Chapter 9

Mass spectrometric techniques

9.1 INTRODUCTION

9.1.1 General

Mass spectrometry (MS) is an extremely valuable analytical technique in which the molecules in a test sample are converted to gaseous ions that are subsequently separated in a mass spectrometer according to their mass-to-charge ratio (m/z) and detected. The mass spectrum is a plot of the (relative) abundance of the ions at each m/z ratio. Note that it is the mass to charge ratios of ions (m/z) and not the actual mass that is measured. If, for example, a biomolecule is ionised by the addition of one or more protons (H^+) the instrument measures the m/z after addition of 1 Da for each proton if, the instrument is measuring positive ions or m/z minus 1 Da for each proton lost if it is measuring negative ions. The mass spectrum allows an accurate measure to be made of the relative molecular mass M_r (see Section 1.2.2 for details of this parameter) of each ionised molecule and in many cases details of its structure. The development of two ionisation techniques, electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI) has enabled the accurate mass determination of high molecular mass compounds as well as low molecular mass molecules and has revolutionised the applicability of mass spectrometry to almost any biological molecule. Applications include the new science of proteomics (Section 8.5) as well as in drug discovery. The latter includes combinatorial chemistry, where a large number of similar molecules (combinatorial libraries) are produced and analysed to find the most effective compounds from a group of related organic chemicals. This chapter will cover the general principles of the technique and will concentrate on the applications of MS to protein structure.

The essential features of all mass spectrometers are therefore:

- production of ions in the gas phase;
- acceleration of the ions to a specific velocity in an electric field;
- separation of the ions in a mass analyser;
- detection of each species of a particular m/z ratio.

The instruments are calibrated with standard compounds of accurately known M_r values. In MS the carbon scale is used with mass of $^{12}C = 12.000000$. This level of

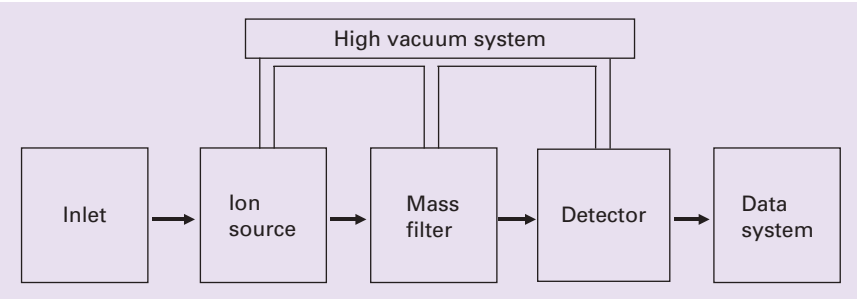


Fig. 9.1. Basic components of mass spectrometers.

accuracy is achievable in high resolution magnetic sector double-focusing and Fourier transform mass spectrometers (Section 9.7).

The mass analyser may separate ions by the use of either a magnetic or an electrical field. Alternatively the time taken for ions of different masses to travel a given distance in space is measured accurately in the time-of-flight (TOF) mass spectrometer (Section 9.3.7). Any material that can be ionised and whose ions can exist in the gas phase can be investigated by MS; remembering that very low pressures, i.e. high vacuum, in the region of 10^{-6} torr are required (1 torr is a measure of pressure that equals 1 mm of mercury (133.3 Pa)). The majority of biological MS investigations on proteins, oligosaccharides and nucleic acids is carried out with quadrupole, quadrupole–ion trap and TOF mass spectrometers. In the organic chemistry/biochemistry area of analysis of lower relative molecular mass compounds, the well-established magnetic sector mass spectrometers still find wide application and their main principles will also be described briefly.

The treatment of MS in this chapter will be strictly non-mathematical and non-technical. However, the intention is to give an overview of the types of instrumentation that are employed, the main uses of each, complementary techniques and the advantages/disadvantages of the different instruments and particular applications most suited to each type. Data analysis and sample preparation to obtain the best sensitivity for a particular type of compound will also be covered.

9.1.2 Components of a mass spectrometer

All mass spectrometers are basically similar (Fig. 9.1). They consist of the following:

- *A high vacuum system (*10^{-6} *torr or* 1 μ*torr):* These include turbomolecular pumps, diffusion pumps and rotary vane pumps.
- *A sample inlet:* This comprises a sample or a target plate; a high performance liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis system; solids probe; electron impact or direct chemical ionisation chamber.
- *An ion source (to convert molecules into gas-phase ions):* This can be MALDI, ESI, fast atom bombardment (FAB), electron impact or direct chemical ionisation.

- *A mass filter/analyser:* This can be: TOF; quadrupole–ion trap; magnetic sector or ion cyclotron Fourier transform (the last is also actually also a detector).
- *A detector:* This can be a conversion dynode; electron multiplier; microchannel plate or array detector.

9.1.3 Vacuum system

All mass analysers operate under vacuum in order to minimise collisions between ions and air molecules. Without a high vacuum, the ions produced in the source will not reach the detector. At atmospheric pressure, the mean free path of a typical ion is around 52 nm; at 1 mtorr, it is 40 mm; and at 1 μtorr, it is 40 m. In most instruments, two vacuum pump types are used, for example a rotary vane pump (to produce the main reduction in pressure) followed by a turbomolecular pump or diffusion pump to produce the high vacuum. The rotary vane pump can be an oil pump to provide initial vacuum (approximately 1 torr), while the turbomolecular pump provides a working high vacuum (1 mtorr to 1 ntorr). This is a high speed gas turbine with interspersed rotors (moving blades) and stators (i.e. fixed or stationary blades) whose rotation forces molecules through the blade system.

9.2 IONISATION

Ions may be produced from a neutral molecule by removing an electron to produce a positively charged cation, or by adding an electron to form an anion. Both positive- and negative-ion MS may be carried out but the methods of analysis in the following sections will be described mainly for positive-ion MS, since this is more common and the principles of separation and detection are essentially the same for both types of ion.

9.2.1 Electron impact ionisation

Electron impact ionisation (EI) is widely used for the analysis of metabolites, pollutants and pharmaceutical compounds, for example in drug testing programmes. EI has major applications as a mass detector for gas chromatography (GC/MS, Section 11.9.3). A stream of electrons from a heated metal filament is accelerated to 70 eV potential. (The electron volt, eV, is a measure of energy.) Sample ionisation occurs when the electrons stream across a high vacuum chamber into which molecules of the substance to be analysed (analyte) are allowed to diffuse (Fig. 9.2). Interaction with the analyte results in either loss of an electron from the substance (to produce a cation) or electron capture (to produce an anion). The analyte must be in the vapour state in the electron impact source, which limits the applicability to biological materials below approximately 400 Da. Before the advent of ESI and MALDI, the method did have some applicability to peptides, for example, whose volatility could be increased by chemical modification. A large amount of fragmentation of the analyte is common, which may or may not be desirable depending on the information required.

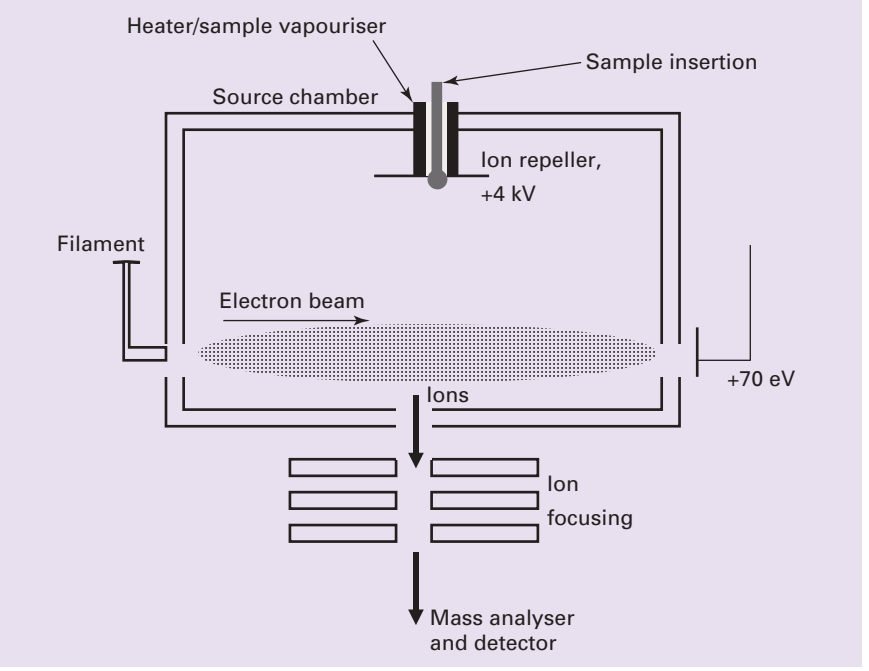


Fig. 9.2. Electron impact source. Electrons are produced by thermionic emission from a filament of tungsten or rhenium. The filament current is typically 0.1 mA. Electrons are accelerated towards the ion source chamber (held at a positive potential equal to the accelerating voltage) and acquire an energy equal to the voltage between the filament and the source chamber, typically 70 eV. The electron trap is held at a fixed positive potential with respect to the source chamber. Gaseous analyte molecules are introduced into the path of the electron beam where they are ionised. Owing to the positive ion repeller voltage and the negative excitation voltage that produce an electric field in the source chamber, the ions leave the source through the ion exit slit and are analysed.

Chemical bonds in organic molecules are formed by the pairing of electrons. Ionisation resulting in a cation requires the loss of an electron from one of these bonds (effectively knocked out by the bombarding electrons), but it leaves a bond with a single unpaired electron. This is a radical as well as being a cation and hence the representation as $M^{+\cdot}$, the (+) sign indicating the ionic state and the (·) a radical. Conversely, electron capture results both in an anion and also the addition of an unpaired electron and therefore a negatively charged radical, hence the symbol $M^{-\cdot}$. Such radical ions are termed molecular ions, parent ions or precursor ions and under the conditions of electron bombardment are relatively unstable. Their energy is in excess of that required for ionisation and has to be dissipated. This latter process results in the precursor ion disintegrating into a number of smaller fragment ions that may be relatively unstable and further fragmentation may occur. This gives rise to a series of daughter ions or product ions, which are recorded as the mass spectrum.

For the production of a radical cation, as it is not known where either the positive charge or the unpaired electron actually reside in the molecule, it is usual practice

to place the dot signs outside the abbreviated bracket sign, '⌉'. When the precursor ion fragments, one of the products carries the charge and the other the unpaired electron, i.e. it splits into a radical and an ion. The product ions are therefore true ions and not radical ions. The radicals produced in the fragmentation process are neutral species and therefore do not take any further part in the MS but are pumped away by the vacuum system. Only the charged species are accelerated out of the source and into the mass analyser. It is also important to recognise that almost all possible bond breakages can occur and any given fragment will arise as both an ion and a radical. The distribution of charge and unpaired electron, however, is by no means equal. The distribution depends entirely on the thermodynamic stability of the products of fragmentation. Furthermore, any fragment ion may break down further (until single atoms are obtained) and hence not many ions of a particular type may survive, resulting in a low signal being recorded. A simple example is given by *n*-butane ($CH_3CH_2CH_2CH_3$) and some of the major fragmentations are shown Fig. 9.3a. The resultant EI spectrum is shown in Fig. 9.3b.

9.2.2 Chemical ionisation

Chemical ionisation (CI) is used for a range of samples similar to those for EI. It is particularly useful for the determination of molecular masses, as high intensity molecular ions are produced owing to less fragmentation. CI therefore gives rise to much cleaner spectra. The source is essentially the same as the EI source but it contains a suitable reagent gas such as methane (CH_4) or ammonia (NH_3) that is initially ionised by EI. The high gas pressure in the source results in ion–molecule reactions between reagent gas ions (such as NH_3^+ and CH_4^+) some of which react with the analyte to produce analyte ions. The mass differences from the neutral parent compounds therefore correspond to these adducts.

9.2.3 Fast atom bombardment

At the time of its development in the early 1980s, FAB revolutionised MS for the biologist. The important advance was that this soft ionisation technique, which led to the formation of ions with low internal energies and little consequent fragmentation, permitted analysis of biomolecules in solution without prior derivatisation. The sample is mixed with a relatively involatile, viscous matrix such as glycerol, thioglycerol or *m*-nitrobenzyl alcohol. The mixture, placed on a probe, is introduced into the source housing and bombarded with an ionising beam of neutral atoms (such as Ar, He, Xe) of high velocity. A later development was the use of a beam of caesium ions (Cs^+) and the term liquid secondary ion mass spectrometry (LSIMS) was introduced to distinguish this from FAB–MS. Pseudomolecular ion species arise as either protonated or deprotonated entities $(M + H)^+$ and $(M - H)^-$ respectively, which allows positive- and negative-ion mass spectra to be determined. The term pseudomolecular implies the mass of the ion formed from a substance of a given mass by the gain or loss of one or more protons. Other charged adducts can also be formed such as $(M + Na)^+$ and $(M + K)^+$.

