 μ m 10.6 μ m | peptides, proteins |
| Glycerine  | 2.79 μ m 2.95 μ m 10.6 μ m | peptides, proteins |

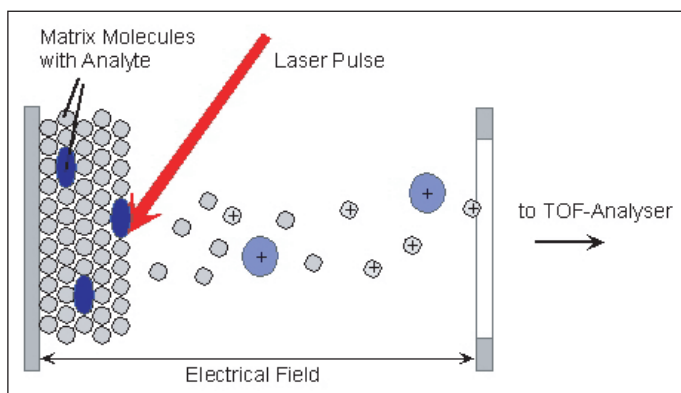


Fig. 4.2. The principle of matrix assisted laser desorption.

focussed onto the crystal. The matrix is chosen such that it absorbs readily at the laser wavelength. The sample, however, should not absorb at this wavelength. When bombarded with the photons from the laser pulse, the matrix molecules are excited rapidly and transferred into the gas phase together with the analyte molecules before energy is transferred to neighbouring molecules. Some matrix and analyte molecules become ionised during this process. Once in the gaseous phase, the ions are accelerated towards the TOF analyser by the applied electrical field. The matrix molecules can also take and donate protons or electrons to the analyte molecules and transfer ionisation energy. The analyte ions obtained are predominantly molecular ions $[M]^+$ or quasi-molecular ions like $[M + H]^+$ as well as adducts with alkali metal ions from buffer solutions like $[M + Na]^+$. Multiply-charged molecular ions also occur. Due to the high matrix concentration the analyte ions are prevented from interacting with each other. MALDI is a very soft ionisation method; large biomolecules like proteins, nucleic acids, polysaccharides and lipids can stay intact. Without the matrix, the analytes could only be desorbed at higher energies, but this would result in their fragmentation.

Nitrogen lasers with a wavelength of $\lambda = 337 \text{ nm}$ are most commonly used for matrices that absorb in the UV-area. Pulses are several ns long with photon energies of 3.68 eV. As an alternative, Nd-YAG lasers at $\lambda = 266$ or 355 nm are also used. IR-lasers are somewhat softer than UV-lasers but there is a limited choice of IR-absorbing matrices. Most frequently used are Er-YAG lasers at $\lambda = 2.94 \mu\text{m}$ with pulses less than 100 ns and photon energies of 0.42 eV or CO₂ lasers with $\lambda = 10.6 \mu\text{m}$. The laser beam passes through optical and electrical components and has a diameter of about 150 μm when it hits the target.

4.2.2 Mass Analysis in Time-of-Flight Analyser

The mass analyser used for MALDI is usually a time of flight (TOF) analyser, which allows for high resolution and accurate mass determination even for high molecular weight species. The ions desorbed by the laser pulse are accelerated in an electric field to a kinetic energy of several keV (Fig. 4.3). They then enter a field free tube in which they drift along with different speeds according to their mass/charge ratios. At the end of the tube, the ions hit a detector and the drift time is measured electronically to a high accuracy.

The kinetic energy of the drifting ions is defined as:

$$E_{kin} = \frac{1}{2} \cdot m \cdot v^2 = z \cdot e \cdot V \quad (\text{equation 4.1})$$

where m is the mass of the ion, v the velocity of the ion after the acceleration region, z is the ion charge, e the elementary charge and V the voltage of the applied electrical field. Light ions are accelerated more than heavier ions and reach the detector first. The velocity, v , can also be defined as the length of the field free drift tube, L , over the time of flight, t :

$$v = \frac{L}{t} \quad (\text{equation 4.2})$$

Substituting the velocity v in equation 4.1 by equation 4.2 leads to:

$$\frac{m}{z} = \frac{2 \cdot e \cdot V}{L^2} \cdot t^2 \quad (\text{equation 4.3})$$

The m/z ratio of the ion is proportional to the square of the drift time. Hence, the mass of an ion can be determined by measuring its drift time once the analyser is

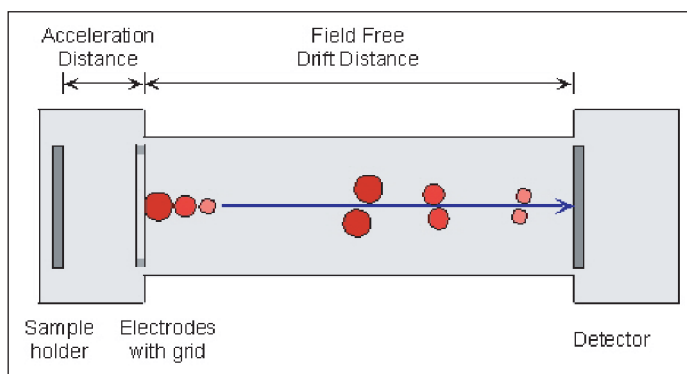


Fig. 4.3. The principle of a linear TOF analyser: the accelerated ions have different velocities according to their m/z .

calibrated with substances of known weight and charge. With this time measurement even heavy ions can be detected accurately, making TOF ideal for molecular weight analysis of biomolecules. Typically, the flight tubes have a length of about two meters, resulting in flight times in the order of microseconds. Very good sensitivities can be obtained, in comparison to other analysers, because all ions that have passed the pin hole into the time of flight analyser reach the detector.