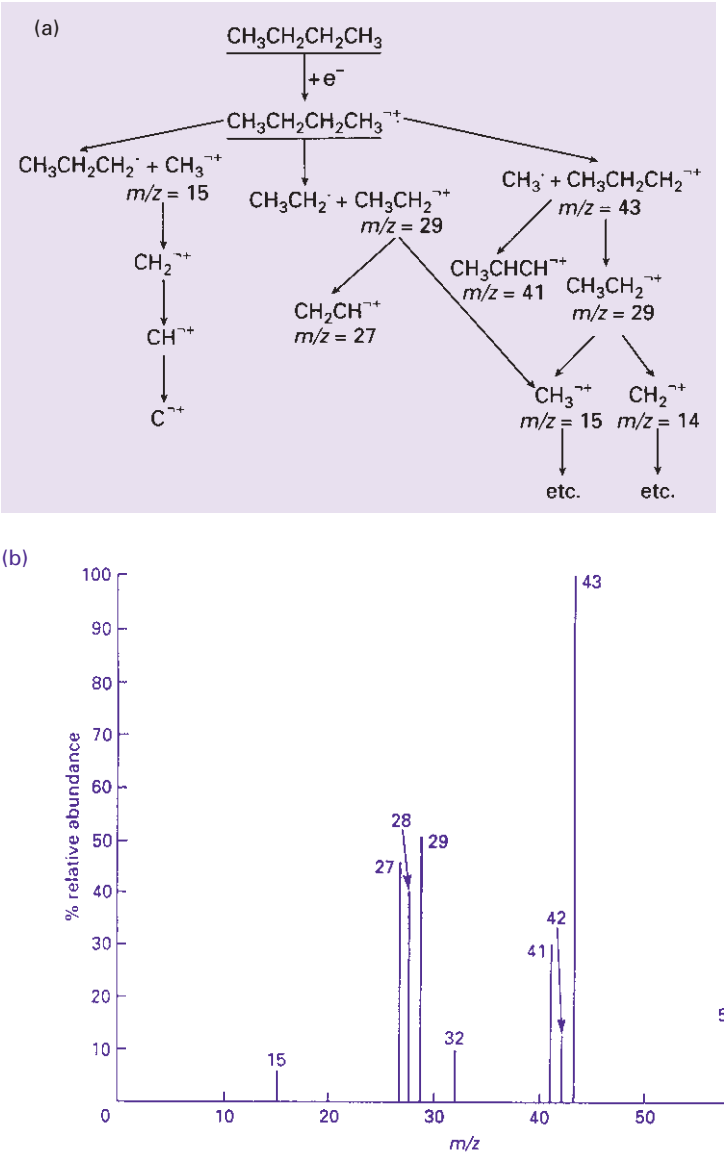


Fig. 9.3. Fragmentation pathways in *n*-butane and the electron impact ionisation spectrum. The pathway for fragmentation of *n*-butane is shown in (a) and the EI spectrum in (b). In the spectrum, the relative abundance is plotted from 0 to 100%, where the largest peak is set at 100% (base peak). Spectra represented in this way are said to be normalised.

9.2.4 Electrospray ionisation

This involves the production of ions by spraying a solution of the analyte into an electrical field. This is a soft ionisation technique and enables the analysis of large, intact (underivatised) biomolecules, such as proteins and DNA. The electrospray (ES) creates very small droplets of solvent-containing analyte. The essential

principle in ES is that a spray of charged liquid droplets is produced by atomisation or nebulisation. Solvent (typically 50:50 water and organic solvent) is removed as the droplets enter the mass spectrometer. ESI is the result of the strong electric field (approximately 4 keV at the end of the capillary and 1 keV at the counter electrode) acting on the surface of the sample solution. As the solvent evaporates in the high vacuum region, the droplet size decreases and eventually charged analyte (free from solvent) remains. Ionisation can occur at atmospheric pressure and this method is also sometimes referred to as atmospheric pressure ionisation (API). The concentration of sample is usually around 1–10 pmol mm^{-3}. Typical solvents are 50:50 acetonitrile (or methanol):H_2O with 1% acetic acid or 0.1% formic acid. Ammonium hydroxide or trifluoroacetic acid (TFA, 0.02%) in 50:50 acetonitrile or methanol): H_2O can also be used. The organic acid (or the NH_4OH) aids ionisation of the analyte. At low pH, basic groups will be ionised. In the example of peptides these are the side groups of lysine, histidine and arginine and the N-terminal amino group. At alkaline pH the carboxylic acid side-chains as well as stronger anions such as phosphate and sulphate groups will be ionised. The presence of organic solvent assists in the formation of small droplets and facilitates evaporation. The flow rate into the source is normally around a few $mm^3\ min^{-1}$, although higher flow rates can be tolerated (up to 1 $cm^3\ min^{-1}$) if the solution is an eluant from on-line HPLC for example.

Smaller molecules usually produce singly charged ions but multiply charged ions are frequently formed from larger biomolecules, in contrast to MALDI, resulting in m/z ratios that are sufficiently small to be observed in the quadruple analyser. Thus masses of large intact proteins, DNA and organic polymers can also be accurately measured in electrospray MS although the m/z limit of measurement is normally 2000 or 3000 Da. For example, proteins are normally analysed in the positive ion mode, where charges are introduced by addition of protons. The number of basic amino acids in the protein (mainly lysine and arginine) determines the maximum number of charges carried by the molecule. The distribution of basic residues in most proteins is such that the multiple peaks (one for each $M + n\mathrm{H})^{n+}$ ion, are centred on an m/z of about 1000. In Fig. 9.6, a large protein with a mass of over 100 000 Da behaves as if it were multiple mass species around 1020 Da. For the species with 100 protons (H^+), i.e. with 100 charges, $z = 100$, $m/z = 1027.6$ therefore $(M + 100\mathrm{H})^{100+} = 1027.6$. When the computer processes the data for the multiple peaks, the average for each set of peaks gives a mass determination to a high accuracy. The peaks can be deconvoluted and presented as a single peak representing the M_r (in this example $M_r = 102\,658$). A diagrammatic representation of the ESI source is shown in Fig 9.4. A curtain or sheath gas (usually nitrogen) around the spray needle at a slow flow rate may be used to assist evaporation of the solvent at or below room temperature. This may be an advantage for thermally labile compounds.

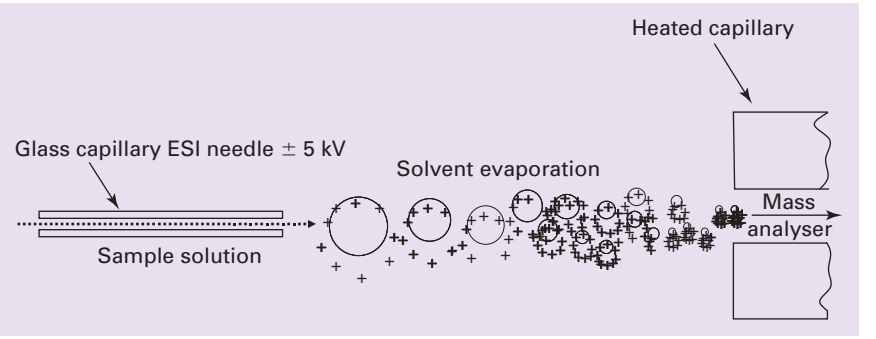


Fig. 9.4. Electrospray ionisation source. The ESI creates very small droplets of solvent-containing analyte by atomisation or nebulisation as the sample is introduced into the source through the fine glass (or other material) hollow needle capillary. The solvent evaporates in the high vacuum region as the spray of droplets enters the source. As the result of the strong electric field acting on the surface of the sample droplets, and electrostatic repulsion, their size decreases and eventually single species of charged analyte (free from solvent) remain. These may have multiple charges depending on the availability of ionisable groups.

Example 1 PROTEIN MASS DETERMINATION BY ESI

Question

A protein was isolated from human tissue and subjected to a variety of investigations. Relative molecular mass determinations gave values of approximately 12 000 by size exclusion chromatography and 13 000 by gel electrophoresis. After purification, a sample was subjected to electrospray ionisation mass spectrometry and the following data obtained.

m/z	773.9	825.5	884.3	952.3	1031.3
Abundance (%)	59	88	100	66	37

Given that $n_2 = (m_1 - 1)/(m_2 - m_1)$ and $M = n_2(m_2 - 1)$ and assuming that the only ions in the mixture arise by protonation, deduce an average molecular mass for the protein by this method.

Answer

M_r by exclusion chromatography = 12 000

M_r by gel electrophoresis = 13 000

Taking ESI peaks in pairs:

$m_1 - 1$	$m_2 - m_1$	n_2	$m_2 - 1$	M(Da)	z
951.3	79.0	12.041	1030.3	12 406.6	12
883.3	68.0	12.989	951.3	12 357.1	13
824.5	58.8	14.022	883.3	12 385.7	14
772.9	51.6	14.978	824.5	12 349.9	15

ΣM = 49 499.3 Da

Mean M = 12 374.8 Da

Note: Relative abundance values are not required for the determination of the mass.

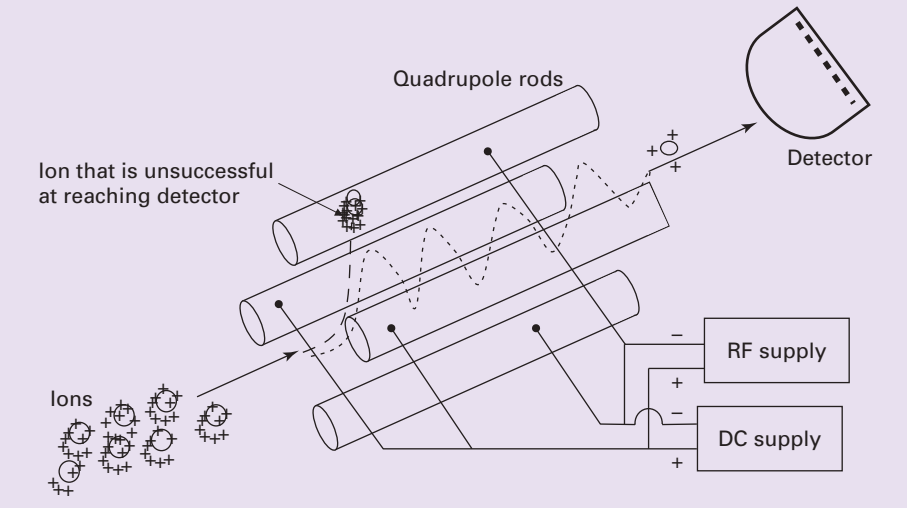


Fig. 9.5. Quadrupole analyser. The fixed (DC) and oscillating (RF) fields cause the ions to undergo complicated trajectories through the quadrupole filter. For a given set of fields, only certain trajectories are stable, which allows only ions of specific m/z to travel through to the detector. The efficiency of the quadrupole is impaired after a build up of ions that do not reach the detector. Therefore a set of pre-filters is added to the quadrupole to remove the ions that would otherwise affect the main quadrupole.

9.3 MASS ANALYSERS

9.3.1 Introduction

Once ions are created and leave the ion source, they pass into a mass analyser, the function of which is to separate the ions and to measure their masses. (Remember, what is really measured is the mass-to-charge ratio (m/z) for each ion.) At any given moment, ions of a particular mass are allowed to pass through the analyser, where they are counted by the detector. Subsequently, ions of a different mass are allowed to pass through the analyser and again the detector counts the number of ions. In this way, the analyser scans through a large range of masses. In the majority of instruments, a particular type of ionisation is coupled to a particular mass analyser that operates by a particular principle. That is, EI, CI and FAB are combined with magnetic sector instruments, ESI and its derivatives with quadrupole (or its variant ion trap) and MALDI is coupled to TOF detection.

9.3.2 Quadrupole mass spectrometry

The quadrupole analyser consists of four parallel cylindrical rods (Fig. 9.5). A direct current (DC) voltage and a superimposed radio frequency (RF) voltage are applied to each rod, creating a continuously varying electric field along the length of the analyser. Once in this field, ions are accelerated down the analyser towards the detector. The varying electric field is precisely controlled so that, during each stage of a scan, ions of one particular mass-to-charge ratio pass down the length of

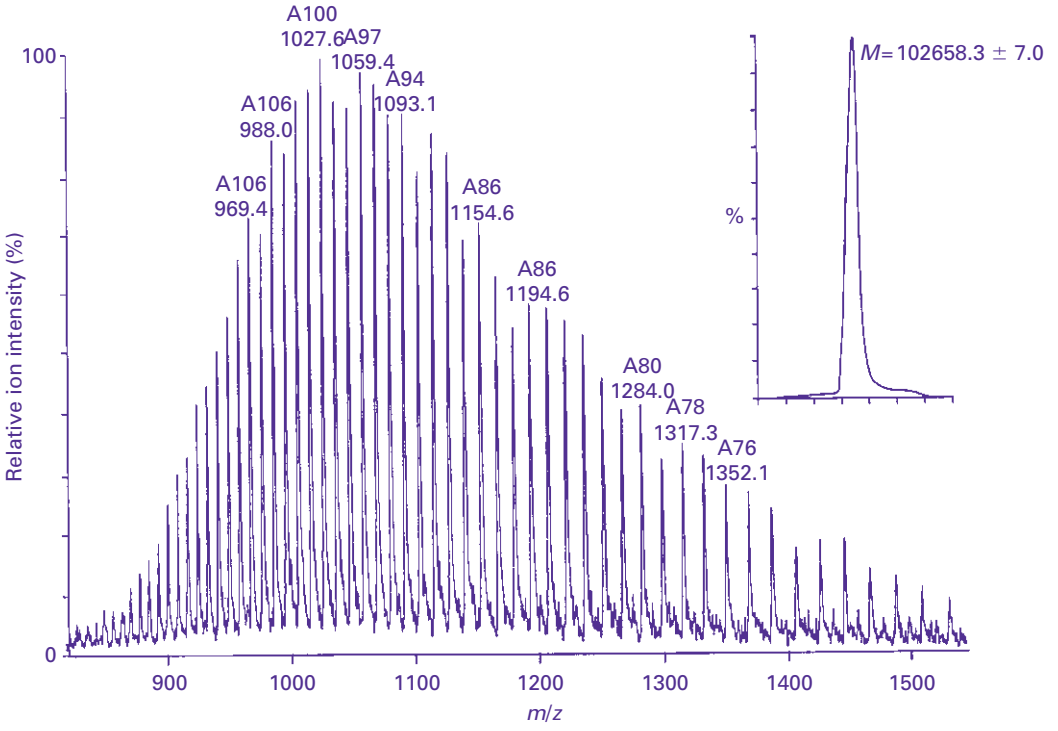


Fig. 9.6. Large intact protein mass accurately measured in electrospray MS. The species of ions are annotated by the charge state, for example with 99, 100, 101 charges, etc., and the associated *m*/*z* value. The inset shows the 'deconvoluted spectrum'.

the analyser. Ions with any other mass-to-charge value impact on the quadrupole rods and are not detected. By changing the electric field (scanning), the ions of different *m*/*z* successively arrive at the detector. Quadrupoles can routinely analyse up to *m*/*z* 3000, which is extremely useful for biological MS, since, as we have seen, proteins and other biomolecules normally give a charge distribution of *m*/*z* that is centred at a value below this (Fig. 9.6). Note that hexapole and octapole devices are also used to direct a beam into the next section of a triple quadrupole or into an ion trap, for example, but the principle is the same.

9.3.3 Ion trap mass spectrometry

Ion trap mass spectrometers use ESI to produce ions, all of which are transferred into, and subsequently measured almost simultaneously (within milliseconds), in a device called an ion trap (Fig. 9.7). The trap must then be refilled with the ions that are arriving from the source. Therefore, although the trap does not measure 100% of all ions produced (it depends on the cycle time to refill the trap then analyse the ions), this results nevertheless in a great improvement in sensitivity relative to quadrupole mass spectrometers, where at any given moment only ions of one particular *m*/*z* are detected. ESI–ion trap mass spectrometers have found

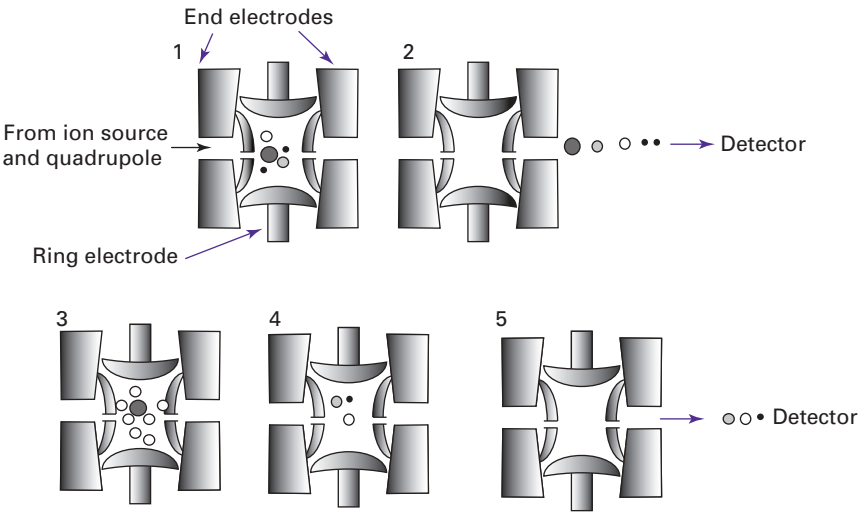


Fig. 9.7. Diagram of an ion trap. The ion trap contains three hyperbolic electrodes which form a cavity in a cylindrical device of around 5 cm diameter, in which the ions are trapped (stored) and subsequently analysed. Each end-cap electrode has a small hole in the centre. Ions produced from the source enter the trap through the quadrupole and the entrance end-cap electrode. Potentials are applied to the electrodes to trap the ions (diagrams 1 and 2). The ring electrode has an alternating potential of constant radio frequency but variable amplitude. This results in a three-dimensional electrical field within the cavity. The ions are trapped in stable oscillating trajectories that depend on the potentials and the m/z of the ions. To detect these ions, the potentials are varied, resulting in the ion trajectories becoming unstable and the ions are ejected in the axial direction out of the trap in order of increasing m/z into the detector. A very low pressure of helium is maintained in the trap, which 'cools' the ions into the centre of the trap by low speed collisions that normally do not result in fragmentation. These collisions merely slow the ions down so that, during scanning, the ions leave quickly in a compact packet, producing narrower peaks with better resolution. In sequencing, all the ions are ejected except those of a particular m/z ratio that has been selected for fragmentation (see diagrams 3, 4 and 5). The steps are: (3) selection of precursor ion, (4) collision induced dissociation of this ion, and (5) ejection and detection of the fragment ions.

wide application for the analysis of peptides and small biomolecules such as in protein identification by tandem MS, liquid chromatography/mass spectrometry (LC/MS), combinatorial libraries and rapid analysis in drug discovery and drug development. Ion trap MS permits structural information to be readily obtained (and sequence information in the case of polypeptides). Not only can tandem MS analysis be carried out but also, owing to the high efficiency of each stage, further fragmentation of selected ions may be carried out to MS to the power n (MS^n) (Fig. 9.8). The instrument still allows accurate molecular mass determination to over 100 000 Da at greater than 0.01% mass accuracy.

The MS^n procedure in an ion trap involves ejecting all ions that are stored in the trap, except those corresponding to the selected m/z value. To perform tandem MS (MS^2) a collision gas is introduced (a low pressure of helium) and collision-induced dissociation (CID) occurs (Fig. 9.7). The fragment ions are then ejected in

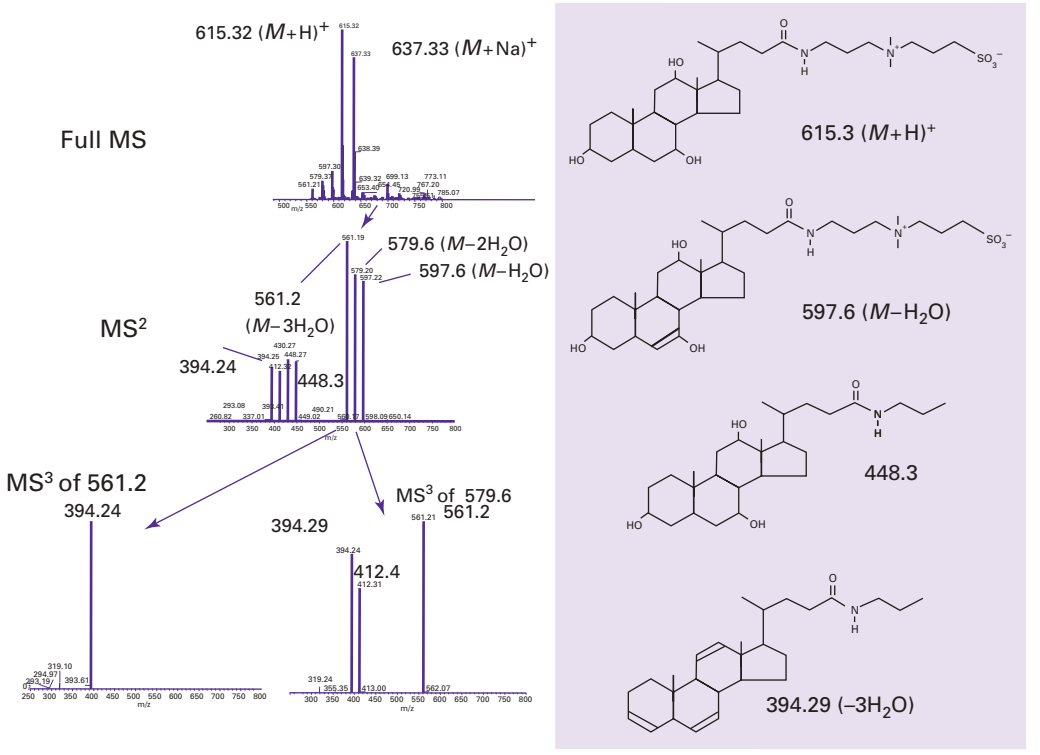


Fig. 9.8. Structural analysis, MS^n in an ion trap. In this example, of a steroid-related compound, the structure can be analysed when the $(M+H)^+$ at 615.3 are selected to be retained in the ion trap. These ions are subjected to collision-induced dissociation (CID) resulting in loss of the aliphatic sulphonate from the quaternary ammonium group and partial loss of some hydroxyl groups in the tandem MS experiment. The major fragment ions (561.2 and 579.6) are further selected for CID (MS^3), resulting in subsequent losses of more hydroxyl groups from specific parts of the steroid ring.

turn and the fragment spectrum determined. The process can be repeated successively where all the fragment ions stored in the trap except those fragment ions corresponding to another selected m/z value are ejected. This fragment ion can then be further fragmented to obtain more structural information, as illustrated for the example shown in Fig. 9.8. This technique has a big advantage, since no additional mass spectrometers or collision cells are required. The limitation is sensitivity, which decreases with each MS experiment, although the claimed record in an ion trap is currently MS^{14}.

9.3.4 Nanospray and on-line tandem mass spectrometry

The sensitivity with ESI can be greatly improved with a reduction in flow rate. Nanospray is therefore the technique of choice for ultimate sensitivity when sample amounts are limited. There are two ways of achieving this. Both static and dynamic nanospray techniques are widely used. Flow rates in both nanospray

techniques are in the order of tens of $nm^3\ min^{-1}$, which leads to low sample consumption and low signal-to-noise ratios.

First, in static nanospray, glass needles are used with a very finely drawn out capillary tip (coated with gold to allow the needle to be held at the correct kilovolt potential; see Fig. 9.4). The needles are filled with 1–2 mm^3 of sample and accurately positioned at the entrance to the source. Closed-circuit television (CCTV) is used to accurately determine the position of the capillary. The solution is drawn into the source by electrostatic pressure, although a low pressure may be applied with an air-filled syringe behind the other (open) end of the needle if necessary. In dynamic nanospray experiments, small diameter microbore HPLC or capillary columns are also used to achieve separation at low flow rates. This can be combined with a stream splitter device, which can further reduce flow rate (Section 11.3.2). The stream splitter can be used to divert a percentage of the solvent flow from the pump, say 99–99.9%, to waste and allow the remainder to pass through the column. This allows for much more accurate flow rates, since it is extremely difficult to directly and accurately pump at 0.5 mm^3 or even 50 $nm^3\ min^{-1}$ with a high pressure pump. Therefore one can use a pump that functions more efficiently at flow rates of 50–500 $mm^3\ min^{-1}$ to pass 0.5 $nm^3\ min^{-1}$ or less into the microcolumn.

Nanospray sources are used in triple quadrupole, ion trap and hybrid MALDI instruments. Computer programs can be set up to perform tandem MS, during the chromatographic separation, on each component as it elutes from the column, if it gives a signal above a threshold that is set by the operator.

9.3.5 Magnetic sector analyser

A magnetic sector analyser is shown diagrammatically in Fig. 9.9. The ions are accelerated by an electric field. The electric sector acts as a kinetic energy filter and allows only ions of a particular kinetic energy to pass, irrespective of the m/z. This greatly increases the resolution, since the ions emerge from the electrostatic analyser (ESA) with the whole range of masses but the same velocity. A given ion with the appropriate velocity then enters the magnetic sector analyser. It will travel in a curved trajectory in the magnetic field with a radius depending on the m/z and the velocity of the ion (the latter has already been selected). Thus only ions of a particular m/z will be detected at a particular magnetic field strength. The trajectory of the ions is through a sector of the circular poles of the magnet, hence the term magnetic sector. Fig. 9.9 shows several possible trajectories for a given ion in the magnetic field. Only one set of ions will be focused on the detector. If the field is changed, these ions will be defocused because they will not be deflected to the correct extent. A new set of ions will be deflected and collected at the detector. By starting at either end of the magnet range, the ions can be scanned from high to low mass or from low to high mass. This magnetic scanning is the most commonly used type of analysis in this instrument. Alternatively, the mass spectrum can be scanned electrically by varying the voltage, V, while holding the magnetic field B constant. This type of instrument is called a two-sector or double-focusing MS and resolving power to parts per million may be obtained.

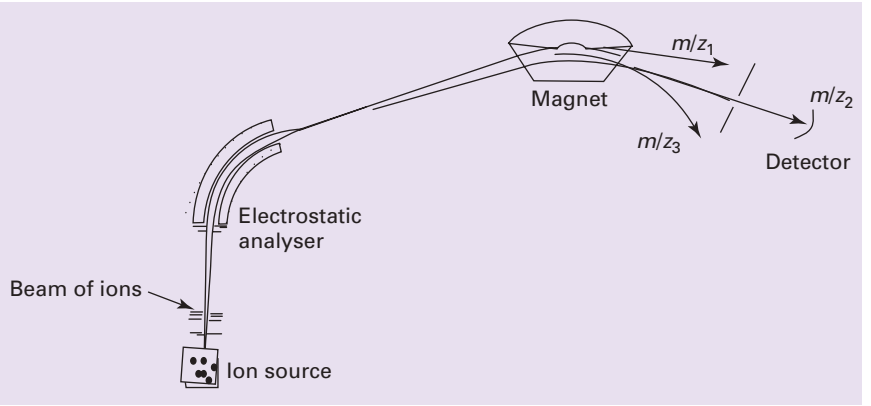


Fig. 9.9. Double-focusing magnetic sector mass spectrometer. The figure shows the 'forward geometry' arrangement where the electrostatic analyser is before the magnetic sector (known as EB; E for electric, B for magnetic). Similar results may be obtained if the reverse geometry (BE) type is used. The radial path followed by each ion is shown by scanning the magnetic field, B, and each ion of a particular m/z value can be brought into the detector slit in turn.