Because not all the molecules get desorbed at the same time in exactly the same place, slightly different velocities are obtained for identical ions which can result in a broad peak and poor resolution. Using a reflector TOF set-up (Fig. 4.4) instead of the linear set-up shown above (Fig. 4.3), can overcome this problem.

In a *reflector TOF* tube, an electrical opposing field is applied at the end of the drift tube at which the ions are forced to change direction. Ions of the same mass but higher kinetic energies (velocities) get deeper into this opposing field and need more time for change of direction but catch up with smaller ions at a certain point in the drift field. When positioning the detector at this focusing point, very sharp signals can be obtained. Another advantage of the reflectron TOF is the capability of detecting ions that decay whilst in the tube, a process which is called *post source decay (PSD)*.

The linear tube allows detection of high molecular weights up to hundreds of kDa, but the resolution decreases as the mass increases. The reflector tube is limited to masses up to tens of kDa; resolution, however, is improved.

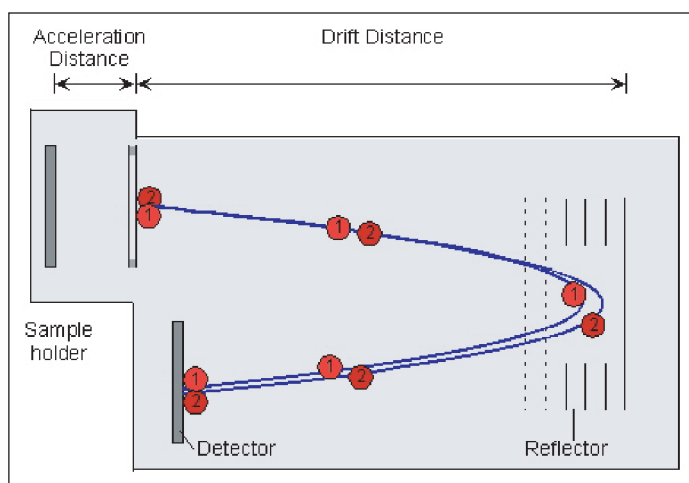


Fig. 4.4. The principle of a reflectron TOF analyser.

4.2.3 Detection of Ions

For ion detection in time of flight analysers, usually secondary electron multipliers are used.

4.2.4 Resolution

An important parameter for the quality of a mass analyser is its capability to separate ions with small mass differences Δm . This is described by the resolution, R_S , the ratio of the mass, m , over the difference Δm of an ion with mass $m + \Delta m$:

$$R_S = \frac{m}{\Delta m} = \frac{m_1}{m_2 - m_1} \quad (\text{equation 4.4})$$

Generally, the higher the resolution the better is the separation. But when are two peaks considered as being separated? This is a question of definition and depends on the analyser. For TOF, Δm is defined as the *full width at half maximum (FWHM)*, i.e. the width of the peak at half its height (Fig. 4.5). With this definition, it is possible to read R_S out of a single peak. Typical resolutions obtained for TOF instruments are $R_S = 15,000$ (FWHM). For other mass analysers, other definitions like the *10 % valley* or *50 % valley* are used (Fig. 4.6). For the 50 % valley definition, two peaks are considered separated if the minimum between them (the valley) is not more than 50 % of the peak height whereas for the 10% valley, the minimum between two peaks must not be more than 10% of the peak height.

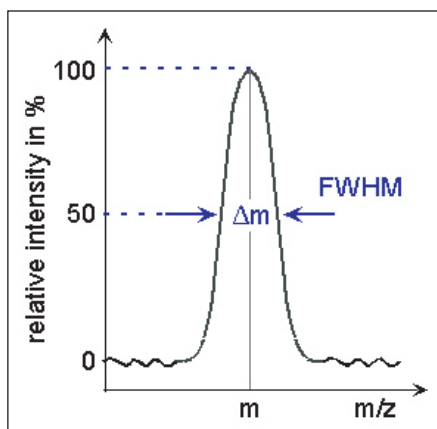


Fig. 4.5. Definition of resolution by full width at half maximum (FWHM).

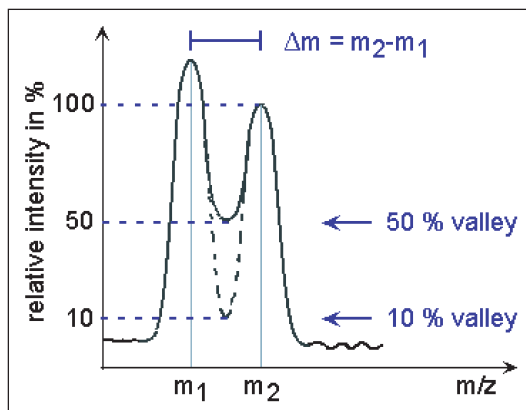


Fig. 4.6. Definition of resolution by 10 % and 50 % valley.

4.2.5 Sample Pretreatment

As mentioned earlier, to achieve MALDI, the sample molecules have to be co-crystallised with a high molar excess of an appropriate matrix. The *function of the matrix* is to absorb and accumulate the energy of the laser radiation and, thus, protect the analytes from destruction and fragmentation. A good matrix material must adsorb strongly at the laser wavelength. Additionally, the matrix must also be chemically inert, stable in vacuum and be able to embed the analyte. Furthermore, the matrix material should promote co-desorption of the analyte upon laser irradiation as well as ionisation of the analyte by donating protons. A number of compounds fulfil these requirements (see Table 4.1), however, their performance varies depending on the analyte, and some trial and error is required to find the optimum matrix for a specific analyte.