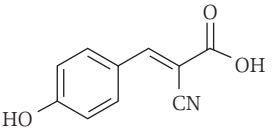
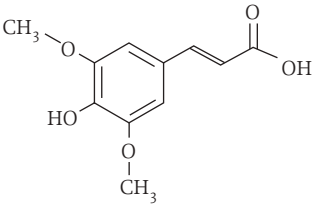
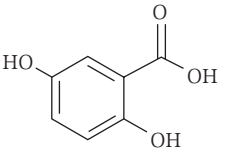
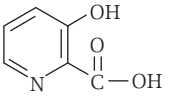
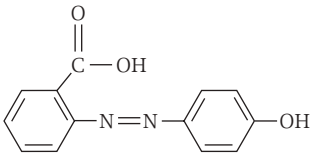
9.3.6 Plasma desorption ionisation

Plasma desorption ionisation mass spectrometry (PDMS) was the first mass spectrometer to be able to analyse proteins and other large biomolecules (although only those of relatively low M_r, less than 35 000). The technique and instruments developed are now obsolete and clearly overtaken by the much more powerful, sensitive and accurate instruments described elsewhere in this chapter. PDMS instruments are, however, still in use in some laboratories and research publications still appear with mass spectra obtained with this instrument. A basic understanding of the principle is therefore worth including. The source of the plasma (atomic nuclei stripped of electrons) is radioactive californium, ^{252}Cf, and two typical emission nuclei are the 100 MeV Ba^{20+} and Tc^{18+}, formed by the decay of the Cf, which are ejected in opposite directions, almost collinearly and with equal velocity. This is a pulsed technique, i.e. particles are emitted at discrete time intervals and require a TOF mass detector. The plasma particle emitted in the direction opposite to that passing through the sample triggers a time counter and the desorbed sample ions are accelerated electrically and detected as for other TOF analysers (Section 9.3.7).

9.3.7 MALDI, TOF mass spectrometry, MALDI–TOF

Matrix-assisted laser desorption ionisation (MALDI) produces gas phase protonated ions by excitation of the sample molecules from the energy of a laser transferred via an ultraviolet (UV) light-absorbing matrix. The matrix is a conjugated organic compound (normally a weak organic acid such as a derivative of cinnamic acid and dihydroxybenzoic acid) that is intimately mixed with the sample. Examples of MALDI matrix compounds and their application for particular

Table 9.1 Examples of MALDI matrix compounds

Compound	Structure	Application
α-Cyano-4-hydroxycinnamic acid (CHCA)		Peptides < 10 kDa (glycopeptides)
Sinapinic acid (3,5-dimethoxy-4-hydoxycinnamic acid, SA)		Proteins > 10 kDa
'Super DHB', mixture of 10% 5-methoxysalycilic acid (2-hydroxy-5-methoxybenzoic acid) with DHB		Proteins, glycosylated proteins
2,5-Dihydroxybenzoic acid (DHB), (gentistic acid)		Neutral carbohydrates, synthetic polymers, (oligos)
3-Hydroxypicolinic acid		Oligonucleotides
2-(4-hydroxy-phenylazo)-benzoic acid (HABA)		Oligosaccharides, proteins

biomolecules are shown in Table 9.1. These are designed to maximally absorb light at the wavelength of the laser, typically a nitrogen laser of 337 nm or a neodymium/yttrium-aluminium-garnet (Nd-YAG) at 355 nm.

The sample (1–10 pmol mm^{-3}) is mixed with an excess of the matrix and dried onto the target plate, where sample and matrix co-crystallise on drying. Pulses of laser light of a few nanoseconds' duration cause rapid excitation and vaporisation of the crystalline matrix and the subsequent ejection of matrix and analyte ions into the gas phase (Fig. 9.10). This generates a plume of matrix and analyte ions that are analysed in a TOF mass analyser. The particular advantage of MALDI is the

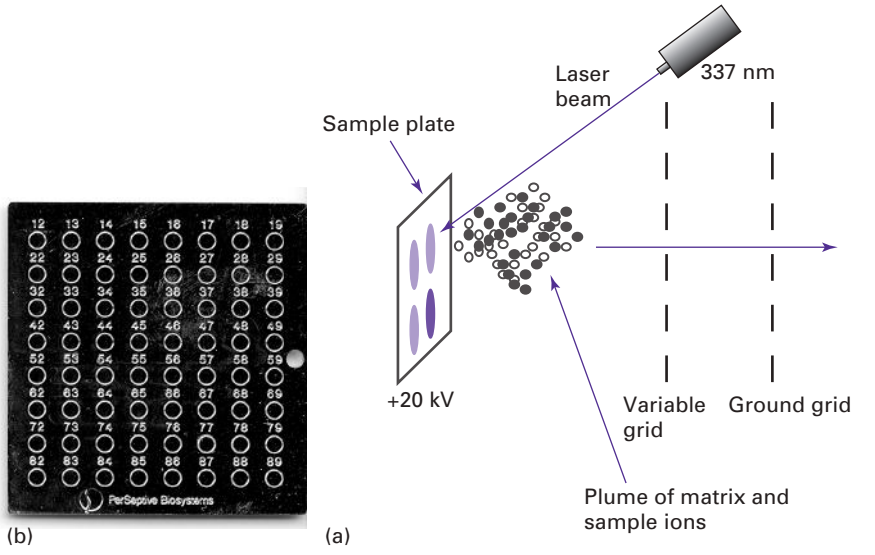


Fig. 9.10. MALDI ionisation mechanism and MALDI–TOF sample plate. (a) The sample (A) is mixed, in solution, with a 'matrix' – the organic acid in excess of the analyte (in a ratio between 1000:1 to10 000:1) and transferred to the MALDI plate. An ultraviolet laser is directed to the sample (with a beam diameter of a few micrometres) for desorption. The laser radiation of a few nanoseconds' duration is absorbed by the matrix molecules, causing rapid heating of the region around the area of laser impact and electronic excitation of the matrix. The immediate region of the sample explodes into the high vacuum of the mass spectrometer, creating gas-phase protonated molecules of both the acid and the analyte.

The laser flash ionises matrix molecules (neutral fragments (*M*) and matrix ions $(M\mathrm{H})^+$, $(M-\mathrm{H})^-$ and sample neutral fragments (A). Sample molecules are ionised by gas-phase proton transfer from the matrix:

$$M\mathrm{H}^+ + \mathrm{A} \rightarrow M + \mathrm{AH}^+$$

$$(M-\mathrm{H})^- + A \rightarrow (A-\mathrm{H})^- + M$$

The matrix serves as an absorbing medium for the ultraviolet light converting the incident laser energy into molecular electronic energy, both for desorption and ionisation and as a source of H^+ to transfer to, and ionise, the analyte molecule. (b) 100-spot MALDI sample plate.

ability to produce large mass ions, with high sensitivity. MALDI is a very soft ionisation technique that does not produce abundant amounts of fragmentation like some other ionisation methods. Since the molecular ions are produced with little fragmentation, it is a valuable technique for examining mixtures (see Fig. 9.14 and compare this to the more complex spectrum in Fig. 9.6).

TOF is the best type of mass analyser to couple to MALDI, as this technique has a virtually unlimited mass range. Proteins and other macromolecules of M_r greater than 400 000 have been accurately measured. The principle of TOF is illustrated in Fig. 9.11 and the main components of the instrument are shown in Fig. 9.12.

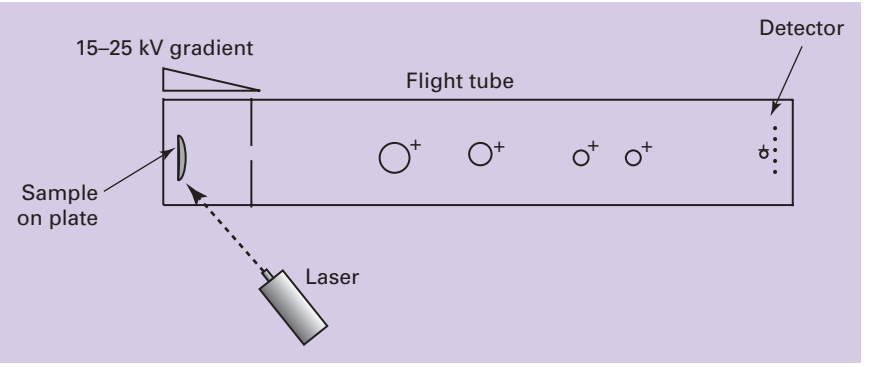


Fig. 9.11. Principle of time-of-flight (TOF). The ions enter the flight tube, where the lighter ions travel faster than the heavier ions to the detector. If the ions are accelerated with the same potential at a fixed point and a fixed initial time, the ions will separate according to their m/z ratios. This time-of-flight can be converted to mass. Typically a few hundred pulses of laser light are used, each of around a few nanoseconds' duration and the information is accumulated to build up a good spectrum. With the benefit of a camera that is used to follow the laser flashes one can move or 'track' the laser beam around the MALDI plate to find so-called sweet spots where the composition of co-crystallised matrix and sample is optimal for good sensitivity.

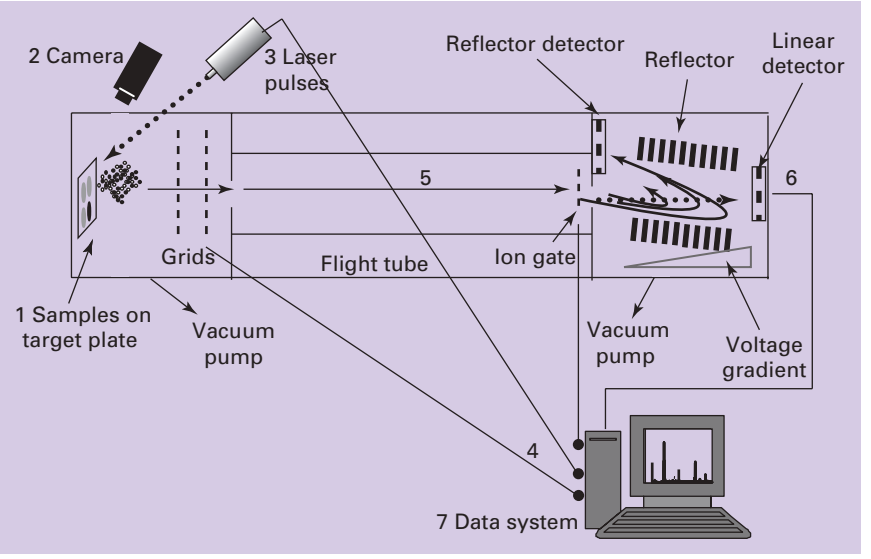


Fig. 9.12. MALDI–TOF instrument components. (1) Sample mixed with matrix is dried on the target plate, which is introduced into high vacuum chamber. (2) The camera allows viewing of the position of the laser beam, which can be tracked to optimise the signal. (3) The sample/matrix is irradiated with laser pulses. (4) The clock is started to measure time-of-flight. (5) Ions are accelerated by the electric field to the same kinetic energy and are separated according to mass as they fly through the flight tube. (6) Ions strike the detector either in linear (dotted arrow) or reflectron (full arrows) mode at different times, depending on their m/z ratio. (7) A data system controls instrument parameters, acquires signal versus time and processes the data.

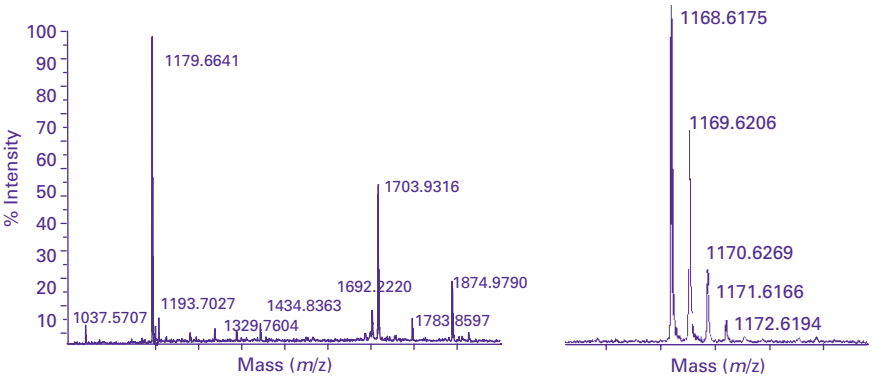


Fig. 9.13. Two examples of MALDI–TOF peptide spectra. The left-hand spectrum is from a protein digest mixture and the right-hand image is an expanded one of a small part of a spectrum showing ^{13}C-containing forms; see Section 9.5.4.