Sample preparation for MALDI is relatively straightforward. The sample could be a commercially obtained protein or peptide, or a band from a dried SDS gel (section 3.2.3) or a spot from a dried 2D gel (section 3.2.5). Solutions of the sample and the matrix are made up and mixed either in a tube prior to placing onto the target plate or on the target plate itself. To obtain good spectra, it is essential to keep the salt concentrations in buffers to a minimum. Two common methods are described below.

The *dried droplet method* is the method originally introduced by Hillenkamp and Karas (Fig. 4.7). A saturated matrix solution, 5–10 g L⁻¹, depending on the solubility of the matrix, is prepared in water, water-acetonitrile, or water-alcohol mixtures. In a second vessel, the sample is diluted to about 100 mg L⁻¹ in a solvent that is miscible with the matrix solution. The matrix and sample solutions are then mixed such that the final molar ratio is 10,000 : 1 with a final volume of a few μL.

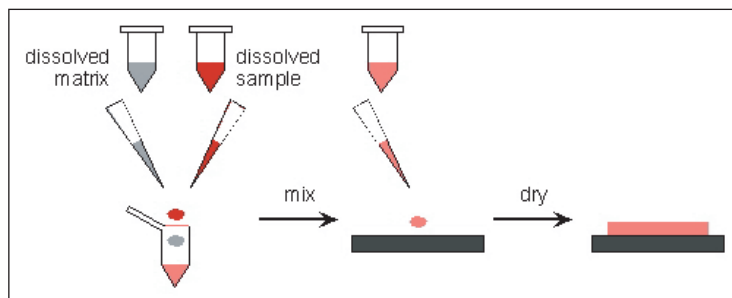


Fig. 4.7. The principle of the dried droplet method.

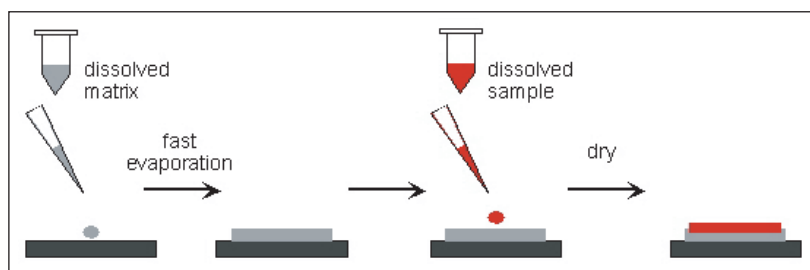


Fig. 4.8. The principle of the fast evaporation method.

A homogenous mixture is essential for obtaining good spectra. A droplet with a volume of 0.5 to 1 μL is placed onto the stainless steel target plate and dried by ambient pressure evaporation, heating with a stream of warm air or under vacuum until crystallisation occurs.

For the *fast evaporation method* (Fig. 4.8), a water-insoluble matrix is used. The matrix is dissolved in an organic solvent like acetone and a drop is applied to the target plate. The solvent evaporates within a few seconds leaving a dry thin film of the matrix on the target. A drop of analyte solution is then applied on top of the dried matrix. The analyte molecules are absorbed into the matrix crystal close to the matrix surface. It is possible to wash the crystal with water several times to remove impurities, especially alkaline metal ions from buffers. With the fast evaporation method, often spectra of high sensitivity and high resolution can be obtained.

4.2.6 Applications of MALDI

MALDI is mainly used for the analysis of proteins and peptides and their mixtures. It is possible to determine the molecular weights, to obtain structural information

and to investigate post-translational processes. Molecular weights of proteins and peptides can be determined accurately with only a small amount of sample. The protein structure can often be determined by digestion with an enzyme and analysing the obtained characteristic peptide fragments. Changes in the protein structure and post-translational processes such as the formation of sulfide bonds or glycosylation can also be identified and localised with MALDI-MS techniques.

The strong points of MALDI include the very *low amount of sample* necessary for analysis, a few fmol are sufficient. Unlike other ionisation methods, MALDI *tolerates* moderate concentrations of *buffer and salts* in the analyte mixture. Sample *preparation is relatively easy* and the spectra obtained are simple, so that even *mixtures can be analysed* without the need to separate the components prior to MALDI analysis. However, in contrary to ESI, MALDI cannot be directly coupled to liquid chromatography (LC) or capillary electrophoresis (CE) as it is not a continuous but a batch ionisation method.

A typical MALDI spectrum is shown in Fig. 4.9. It is the spectrum of r-hirudin, a protein consisting of 64 amino acids. As little or no fragmentation occurs, only one *major peak*, $[M+H]^+$, and two minor peaks, $[M+2H]^{2+}$ and the agglomerate $[2M+H]^+$ are observed. This peak-pattern is very typical of MALDI-TOF spectra. Depending on the salt concentrations in the buffers used for sample preparation, peaks like $[M+Na]^+$ and $[M+K]^+$ are also observed.

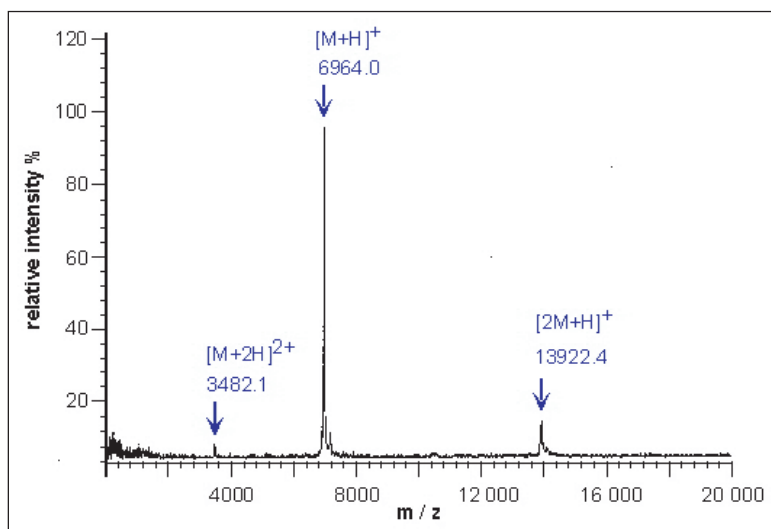


Fig. 4.9. Positive ion mass spectrum of the protein r-hirudin with MW 6963.5 Da. The matrix used was sinapinic acid (courtesy of Olaf Börnsen, Novartis).

The spectrum in Fig. 4.9 demonstrates that MALDI-TOF is a powerful method for accurate *molecular weight determination* of peptides and proteins. As there is almost no fragmentation, *mixtures* of peptides and proteins can be analysed without having to separate the compounds prior to analysis. In this respect, MALDI-TOF has to be regarded as a very fast separation method and is in many ways more powerful than chromatography or electrophoresis. In Fig. 4.10, a spectrum of low fat bovine milk is shown. The milk sample was added to the matrix without any pre-treatment and the different components present in the sample are resolved in the obtained mass spectrum.

Identification of proteins can also be achieved with MALDI-TOF by measuring a “*peptide fingerprint*” of the protein and comparing it to a database. The protein, usually taken from a 2D gel electrophoresis-plate (see section 3.2.5) is reacted with an enzyme, which cleaves the amino acid chain in specific places (Fig. 4.11). For example, trypsin, the most commonly used enzyme, cleaves the protein after Lysines and Arginines resulting in peptide fragments of several hundred to several thousand Da (see section 7.6.1). This fragment mixture, the peptide fingerprint, is very specific for a given protein (Fig. 4.12). Data obtained from the MALDI spectrum can be compared to a database containing theoretically calculated fingerprints for thousands of proteins. Often, the protein of interest can be identified unambiguously.

MALDI is more reliable for protein identification than other, commonly used methods, such as identification due to migration patterns in 2D gel electrophoresis

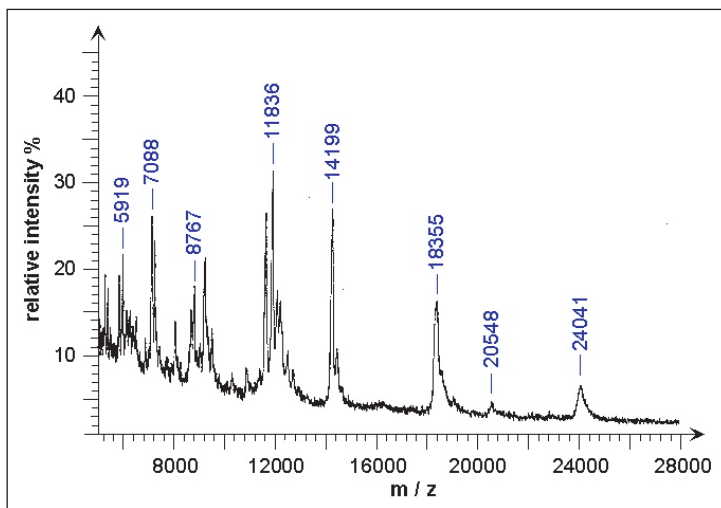


Fig. 4.10. MALDI spectrum of low fat bovine milk (courtesy of Olaf Börnsen, Novartis).

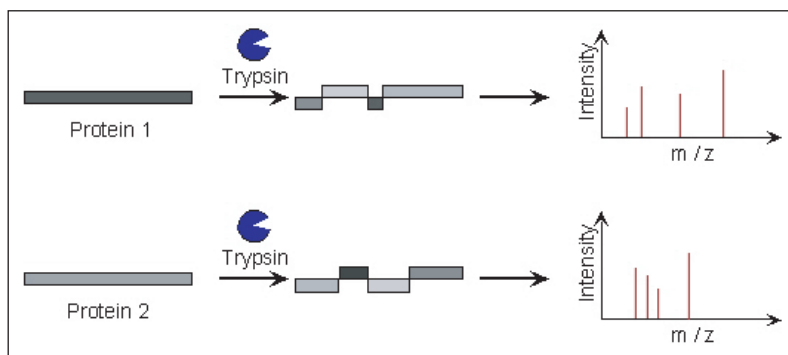


Fig. 4.11. Principle of protein digestion with trypsin to obtain a peptide fingerprint.

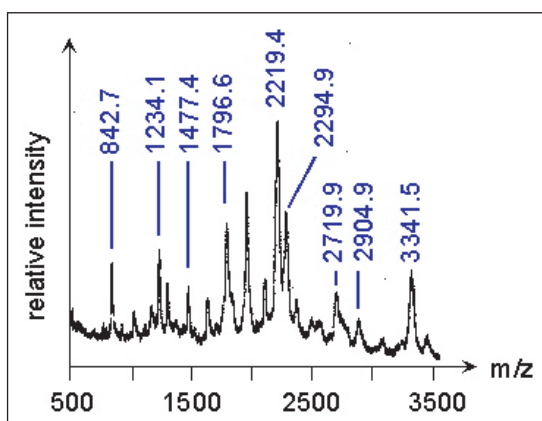


Fig. 4.12. Example of a peptide fingerprint: a tryptic digest of bovine serum albumin (BSA); (redrawn with permission, W.J. Henzel et al., PNAS 1993, 90: 5011–5015, copyright 1993, National Academy of Sciences, U.S.A.).

(section 3.2.5) and retention times in liquid chromatography (chapter 2). MALDI can, however, only be used, if the protein of interest is already known and kept in a database. If the protein is completely unknown, *de novo* sequencing of the amino acid chain becomes necessary (see chapter 6.3).