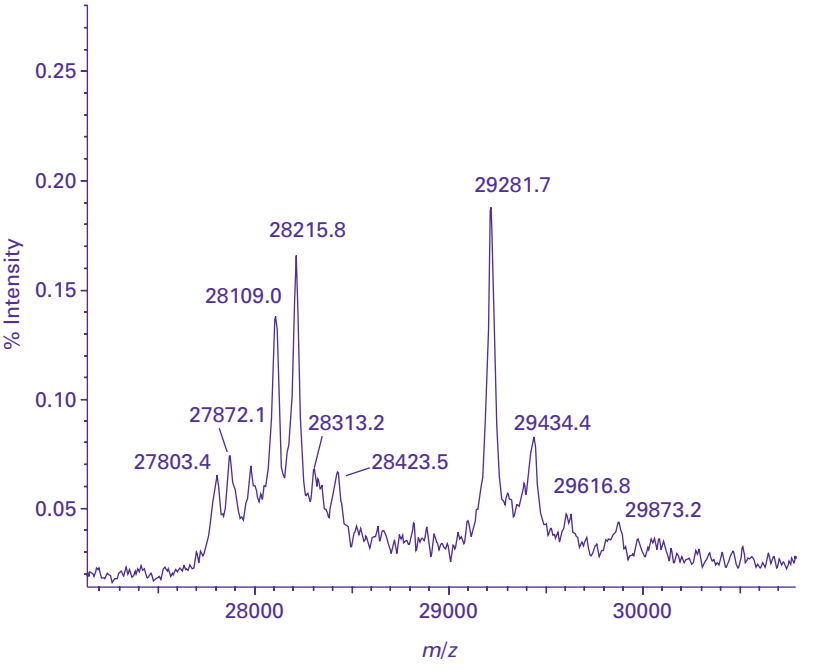


Fig. 9.14. MALDI–TOF spectrum of protein isoforms. The spectrum is almost exclusively singly charged ions representing the molecular ion species of the constitutent proteins. Compare this spectrum with the electrospray spectrum of another protein (Fig. 9.6) where the multiply charged ions result in multiple peaks that would make it harder to interpret masses of mixtures. (I acknowledge the assistance of Bruker Daltonics, who carried out the analysis.)

Sample concentration for MALDI

Maximum sensitivity is achieved in MALDI–TOF if samples are diluted to a particular concentration range. If the sample concentration is unknown a dilution series may be needed to produce a satisfactory sample/matrix spot of suitable concentration on the MALDI plate. Peptides and proteins seem to give best spectra at around 0.1–10 pmol mm^{-3} (Figs. 9.13 and 9.14). Some proteins, particularly glycoproteins, may yield better results at concentrations up to 10 pmol mm^{-3}. Oligonucleotides give better spectra at around 10–100 pmol mm^{-3}, whilst polymers require a concentration around 100 pmol nm^3. (Note: 1 pmol mm^{-3} = 10^{-6} mol dm^{-3}.)

Example 2 PEPTIDE MASS DETERMINATION(I)

Question

A peptide metabolite and an enzyme digest of it were analysed by a combination of mass spectrometric techniques giving the data listed below:

(i) The peptide showed two signals at 3841.5 and 1741 in the MALDI–TOF.

(ii) Five signals could be discerned when the peptide was introduced into a mass spectrometer via an electrospray ionization source:

m/z	498.2	581.1	697.1	871.2	1161.2

(iii) HPLC-MS of the digest indicated *four* components; the $[M + H]^+$ data for the components being $m/z = 176, 625, 1229$ and 1508. The ions corresponding to the MS of the '625' component appeared at $m/z = 521, 406, 293, 130$ and 113.

(iv) HPLC-MS–MS of the $m/z = 406$ ion of the '625' component identified two ions at $m/z = 378$ and 336 and of the $m/z = 113$ ion gave $m/z = 85$ and 57, in the product ion spectra.

Use the above data to compare and contrast the different ionisation methods, deduce a molecular mass for the peptide and determine a sequence for the '625' component.

Use the amino acid residue mass values in Table 9.2.

Answer

The data in (i) are $m/z = 3481.5$ and $m/z = 1741$. These data could represent either of the following possibilities:

(a) $m/z = 3481.5 \equiv (M + H)^+$
when $m/z = 1741 \equiv (M + H)^{2+}$, giving $M = 3480.5$

(b) $m/z = 3481.5 \equiv (2M + H)^+$
when $m/z = 1741 \equiv (M + H)^+$, giving $M = 1740$

Consideration of the data in (ii) allows a choice to be made between these two alternatives, using $n_2 = (m_1 - 1)/(m_2 - m_1)$ and $M = n_2(m_2 - 1)$.

Example 2 (*Cont.*)

$m_1 - 1$	$m_2 - m_1$	n_2	$m_2 - 1$	M(Da)	z
870.2	290	3.0006	1160.2	3481.2	3
696.1	174.1	3.9982	870.2	3479.3	4
580.1	116	5.0000	696.1	3481.1	5
497.2	82.9	5.9975	580.1	3479.2	6

$\Sigma M = 13920.8$ Da

Mean $M = 3480.2$ Da

The mean M result confirms set (a) of the conclusions above concerning the data obtained from the MALDI experiments.

The data in (iii) indicate that four products arise from the enzymatic digest of the original peptide. As these products arise directly from the original, the sum of these masses will be related to the M of the pepide.

Therefore

$176 + 625 + 1229 + 1508 = 3538$ Da

The difference between this mass and the M determined above is

$3538 - 3480.2 = 57.8 \approx 58$ Da

The difference of 58 mass units is explained as follows.

Each of the enzyme digest products is protonated (to be 'seen' in the mass spectrometer). Hence this accounts for 4 units. The remaining 54 unit increase arises from the enzymic hydrolysis. From a linear peptide, four products arise from three cleavage points (three cuts in a piece of string give four pieces). Each cleavage point requires the input of one water molecule (hydrolysis, $H_2O = 18$). Three cleavage points require $3 \times 18 = 54$.

The $m/z = 625$, $(M + H)^+$, peak was subjected to further mass spectrometry and sequence ions were observed.

m/z	624	521	406	293	130	113
Δ	103	115	113	163	17	
aa	Cys	Asp	Ile/Leu	Tyr	Ile/Leu	

The loss of 113 from the $m/z = 406$ ion indicates either Ile or Leu. MS^2 shows consecutive losses of 28 (CO) and 42 ($CH_2 = CH = CH_3$) which is indicative of Leu. The loss of 17 (not a sequence ion) from 130 confirms this as the C-terminal amino acid.

The predicted sequence, from the N-terminal end is

Cys-Asp-Leu-Tyr-Ile

Delayed extraction

In the first MALDI–TOF instruments, the ions in the plume of material generated by the laser pulse were continuously extracted by a high electrostatic field. Since this plume of material occupies a small but finite volume of space, ions arising at different places could have different energies. This energy spread (and

Example 3 PEPTIDE MASS DETERMINATION (II)

Question

Consider the following mass spectrometric data obtained for a peptide metabolite.

(i) The MALDI spectrum showed two signals at $m/z = 1609$ and 805.
(ii) There were two significant signals in positive ion trap MS mass spectrum at $m/z = 805$ and 827, the latter signal being enhanced on addition of sodium chloride.
(iii) Signals at $m/z = 161.8$, 202.0, 269.0 and 403.0 were observed when the sample was introduced into the mass spectrometer via an electrospray ionization source.

Use these data to give an account of the ionisation methods used. Discuss the significance of the data and deduce a relative molecular mass for the metabolite.

Use the amino acid residue mass values in Table 9.2.

Answer

(i) Signals in the MALDI spectrum were observed at $m/z = 1609$ and 805. These data could represent the following possibilities:

(a) $m/z = 1609 \equiv (M + H)^+$

when $m/z = 805 \equiv (M + 2H)^{2+}$
and $m/z = 403 \equiv (M + 4H)^{4+}$, giving $M = 1608$ Da

(b) $m/z = 1609 \equiv (2M + H)^+$

when $m/z = 805 \equiv (M + H)^+$
and $m/z = 403 \equiv (M + 2H)^{2+}$, giving $M = 804$ Da

(ii) The distinction between the above options can be made by considering the ion trap data. This mode of ionisation gave peaks at $m/z = 805$ and 827, the latter being enhanced on the addition of sodium chloride. This evidence suggests:

$m/z = 805 \equiv (M + H)^+$
$m/z = 827 \equiv (M + Na)^+$

giving $M = 804$ Da and supports option (b) from the MALDI data.

(iii) The multiply charged ions observed in the electrospray ionization method allow an average M to be calculated. Using the standard formula;

$m_1 - 1$	$m_2 - m_1$	n_2	$m_2 - 1$	M(Da)	z
268.0	134	2.0	402.0	804	2
201.0	67	3.0	268.0	804	3
160.8	40.2	4.0	201.0	804	4

The molecular mass is clearly 804 Da, confirming the above conclusions.

fragmentation occurring during this initial extraction period) usually broadens the peak corresponding to any particular ion, which leads to lower mass accuracy. However, if extraction is delayed until all ions have formed, this spread is minimised. The procedure is known as delayed extraction (DE), whereby the ions are formed in either a weak field or no field during a predetermined time

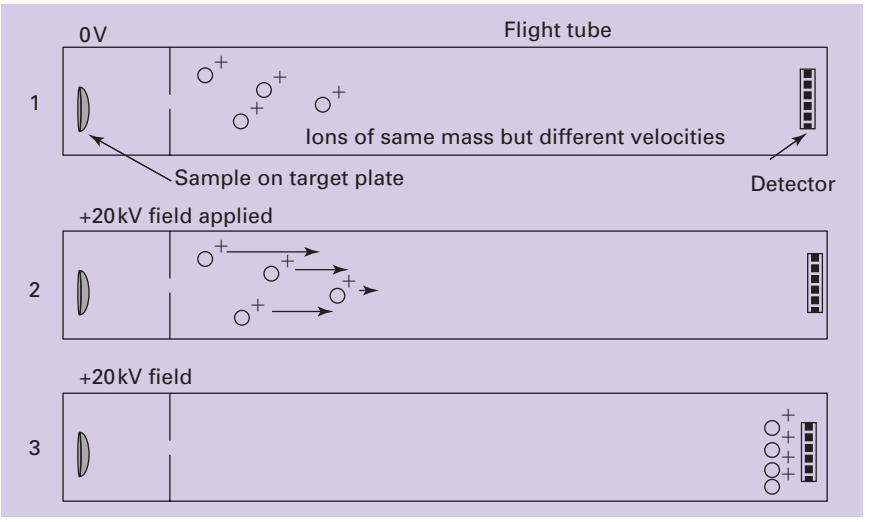


Fig. 9.15. Delayed extraction (DE). (1) No applied electric field. The ions spread out. (2) Field applied. The potential gradient accelerates slow ions more than fast ones. (3) Slow ions catch up with faster ones at the detector.

delay, and then extracted by the application of a high voltage pulse. The degree of fragmentation of ions (Section 9.3.8) can also be controlled, to some extent, by the length of the time delay. Delayed extraction is illustrated in Fig. 9.15.

9.3.8 Post-source decay

Post-source decay (PSD) is the process of fragmentation that may occur after an ion (the precursor ion) has been extracted from the source. Many biological molecules, particularly peptides, give rise to ions that dissociate over a time span of microseconds and most precursor ions will have been extracted before this dissociation is complete. The fragment ions generated will have the same velocity as the precursor and cause peak broadening and loss of resolution in a linear TOF analyser (Fig. 9.16). The problem is overcome by the use of a reflector.

The reflector

A reflector (or reflectron) is a type of ion mirror that provides higher resolution in MALDI–TOF. The reflector increases the overall path length for an ion and it corrects for minor variation in the energy spread of ions of the same mass. Both effects improve resolution. The device has a gradient electric field and the depth to which ions will penetrate this field, before reversal of direction of travel, depends upon their energy. Higher energy ions will travel further and lower energy ions a shorter distance. The flight times thus become focused, while neutral fragments are unaffected by the deflection. Fig. 9.16 shows a diagrammatic representation of a MALDI–TOF instrument that includes the facility for both linear and reflectron modes of ion collection. The reflectron improves resolution and mass accuracy and also allows structure and sequence information (in the case of peptides) to be obtained by PSD analysis.

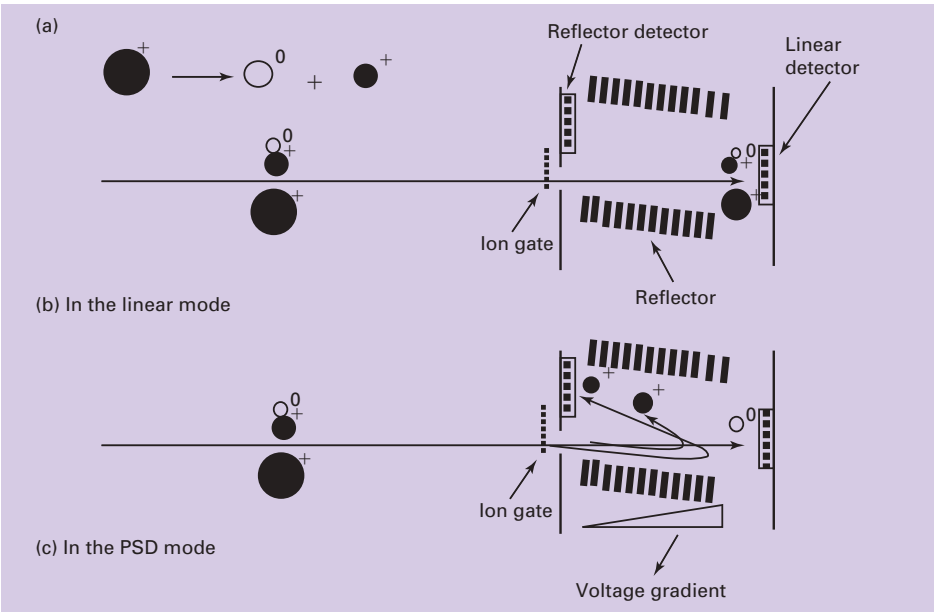


Fig. 9.16. The MALDI–TOF reflector. Post-source decay (PSD) theory. (a) Fragmentions arising by PSD as well as the neutral fragments and the precursor ions have the same velocity and reach the detector simultaneously. This prevents a distinction between precursor and PSD fragment. (b) In the linear mode the charged fragments are not separated. (c) In the reflector mode, the fragment that does not retain the charge (neutral, denoted by $\bigcirc^0$) is not deflected in the reflector but the charged fragments ($\bullet^+$) are deflected according to their m/z ratios and a spectrum of the fragment (daughter) ions is recorded, albeit of a limited m/z range for each setting of the reflector voltages.