4.3 Electrospray Ionisation Mass Spectrometry (ESI-MS)

Electrosprays are generated by dispersing a liquid into small droplets via an electric field. This method has been known for a long time and is used for a variety of tasks

ranging from metal spraypainting to ionisation of samples in mass spectrometry. First experiments on electrospray ionisation (ESI) of polymers were undertaken by Malcolm Dole in the late 1960s. ESI for mass spectrometry as used in modern instruments today was developed by John Fenn in the 1980s. In 2002, Fenn was awarded the Nobel Prize for his invention.

ESI enables the production of molecular ions directly from samples in solution. It can be used for small as well as large biopolymers up to about 200,000 Da including peptides, proteins, carbohydrates, DNA fragments and lipids. Unlike MALDI, ESI is a *continuous ionisation method* and suitable for coupling with liquid separation methods like HPLC (chapter 2) or CE (chapter 3.3).

4.3.1 Ionisation Principle

Electrospray ionisation is based on the dispersion of a liquid with the help of an electric field. The sample solution, containing analyte ions, is pumped into a heated chamber through a capillary or needle. A potential difference of several kilovolts is applied between the capillary and the opposing chamber wall (Fig. 4.13), creating an intense electric field at the capillary exit. If the capillary has a positive potential, negative ions are held back and positive ions are drawn away from the capillary towards the opposing chamber wall. This leads to the formation of a liquid cone at the end of the capillary. Droplets with positively charged analyte ions form at the tip of this cone. These are dragged through the chamber by the electric field whilst continuously losing solvent due to evaporation. The droplets shrink which leads to an increase of charge density on the droplet surface. The repulsive forces on the droplet surface move eventually so close together, that the droplet bursts into

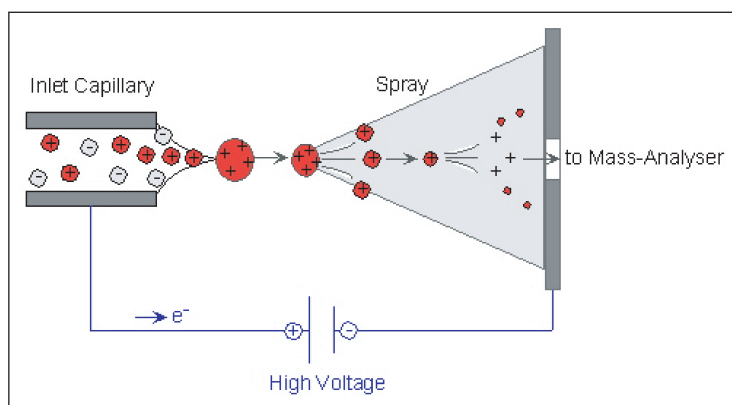


Fig. 4.13. The principle of electrospray ionisation.

a mist of finer droplets. This process of shrinking and bursting occurs repeatedly until, eventually, the analyte is completely desolvated and transferred into the mass analyser.

A typical feature of electrospray ionisation is the formation of multiply charged ions. For larger biomolecules a series of signals is obtained, consisting of $[M + H]^+$, $[M + 2H]^{2+}$, $[M + 3H]^{3+}$ to $[M + nH]^{n+}$ signals. As these highly charged ions appear at relatively low m/z values in the mass spectrum, ESI-MS allows observation of very high molecular weights, which are not accessible by other techniques. Samples suitable for ESI have to be soluble and stable in solution and need to be relatively clean. Ion formation in the spray is hindered by buffers, salts and detergents. These have to be kept to an absolute minimum.

The potential difference between the capillary and the channel wall can be applied in two ways, depending on whether cations or anions are to be analysed. (1) In the *positive ion mode*, the capillary has a positive potential. Negatively charged ions are held back by the capillary and cations are dragged through the chamber into the mass analyser and detected. Often low pH values of the sample solution are used to promote formation of cations. (2) In the *negative ion mode*, the potential difference is reversed – the capillary is negative. Cations are held back whereas anions are drawn towards the analyser. In this mode, high pH values, $pH > pI$, are employed.

4.3.2 ESI-Source and Interface

Electrospray ionisation is achieved at atmospheric pressure, the mass analyser, however, operates under high vacuum. A special *interface* is therefore necessary to transfer the ions from the ionisation chamber into the mass spectrometer. A schematic of such an interface is shown in Fig. 4.14. Usually a zone of intermediate pressure separates the ionisation chamber and the mass analyser. The liquid sample together with a *curtain* or *nebulising gas* is introduced into the heated ionisation chamber. An electrospray is generated by applying a potential difference between the needle and the opposite interface plate. A small proportion of the desolvated analyte ions exit the ionisation chamber through a submillimeter orifice and enter the zone of intermediate pressure. The analyte ions then pass via another small orifice into the mass analyser. This is usually a quadrupole which is operated under high vacuum.

A characteristic feature of ESI is that the sample can be pumped into the mass analyser *continuously*. MALDI, on the other hand, is a pulsed method which requires a dry sample. Thus, ESI-MS can be coupled directly to liquid separation methods such as RP-HPLC (section 2.3.1) and CE (section 3.3). As the sample emerges from the separation column it is directly pumped into the electrospray chamber. As outlined earlier, MALDI-TOF is capable of separating

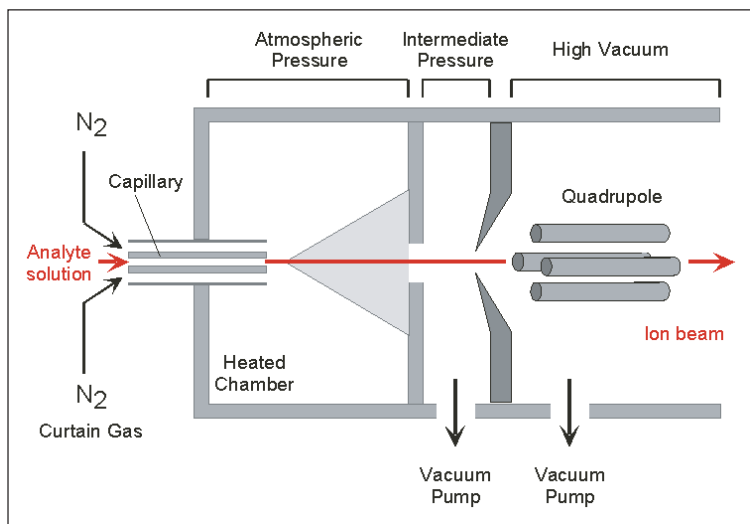


Fig. 4.14. ESI interface, connecting the ionisation chamber at atmospheric pressure to the mass analyser under high vacuum.

sample components directly from the sample mixture (Fig. 4.10); ESI-MS has to be coupled to LC or CE for separation of sample components.

Depending on the amount of sample available, different *flow rates* are used. A low flow rate allows for long measurement times to optimise instrument parameters. With the *pneumatically assisted electrosprays* as shown in Fig. 4.13, rather large capillaries of 50–100 μm and flow rates of 5–200 $\mu\text{L min}^{-1}$ are used. In *micro-electrospray*, capillaries with 10–25 μm diameter and flow rates of 0.2–1 $\mu\text{L min}^{-1}$ are employed. For bioanalysis often only a limited amount of sample is available, requiring very low flow rates in the nanolitre per minute range. *Nano-electrosprays* can be operated at 5–20 nL min^{-1} by using 3–5 μm diameter capillaries.

4.3.3 Quadrupole Analyser

The mass analyser most commonly used with ESI is the *quadrupole analyser*. The quadrupole is essentially a mass filter. At a given set of parameters only ions with a specific m/z value pass through the quadrupole and reach the detector. By scanning over an m/z range, whole spectra can be obtained.

The quadrupole analyser consists of four parallel rod-like metal electrodes. A direct current (DC) and an alternating current (AC) field are applied to these electrodes (Fig. 4.15). At a given field, ions of one defined m/z ratio can pass

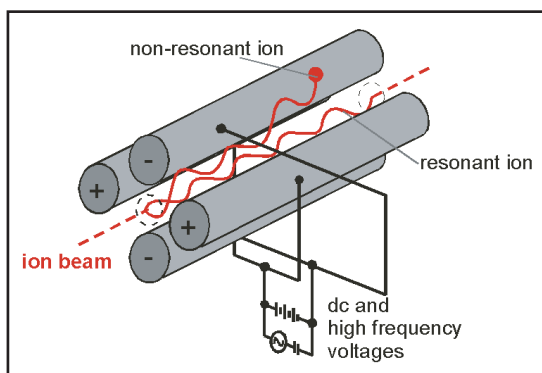


Fig. 4.15. Principle of the quadrupole analyser.

through the quadrupole following an oscillating pathway. These resonant ions reach the detector. All other ions are non-resonant and stopped by the quadrupole. To obtain a spectrum with all m/z ratios, the potential of the direct current and the amplitude of the alternate current are increased and all ions sequentially become resonant and reach the detector. This explains the low sensitivity of quadrupole analysers in comparison to time-of-flight analysers. In a quadrupole analyser, only a very small proportion ($<1\%$) of the ions reaches the detector. It can be shown that the ion-mass is proportional to the potential of the dc and the amplitude of the ac. Thus, a mass spectrum can be obtained directly from the electrical field values. Quadrupole analysers are said to be easy to handle and robust. They can measure up to m/z values of 4,000, and resolutions between $R_S = 500$ and $R_S = 5,000$ can be achieved.

4.3.4 Applications of ESI-MS

ESI is suitable for almost all kinds of biomolecules, as long as they are polar and soluble in a solvent system that can be used for spraying. Peptides, proteins, carbohydrates, DNA fragments and lipids are all commonly analysed via ESI-MS. Molecular weight determination is one of the main applications. Furthermore, sequencing of peptides and DNA fragments (section 6.3) is possible with ESI connected to a *tandem mass spectrometer* (ESI-MS/MS).

ESI is a soft ionisation technique capable of ionising large biomolecules with little to no fragmentation; even non-covalent complexes remain intact and can be analysed. Fragmentation, if desired, can be controlled by changing the spray settings. As mentioned earlier, ESI can readily be coupled to liquid separation methods such as chromatography and capillary electrophoresis.

A problem with electrospray ionisation is its low tolerance for impurities or additives. Buffer and salt concentrations of more than 0.1 mM can prevent sufficient ion formation in the electrospray process, as can certain detergents at concentrations of more than 10 μM . Buffers commonly used in bioanalysis contain 100 mM phosphate and 150 mM NaCl and are thus unsuitable for ESI-MS.

Volatile organic solvents such as methanol, ethanol and acetonitrile are typically contained in the sample solution for electro spraying. Sometimes a volatile organic acid such as formic acid is added to promote cation formation. In almost every case it is necessary to clean the sample from salt contents and impurities prior to introduction into the electrospray chamber. Commonly used techniques for desalting include microdialysis and solid phase microextraction, which are quite labour intensive. Reversed phase liquid chromatography (RP-LC) (section 2.3.1) can be used for preconcentrating and isolating the sample compounds of interest. It can be coupled directly to ESI-MS as the organic solvents used in RP-LC are compatible with electrospray ionisation. At low flow rates, the sample can be injected directly from the column into the ionisation chamber; at higher flow rates the sample stream is split and only a fraction is directed into the mass spectrometer.