Sequencing peptides by PSD analysis in MALDI–TOF is less straightforward (and in a large percentage of experiments is unsuccessful) than tandem MS on a quadrupole ESI or ion trap instrument. At any given setting of the reflector/ion mirror, charged fragments of a particular range of m/z are focused in the reflector (Fig. 9.16). Fragment ions of m/z above and below this narrow range are poorly focused. Therefore, since only fragment ions of a limited mass range are focused for a given mirror ratio in the reflector, a number of spectra are run at different settings and stitched together to generate a composite spectrum.

Types of MALDI sample plates

MALDI sample plate types that are available include 100-well stainless steel flat plates. These are good for multiple sample analysis where close external calibration is used; that is, the use of a compound or compounds of known molecular mass placed on an adjacent spot to calibrate the instrument. It is also easier to see crystallisation of the matrix on this type of surface. Four-hundred-spot Teflon-coated plates have particular application for concentrating samples for increased sensitivity. Owing to the very small diameter of the spots, it is difficult to spot accurately manually but these plates are good for automated sample spotting. Only in the centre of each spot is the surface of the plate exposed, therefore the

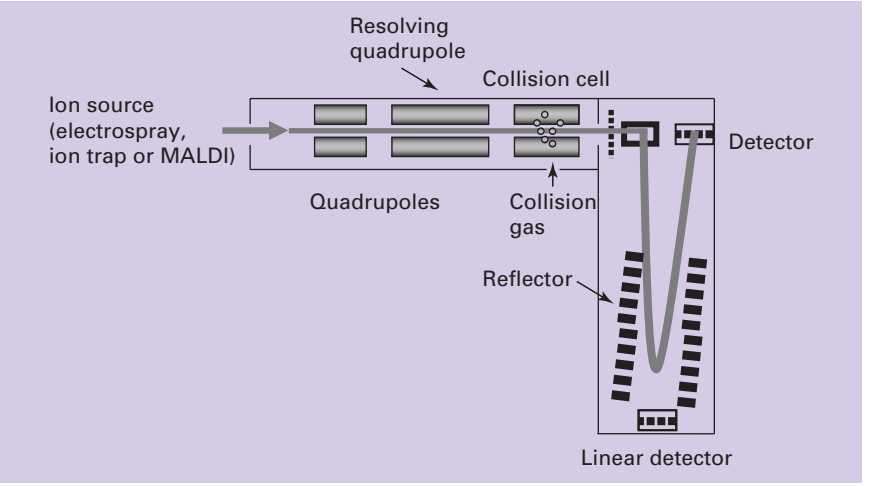


Fig. 9.17. Diagram of a hybrid quadrupole TOF MS. The diagram shown here does not represent any specific instrument from a particular manufacturer. The source may be an ion trap device, an electrospray or even a MALDI source (in the 'MALDI Q-TOF' from Micromass). Other hybrid instruments include the Bruker Daltonics 'BioTOF III, ESI-Q-q-TOF System' and the 'QSTAR' Hybrid LC-MS/MS from Applied Biosystems with an electrospray, nanospray or an optional MALDI source. The Shimadzu Biotech 'AXIMA MALDI QIT TOF' combines a MALDI source with an ion trap and reflectron TOF mass analyser.

sample does not 'wet' over the whole surface but concentrates itself into the centre of each spot as it dries. Gold-coated plates with wells (2 mm diameter, see Fig. 9.10b) are good surfaces on which to contain the spread of sample and matrix when used with high organic solvents, for example tetrahydrofuran preparations for polymers. They also allow on-plate reactions within the well with thiol-containing reagents that bind to the gold surface.

9.3.9 Novel hybrid instruments

There are a number of commercial developments of hybrid MS instruments that involve coupling an electrospray, ion trap or MALDI ion source with a hybrid quadrupole orthogonal acceleration TOF mass spectrometer (Fig. 9.17). This potentially leads to improved tandem MS performance from MALDI phase samples. The intention of the development of these instruments is to combine the best features of both types of ion source with the best features all types of analyser in order to improve tandem MS capability and increase sensitivity. Hybrid magnetic sector instruments are also manufactured where the first mass spectrometer is a two-sector device and the second mass spectrometer is a quadrupole.

9.3.10 Fourier transform ion cyclotron resonance mass spectrometry

The recent development of Fourier transform ion cyclotron resonance (FT-ICR) MS has great potential in analysis of a wide range of biomolecules. It is potentially the

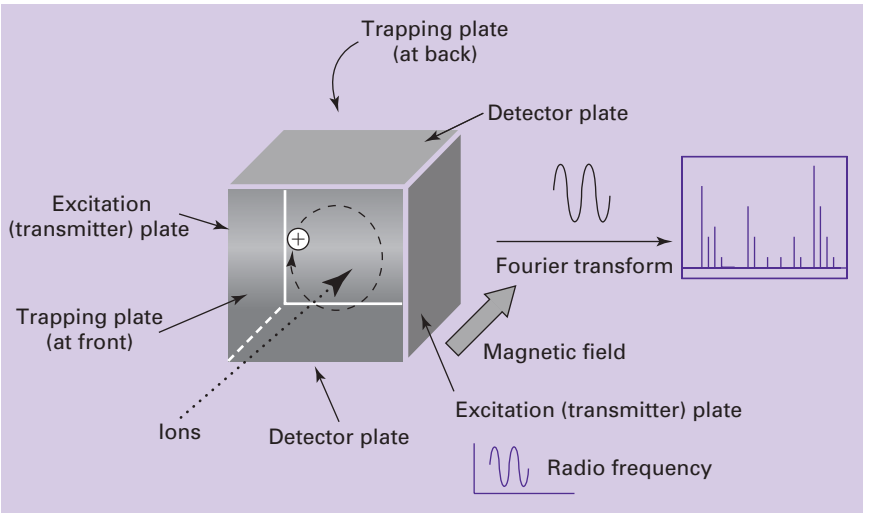


Fig. 9.18. Schematic diagram of the Fourier transform ion cyclotron resonance (FT-ICR) instrument. The technique involves trapping, excitation and detection of ions to produce a mass spectrum. The trapping plates to maintain the ions in orbit are at the front and back in the schematic. The excitation or transmitter plates, where the radio frequency pulse is given to the ions, are shown at each side and the detector plates that detect the image current, which is Fourier transformed, are shown at the top and bottom. The sample source is normally electrospray or MALDI. The ions are focused and transferred into the analyser cell under high vacuum. The analyser cell is a type of ion-trap in a spatially uniform static magnetic field which constrains the ions in a circular orbit, the frequency of which is determined by the mass, charge and velocity of the ion. Whilst the ions are in these stable orbits between the detector electrodes they will not give a measurable signal. In order to achieve this, ions of a given *m/z* ratio are excited to a wider orbital radius by applying a radio frequency signal of a few milliseconds' duration. One frequency excites ions of one particular *m/z* value, which results in the ions producing a detectable image current. This time-dependent image current is Fourier transformed to obtain the component frequencies, which correspond to the *m/z* ratios of the different ions. The angular frequency measurements produce values for *m/z*. Therefore the mass spectrum is determined to a very high mass resolution since frequency can be measured more accurately than any other physical property. After excitation, the ions relax back to their previous orbits and high sensitivity can be achieved by repeating this process many times.

most sensitive mass spectrometric technique and has almost unlimited mass resolution, $>10^6$ is observable with most instruments. The instrument also allows tandem MS to be carried out. The ions can be generated by a variety of techniques, such as an ESI or a MALDI source. FT-ICR MS is based on the principle of ions, which, whilst orbiting in a magnetic field, are excited by radio frequency signals. As a result, the ions produce a detectable image current on the cell in which they are trapped. The time-dependent image current is Fourier transformed to obtain the component frequencies of the different ions, which correspond to their *m/z* (Fig. 9.18).

9.4 DETECTORS

9.4.1 Introduction

The ions from the mass analyser impinge on a surface of a detector where the charge is neutralised, either by collection or donation of electrons. An electric current flows that is amplified and ultimately converted into a signal that is processed by a computer. The total ion current (TIC) is the sum of the current carried by all the ions being detected at any given moment and is a very useful parameter to measure during on-line MS. A plot of ion current versus time complements the ultraviolet trace that is also normally recorded during the chromatography run. Unlike the ultraviolet trace, which depends on the absorbance of each component at the particular wavelength(s) set on the ultraviolet detector, the TIC is of course independent of the light-absorbing properties of a substance and depends only upon its ionisability in the instrument.

9.4.2 Electron multiplier and conversion dynode

Electron multipliers are used as detectors for all types of mass spectrometers. These are frequently combined with a conversion dynode, which is a device to increase sensitivity. The ion beam from the mass analyser is focused onto the conversion dynode, which emits electrons in direct proportion to the number of bombarding ions. A positive ion or a negative ion hits the conversion dynode, causing the emission of secondary particle containing secondary ions, electrons and neutral particles (Fig. 9.19). These secondary particles are accelerated into the dynodes of the electron multiplier. They strike the dynodes with sufficient energy to dislodge electrons, which pass further into the electron multiplier, colliding with the dynodes, producing more and more electrons.

9.5 STRUCTURAL INFORMATION BY TANDEM MASS SPECTROMETRY

9.5.1 Introduction

As mentioned above, the newer ionisation techniques ESI and MALDI are soft ionisation techniques (as is FAB and its derivative techniques). In contrast to EI, they do not produce significant amounts of fragment ions. Therefore in order to obtain structural information on biomolecules and sequence information (in the case of proteins and peptides), tandem MS has been developed. The technique can also be applied to obtain sequence information on oligosaccharides (Section 9.5.6) and oligonucleotides (Fig. 9.20). Although it is unlikely that this method will ever replace DNA sequencing gels, it can be used to identify positions of modified or labelled bases that might not be picked up by the Sanger dideoxy sequencing method.

Structural information can be obtained on almost any type of organic molecule, on an instrument that is suitable for that type of sample. This includes

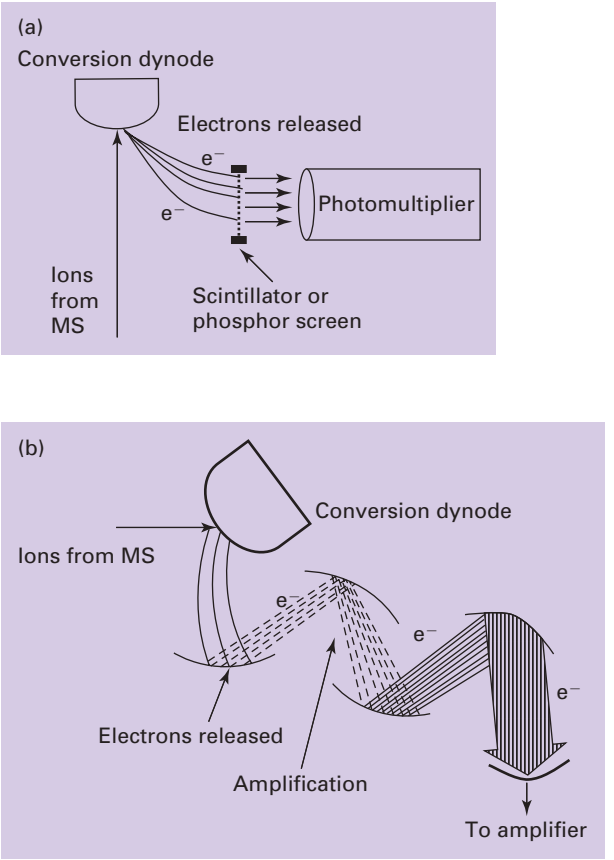


Fig. 9.19. Conversion dynode and electron multiplier. (a and b) Each ion strikes the conversion dynode (which converts ions to electrons), which emits a number of electrons that travel to the next, higher voltage dynode. The secondary electrons from the conversion dynode are accelerated and focused onto a second dynode, which itself emits secondary electrons. Each electron then produces several more electrons. Amplification is achieved through the 'cascading effect' of secondary electrons from dynode to dynode that finally results in a measurable current at the end of the electron multiplier. The cascade of electrons continues until a sufficiently large current for normal amplification is obtained. A series of up to 10–20 dynodes (set at different potentials) provides an amplification gain of 10^6 or 10^7.

investigation of organic compounds on a magnet sector MS where two double-focusing magnetic sector machines can be combined into a four-sector device coupled through a collision cell. The general procedure is that a mixture of ions is generated in the ion source of the mass spectrometer as normal and the ions are allowed to pass through the first mass analyser, where an ion of a particular m/z is selected (but not detected). This ion then enters the collision cell and collides with an inert collision gas such as helium or argon. The kinetic energy of this ion is converted to vibrational energy and the ion fragments. This is known as