The ESI-MS spectrum of neurotensin, a peptide consisting of 13 amino acids with a molecular weight of 1,672 Da is shown in Fig. 4.16. Due to the soft ionisation, no fragments are observed. As mentioned earlier, ESI promotes the formation of multiply charged ions. Peptides and proteins, thus, give a series of signals with $[\text{M} + \text{H}]^+$, $[\text{M} + 2\text{H}]^{2+}$, $[\text{M} + 3\text{H}]^{3+}$ to $[\text{M} + n\text{H}]^{n+}$.

The number of peaks depends on the size of the molecule as well as the number of acidic and basic groups. Larger proteins can have a signal series with ions of up to $[\text{M} + 100\text{H}]^{100+}$. Isotopes are detected in addition to these peaks, leading to an overall rather complex spectrum with a large number of signals. How is it possible to determine which peak refers to the $[\text{M} + \text{H}]^+$ ion and, thus, the molecular

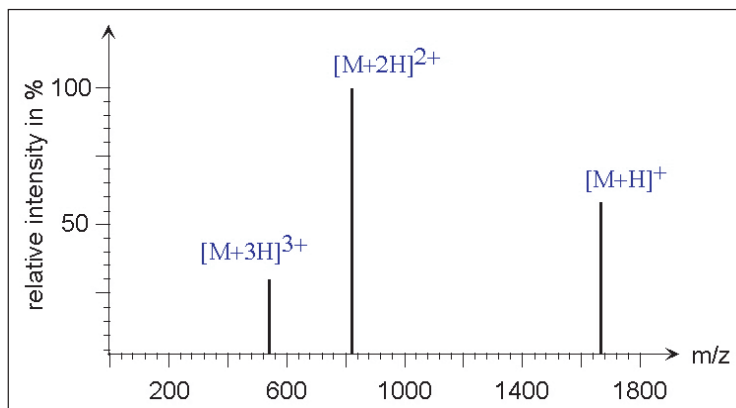


Fig. 4.16. ESI-spectrum of neurotensin (MW 1672 Da).

weight of the analyte molecule? If the resolution is good enough to see different isotopes, these can be used. In case of singly charged ions, the difference between the isotope peaks is exactly 1 Da, whereas for doubly charged ions, the difference between isotope peaks is only 0.5 Da. Often, the isotopes cannot be resolved, as in the spectrum shown in Fig. 4.17. The molecular weight is then calculated by software algorithms included within the instrument software. These algorithms produce a so-called *deconvoluted spectrum* (Fig. 4.18), that give the molecular weight in the form of a peak.

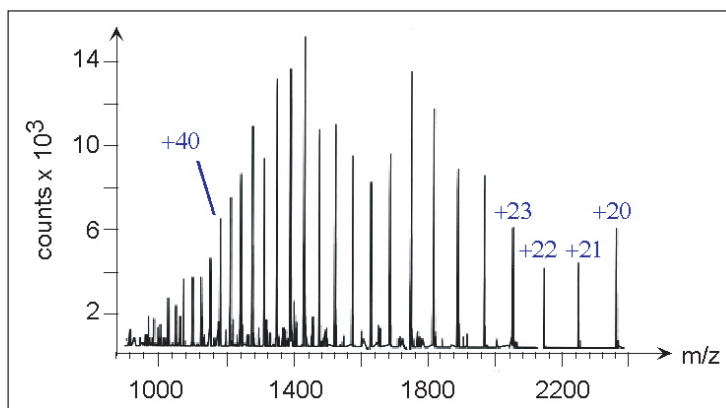


Fig. 4.17. ESI-MS spectrum of lactose permease showing differently ions carrying 20 to more than 50 positive charges (redrawn with permission from J.P. Whitelegge et al. PNAS, 1999, 96: 10695–10698, copyright 1999, National Academy of Sciences, U.S.A.).

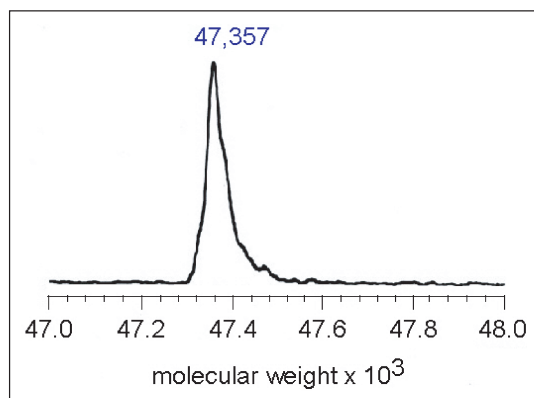


Fig. 4.18. The deconvoluted spectrum of lactose permease showing a molecular weight of 47,357 Da (redrawn with permission from J.P. Whitelegge et al. PNAS, 1999, 96: 10695–10698, copyright 1999, National Academy of Sciences, U.S.A.).

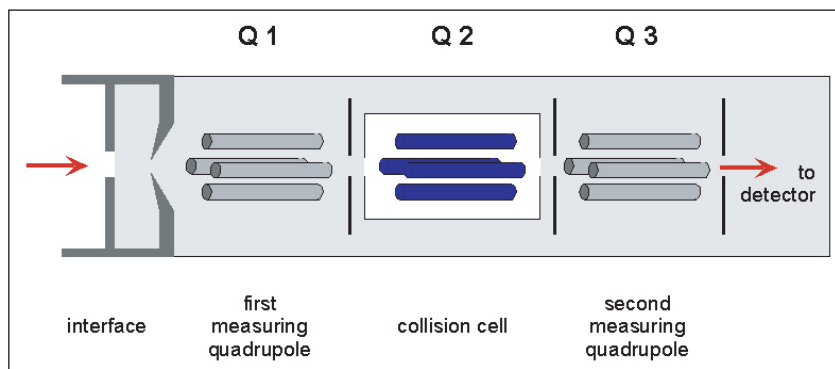


Fig. 4.19. Schematic of a tandem mass spectrometer.