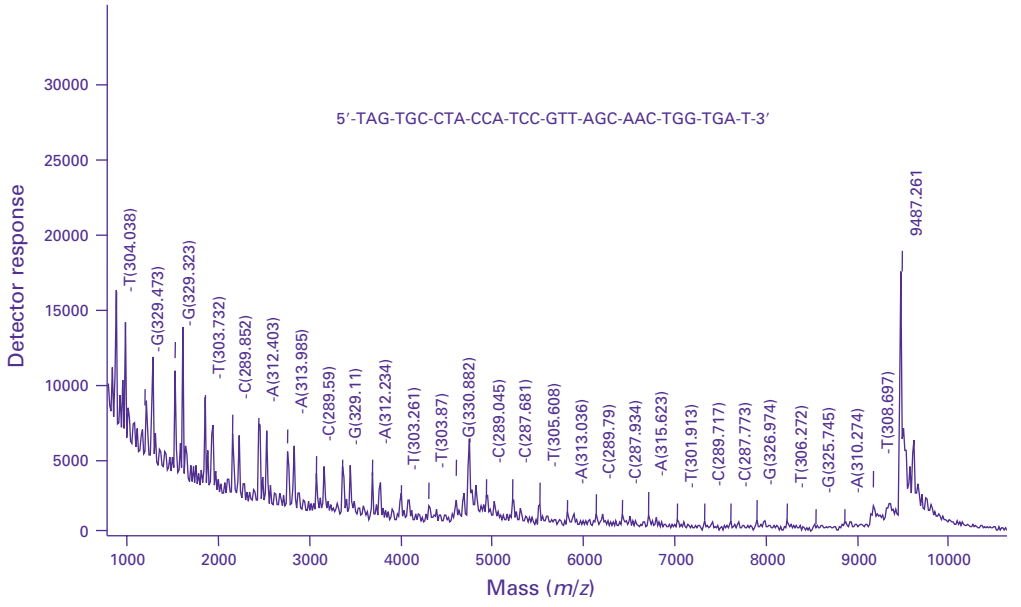


Fig. 9.20. DNA sequencing by MS. This shows an example of a 36-mer oligonucleotide sequenced by in-source decay on MALDI–TOF. The sequence is reconstructed from the mass differences between the peaks, which correspond to consecutive loss of a particular nucleotide at each fragmentation. (I acknowledge the use of the spectrum from Applied Biosystems.)

collision-induced dissociation (CID) or collision-activated dissociation (CAD). The m/z values of the fragment ions are then determined in a second mass spectrometer (see Fig. 9.21 for an illustration of the principle in a quadrupole mass spectrometer). Collision cells may be placed in any of the field-free regions, leading to a wide variety of experimental methodologies for many different applications. For example, as well as in the triple quadrupole MS this can be done in a hybrid instrument such as the Q-TOF (Section 9.3.9). Since the principles of tandem MS are similar for most instrument configurations, further discussion will focus on electrospray tandem MS. The procedure for obtaining structural and sequence information on polypeptides in ion trap MS has been described above (Section 9.3.3).

9.5.2 Sequencing of proteins and peptides

The structural identification of proteins involves protease cleavage, mostly by trypsin. Owing to the specificity of this protease, tryptic peptides usually have basic groups at the N and C termini. Trypsin cleaves after lysine and arginine residues, both of which have basic side-chains (an amino and a guanidino group, respectively). This results in a large proportion of high energy doubly charged positive ions that are more easily fragmented. The digestion of the protein into peptides is followed by identification of the peptides by (m/z), either as very accurate masses alone or by using a second fragmentation that gives ladders of

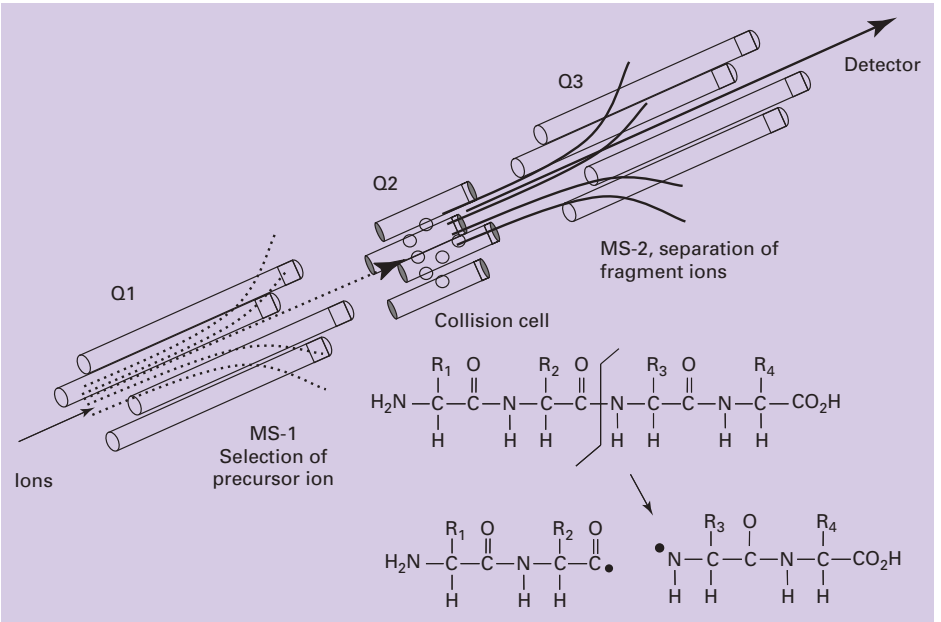


Fig. 9.21. Quadrupole MS sequencing. An ion of a particular *m*/*z* value is selected in the first quadrupole, Q1, as in Fig. 9.5, but instead of being detected, it passes through the second quadrupole, Q2, where it is subjected to collision with the collision gas. The Q3 acts like a second quadrupole mass spectrometer, MS-2, to scan *m*/*z* to obtain a spectrum of the fragment ions. The collision cell, Q2, is frequently a radio frequency (RF)-only quadrupole containing the appropriate collision gas. No mass filtering occurs here, the RF merely constrains the ions to allow a greater number of collisions to occur. The fragmentation depicted here is at the peptide bond and one of the fragments will retain the charge, resulting in either a y-series or a b-series ion, see Fig. 9.22.

fragments cleaved at the peptide bonds. Although a wide variety of fragmentations may occur, there is a predominance of peptide bond cleavage, which gives rise to peaks in the spectrum that differ sequentially by the residue mass. The mass differences are thus used to reconstruct the amino acid sequence (primary structure) of the peptide (Table 9.2).

Different series of ions, a, b, c and x, y, z, may be recognised, depending on which fragment carries the charge. Ions x, y and z arise by retention of charge on the C-terminal fragment of the peptide. For example, the z_1 ion is the first C-terminal residue; y_1 also contains the NH group (15 atomic mass units greater) and x_1 includes the carbonyl group; y_2 comprises the first two C-terminal residues, and so on. The a, b, and c ion series arise from the N-terminal end of the peptide, when the fragmentation results in retention of charge on these fragments. Fig. 9.22a shows an idealised peptide subjected to fragmentation. Particular series will generally predominate so that the peptide may be sequenced from both ends by obtaining complementary data (Fig. 9.22b). In addition, ions can arise from side-chain fragmentation, which enables a distinction to be made between isomeric amino

Table 9.2 Symbols and residue masses of the protein amino acids

Name	Symbol	Residue mass[a]	Side-chain
Alanine	A, Ala	71.079	CH_3-
Arginine	R, Arg	156.188	$HN{=}C(NH_2){-}NH{-}(CH_2)_3$-
Asparagine	N, Asn	114.104	$H_2N{-}CO{-}CH_2$-
Aspartic acid	D, Asp	115.089	$HOOC{-}CH_2$-
Cysteine	C, Cys	103.145	$HS{-}CH_2$-
Glutamine	Q, Gln	**128.131**	$H_2N{-}CO{-}(CH_2)_2$-
Glutamic acid	E, Glu	129.116	$HOOC{-}(CH_2)_2$-
Glycine	G, Gly	57.052	H-
Histidine	H, His	137.141	Imidazole-CH_2-
Isoleucine	I, Ile	**113.160**	$CH_3{-}CH_2{-}CH(CH_3)$-
Leucine	L, Leu	**113.160**	$(CH_3)_2{-}CH{-}CH_2$-
Lysine	K, Lys	**128.17**	$H_2N{-}(CH_2)_4$-
Methionine	M, Met	131.199	$CH_3{-}S{-}(CH_2)_2$-
Metsulphoxide	Met.SO	**147.199**	$CH_3{-}S(O){-}(CD_2)_2$-
Phenylalanine	F, Phe	**147.177**	Phenyl-CH_2-
Proline	P, Pro	97.117	Pyrrolidone-CH-
Serine	S, Ser	87.078	$HO{-}CH_2$-
Threonine	T, Thr	101.105	$CH_3{-}CH(OH)$-
Tryptophan	W, Trp	186.213	Indole-$NH{-}CH{=}C{-}CH_2$-
Tyrosine	Y, Tyr	163.176	4-OH-Phenyl-CH_2-
Valine	V, Val	99.133	$CH_3{-}CH(CH_2)$-

[a]Residue mass is the mass in a peptide bond, i.e. after loss of H_2O when the peptide bond is formed. The numbers in bold in the residue mass column indicate amino acids that may be ambiguous in a sequence determined by tandem MS due to close similarity or identity in mass.

acids such as leucine and isoleucine. The protein is identified by searching databases of expected masses from all known peptides from every protein (or translations from DNA) and theoretical masses from fragmented peptides. The sensitivity of tandem MS has been claimed down to zeptomole level (see Table 9.3).

9.5.3 Comparison of mass spectrometry and Edman sequencing

Edman degradation to obtain the complete sequence of a protein is uncommon nowadays, since genomes are available to search with fragmentary sequences. Most intact proteins, if they are not processed from a secretory or pro-peptide form, are blocked at the N terminus, most commonly with an acetyl group. Other N-terminal blocking includes fatty acylation, most commonly with a myristoyl, C_{12} fatty acid, attached through a glycine residue, but the presence of many shorter chain fatty acids is known to occur. Cyclisation of glutamine to a pyroglutamyl residue and post-translational modification to N-terminal trimethylalanine and dimethylproline also occur. In the case of recombinant proteins overexpressed in *Escherichia coli*, the initiator residue *N*-formylmethionine is often incompletely removed. All these modifications leave the N-terminal residue without a free

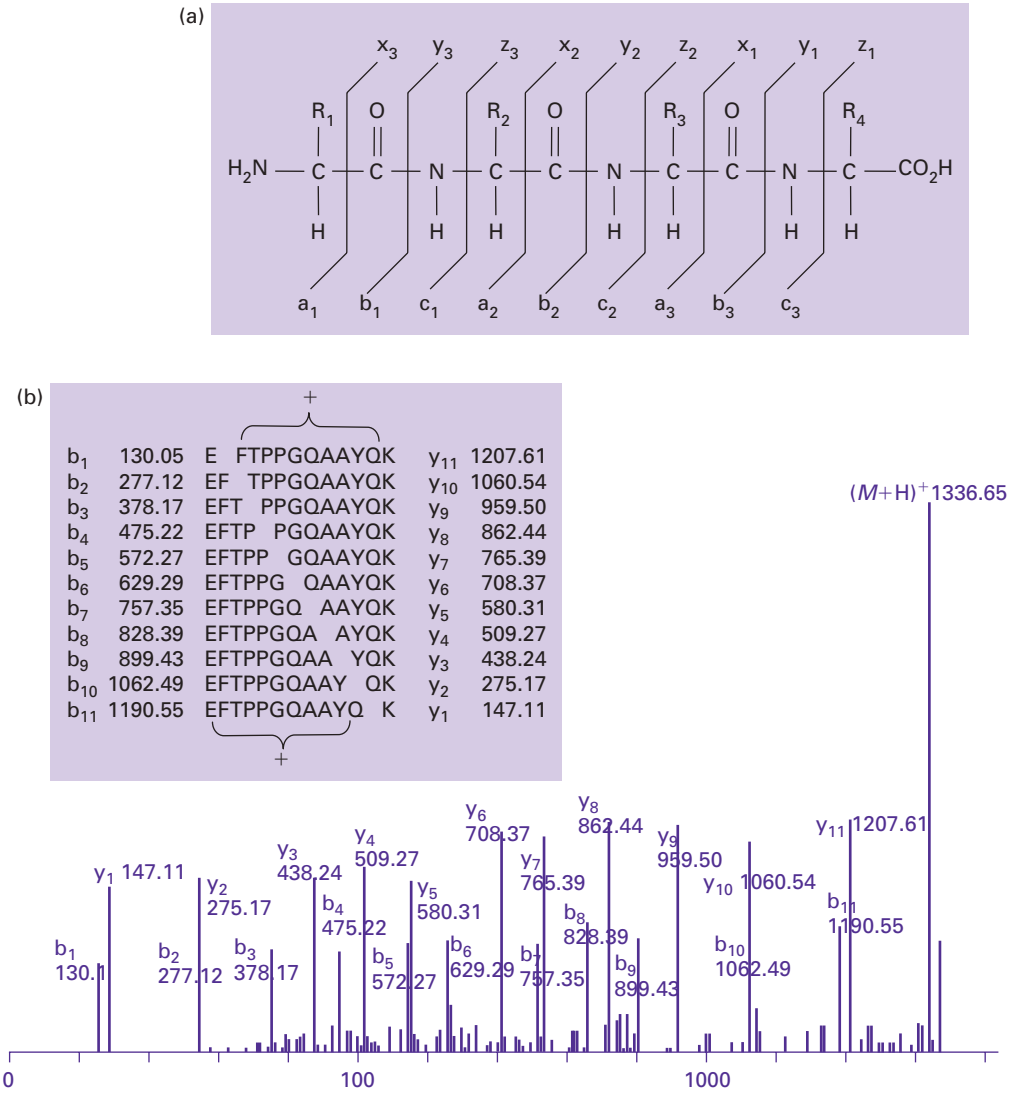


Fig. 9.22. Peptide fragment ion nomenclature and tandem MS spectrum of a peptide. (a) Charge may be retained by either the N- or C-terminal fragment, resulting in the a, b and c series of ions or x, y and z series, respectively. b- and y-series ions frequently predominate. Corresponding neutral fragments are of course not detected. The sequence of the peptide, from a mutant haemoglobin, is EFTPPGQAAYQK. The figure shows the tandem mass spectrum from collision-induced dissociation of the doubly charged $(M + 2H)^{2+}$ precursor, $m/z = 668.3$. Cleavage at each peptide bond results in the b or y ions when the positive charge is retained by the fragment containing the N or C terminus of the peptide, respectively (see inset).

proton on the alpha nitrogen and Edman chemistry cannot proceed. Mass spectrometry has therefore been essential for their correct structural identification. The protein-sequencing instruments are still important for solid-phase sequencing to identify post-translational modifications; in particular, sites of phosphorylation

Example 4 PEPTIDE SEQUENCING (I)

Question

An oligopeptide obtained by tryptic digestion was investigated by ESI–MS and ion trap MS–MS both in positive mode, and gave the following *m/z* data:

ESI	223.2	297.3								
Ion trap	146	203	260	357	444	591	648	705	802	890

(i) Predict the sequence of the oligopeptide. Use the amino acid residual mass values in the table below.
(ii) Determine the average molecular mass.
(iii) Identify the peaks in the ESI spectrum.