Structural analysis of peptides (chapter 7), nucleic acids (chapter 6.3) and oligosaccharides can be performed with a *tandem mass spectrometer*, for example an ESI-MS/MS. In such a tandem mass spectrometer, three quadrupoles are arranged in series (Fig. 4.19). The first quadrupole ($Q1$) is a measuring quadrupole for determining the m/z of the introduced sample. The second quadrupole ($Q2$) acts as a reaction zone. It is a cell filled with an inert gas such as nitrogen, helium or argon. The analyte ions collide with the gas molecules and become fragmented, a process called *collision induced dissociation (CID)*. These fragments are then introduced into the third quadrupole ($Q3$) for mass analysis.

The measuring quadrupoles can be run in a *static* or *scanning mode*. In the *static mode*, the electric fields are kept constant and only ions with one defined m/z -value can pass. In the *scanning mode*, the quadrupole sequentially allows ions within a defined m/z range to pass through. With these two options and two measuring quadrupoles, several modes of operation are possible (Table 4.2). The *daughter ion analysis* method is the most commonly used approach. For this method, the first quadrupole is set in a static mode and only ions with one specific m/z -value can pass. They are then reacted and fragmented in the collision cell. Ions resulting from this fragmentation, the so-called daughter ions are then analysed in the third quadrupole.

Tandem mass spectrometry can be applied for analysis of peptide mixtures. The first quadrupole only passes one specific peptide ion, which is then fragmented in the collision chamber, i.e. amino acids are cleaved from the peptide chain. In the third quadrupole, the difference between mass peaks gives information about the amino acid sequence in the peptide. An example of peptide sequencing with ESI-MS/MS is shown in Fig. 4.20. Oligonucleotides and oligosaccharides can be analysed in a similar fashion.

Table 4.2. Modes of operation for tandem mass spectrometry.

| Experiment | Mode of $Q1$ | Mode of $Q3$ |
|--------------------------------|---------------------------------------|---------------------------------------|
| daughter ion analysis | static (parent mass selection) | scanning |
| parent ion analysis | scanning | static (daughter mass selection) |
| multiple reaction monitoring | static (parent mass selection) | static (daughter mass selection) |
| constant neutral loss spectrum | scanning (synchronised with $Q3$) | scanning (synchronised with $Q1$) |

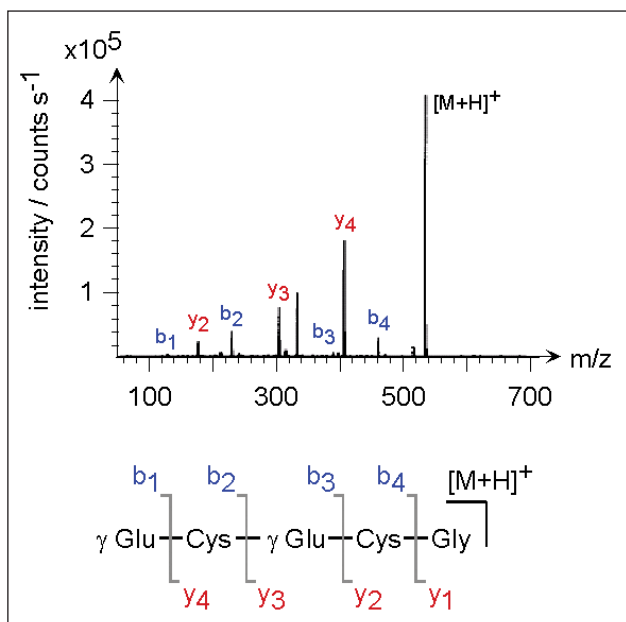


Fig. 4.20. Peptide sequencing of phytochelatin with ESI-MS/MS (V. Vacchina et al., *Analyst*, 1999, 124: 1425–1430; reproduced with permission of the Royal Society of Chemistry).

Summary

MALDI and ESI mass spectrometry are both powerful techniques for the analysis of high molecular weight biomolecules (Table 4.3) with applications including high accuracy determination of molecular weights, fingerprinting of peptides and structural analysis of peptides, oligonucleotides and oligosaccharides.

Table 4.3. Comparison of MALDI and ESI.

| | MALDI | ESI |
|-----------------------------|--------------|---|
| analysis of mixtures | possible | pure compound required |
| limit of detection | very low | higher due to losses in spray and analyser |
| coupling | not possible | possible to LC and CE |
| fragmentation | no fragments | some fragments, depending on applied voltages |

For MALDI, samples are co-crystallised with a matrix and desorbed by laser pulses. The desorption process is very mild and spectra contain hardly any fragments. The method allows the analysis of relatively crude samples with very low limits of detection. Coupling to liquid separation methods is not possible; however, the time of flight analyser separates ions according to their m/z ratio in microseconds and thus allows analysis of mixtures without any sample pretreatment.

In ESI, the sample is dissolved in a volatile solvent. Sample pretreatment is more labour-intensive as impurities and salt concentrations have to be kept to a minimum. Detection limits are not as low as with MALDI due to the loss of sample during the electrospraying process as well as in the quadrupole analyser. Multiply charged molecular ions allow molecular weight determination of very large biomolecules. Fragments can be observed and enable sequencing and identification. Because ESI is a continuous ionisation method, direct coupling to chromatography and electrophoresis is possible.

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