(See table 9.2 for amino acid residue mass.)
Note: Trypsin cleaves on the C-terminal side of arginine and lysine.

Answer

(i) The highest mass peak in the ion trap MS spectrum is $m/z = 890$, which represents $(M + H)^+$.

Hence $M = 889$ Da.

m/z	146	203	260	357	444	591	648	705	802	889
Δ		57	57	97	87	147	57	57	97	87
aa		Gly	Gly	Pro	Ser	Phe	Gly	Gly	Pro	Ser

The mass differences (Δ), between sequence ions, represent the amino acid(aa) residue masses. The lowest mass sequence ion, $m/z = 146$, is too low for arginine and must therefore represent Lys + OH. The sequence in conventional order from the N-terminal end would be:
Ser-Pro-Gly-Gly-Phe-Ser-Pro-Gly-Gly-Lys

(ii) The summation of the residues = 889 Da, which is a check on the mass spectrometry value for *M*.
(iii) The *m/z* values in the ESI spectrum represent multiply charged species and may be identified as follows:

$m/z = 223.2 \equiv (M + 4H)^{4+}$ from $889/223.2 = 3.98$
$m/z = 297.3 \equiv (M + 3H)^{3+}$ from $889/297.3 = 2.99$

Remember that *z* must be an integer and hence values need to be rounded to the nearest whole number.

and a combination of microsequencing and MS techniques are now commonly employed for complete covalent structure determination of proteins.

9.5.4 Carbon isotopes and finding the charge state of a peptide

Since the mass detector operates on the basis of *m*/*z*, mass assignment is normally made assuming a single charge per ion (i.e. $m/z = m + 1$ in positive ion mode).

Example 5 PEPTIDE SEQUENCING (II)

Question

Determine the primary structure of the oligopeptide that gave the following, positive mode, MS–MS data:

m/z	149	305	442	529	617

Use the amino acid residual mass values in Table 9.2.

Answer

$m/z = 617 \equiv (M + H)^+$

m/z	149	305	442	529	616
Δ	156	137	87	87	
aa	Arg	His	Ser	Ser	

Conventional order for the sequence would be:
Ser-Ser-His-Arg-?
It is important to note that no assignment has been given for the remaining $m/z =$ 149. It may not in fact be a sequence ion and more information would be required, such as an accurate molecular mass of the oligopeptide, in order to proceed further. It is, however, possible to speculate as to the nature of this ion. If the $m/z = 149$ ion is the C-terminal amino acid then it would end in-OH and be 17 mass units greater than the corresponding residue mass. The difference between 149 and 17 is 132, which is extremely close to methionine, so this amino acid remains a possibility to end the chain.

However, since there is around 1.1% ^{13}C natural abundance, with increasing size, peptides will have a greater chance of containing at least one ^{13}C and two ^{13}C, etc. A peptide of 20 residues has approximately equal peak heights of the 'all ^{12}C peptide' and of the peptide with one ^{13}C. A singly charged peptide will show adjacent peaks differing in one mass unit; a doubly charged peptide will show adjacent peaks differing in half a mass unit and so on (Fig. 9.23 and Table 9.3). In the example illustrated (Fig. 9.23), angiotensin has an M_r calculated from its sequence as 1295.69. The experimentally derived values are, for the singly charged ion, $[(M + H)/1] = 1296.65$ and for the doubly charged ion, $[(M + 2H)/2] = 648.82$. For elements such as chlorine, the isotopic abundance ^{35}Cl: ^{37}Cl is approximately 3:1. If a compound contains a single chlorine atom, two ion species will be observed, with peak intensities in an approximate ratio of 3:1. If a compound contains two chlorine atoms then three peaks will be seen. The technique is particularly useful for determining which are the high energy doubly charged tryptic peptides, for tandem MS.

9.5.5 Ladder sequencing

This technique is an alternative to tandem MS and involves the generation of a set of fragments of a polypeptide chain followed by analysis of the mass of each component. Each component in the polypeptide mixture differs from the next by

Example 6 PEPTIDE MASS DETERMINATION (III)

Question

An unknown peptide and an enzymatic digest of it were analysed by mass spectrometric and chromatographic methods as follows:

(i) MALDI–TOF mass spectrometry of the peptide gave two signals at m/z = 3569 and 1785;
(ii) MALDI–TOF of the hydrolysate showed signals at m/z = 766, 891, 953 and 1016;
(iii) the data obtained from analysis of the peptide using coupled HPLC–MS operating through an electrospray ionisation source were m/z = 510.7, 595.7, 714.6, 893.0 and 1190.3;
(iv) when the hydrolysate was analysed by HPLC, four distinct components could be discerned.

Explain what information is available from these observations and determine a molecular mass, using the amino acid residue mass values in Table 9.2, for the unknown peptide.

Answer

(i) Signals from MALDI–TOF were observed at m/z = 3569 and 1785. These data could represent either of the following possibilities:

(a) $m/z = 3569 \equiv (M + H)^+$
when $m/z = 1785 \equiv (M + 2H)^{2+}$, giving $M = 3568$

(b) $m/z = 3569 \equiv (2M + H)^+$,
when $m/z = 1785 \equiv (M + H)^+$, giving $M = 1784$

(ii) It is possible to distinguish between these two options by considering the MALDI–TOF of the products of hydrolysis. Four m/z values were obtained: 766, 891, 953 and 1016.
Each is a protonated species and the sum of these masses, 3626, will be of the order of the M of the original peptide. The value of this sum supports option (a) in (i) above.

(iii) Electrospray ionisation data represent multiply charged ions. Using the standard formula the mean M may be obtained.

$m_1 - 1$	$m_2 - m_1$	n_2	$m_2 - 1$	M (Da)	z
892.0	297.3	3.0003	1189.3	3568.3	3
713.6	178.4	4.0000	892.0	3568.0	4
594.7	118.9	5.0016	713.6	3569.2	5
509.7	85	5.9964	594.7	3566.1	6

ΣM = 14271.6 Da
Mean M = 3567.9 Da

This more precise value confirms the conclusions found above. For an explanation of the mass difference between M_r and the sum of the hydrolysate products, refer to the answer to Example 2.

The data in (iv) are confirmatory chromatographic evidence that only four hydrolysis products were obtained.

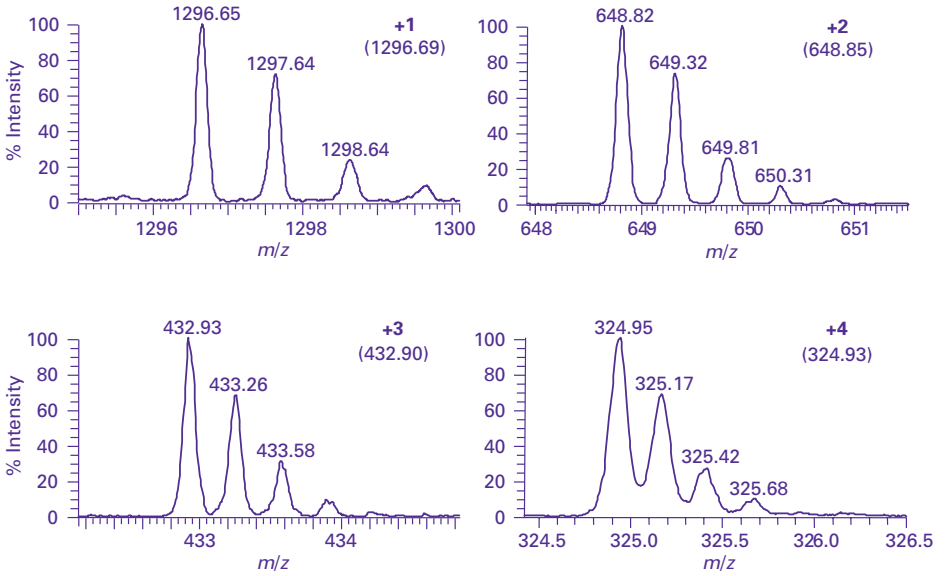


Fig. 9.23. Spectra of a multiply charged peptide. Finding the charge state of a peptide involves zooming in on a particular part of the mass spectrum to obtain a detailed image of the mass differences between different peaks that arise from the same biomolecule, due to isotopic abundance. This is due mainly to ^{12}C and its ^{13}C isotope, as described in the text.

Table 9.3 Mass differences due to isotopes in multiply charged peptides

Charge on peptide	Apparent mass	Mass difference between isotope peaks
Single charge	$[(M + H)/1]$	1 Da
Double charge	$[(M + 2H)/2]$	0.5 Da
Triple charge	$[(M + 3H)/3]$	0.33 Da
n charges	$[(M + nH)/n]$	$1/n$ Da

loss of a mass that is characteristic of the residue weight (which may involve a modified side-chain) thus enabling the sequence of the polypeptide to be read. The ladder of degraded peptides can be generated by Edman chemistry (Section 8.4.3) or by exopeptidase digestion from the N or the C terminus. The Edman chemistry is modified to carry out the coupling step with phenylisothiocyanate (PITC) in the presence of a small amount of phenylisocyanate, which acts as a chain-terminating agent. This has some analogy to the dideoxy Sanger DNA sequencing methodology. This is essentially a subtractive technique (one looks at the mass of the remaining fragment after each cycle). For example when a phosphoserine residue is encountered, a loss of 167.1 Da is observed in place of 87.1 for loss of a serine residue. This technique therefore avoids one of the major problems of analysing post-translational modifications, since the majority of modifications are stable during the Edman chemistry. The technique of ladder sequencing has particular application in MALDI–TOF MS, which has high sensitivity and greater ability to analyse mixtures.

9.5.6 Post-translational modification of proteins

Many chemically distinct types of post-translational modification of proteins are known to occur. These include the wide variety of acylations at the N terminus of proteins (mentioned above) as well as acylations at the C terminus and at internal sites. In this section, examples of the application of MS techniques employed for analysis of glycosylation, phosphorylation and disulphide bonds are given. An up-to-date list of the broad chemical diversity of known modifications and the side-chains of the amino acids to which they are attached is in the website 'Delta Mass', which is a database of protein post-translational modifications that can be found at <http://www.abrf.org/index.cfm/dm.home>. There are hyperlinks to references to the modifications.

Protein phosphorylation and identification of phosphopeptides

The reversible covalent phosphorylation of eukaryotic proteins is a common regulatory mechanism for protein activity (Sections 15.5.4 and 16.5.3). The modified residues are *O*-phosphoserine, *O*-phosphothreonine and *O*-phosphotyrosine but many other amino acids in proteins can be phosphorylated: *O*-phospho-Asp; *S*-phospho-Cys; *N*-phospho-Arg; *N*-phospho-His and *N*-phospho-Lys. Analysis of modified peptides by MS is essential to confirm the exact location and number of phosphorylated residues, especially if no ^{32}P or other radiolabel is present. The identification of either positive or negative ions may yield more information, depending on the mode of ionisation and fragmentation of an individual peptide. Phosphopeptides may give better spectra in the negative-ion mode, since they have a strong negative charge due to the phosphate group. Phosphopeptides may not run well on MALDI–TOF and methods have been successfully developed for this type of instrument that employ examination of spectra before and after dephosphorylation of the peptide mixture with phosphatases.

Mass spectrometry of glycosylation sites and structures of the sugars

The attachment points of N-linked (through asparagine) and O-linked (through serine) glycosylation sites and the structures of the complex carbohydrates can be determined by MS. The loss of each monosaccharide unit of distinct mass can be interpreted to reconstruct the glycosylation pattern (Fig. 9.24).

The 'GlycoMod' website, part of the 'ExPASy' suite, provides valuable assistance in interpretation of the spectra. GlycoMod is a tool that can predict the possible oligosaccharide structures that occur on proteins from their experimentally determined masses. The program can be used for free or derivatised oligosaccharides and for glycopeptides. Another algorithm 'GlycanMass', also part of the ExPASy suite, can be used to calculate the mass of an oligosaccharide structure from its oligosaccharide composition. GlyocoMod and GlycanMass may be found at <http://us.expasy. org/tools/glycomod/> and <http://us.expasy.org/tools/glycomod/glycanmass.html>, respectively.

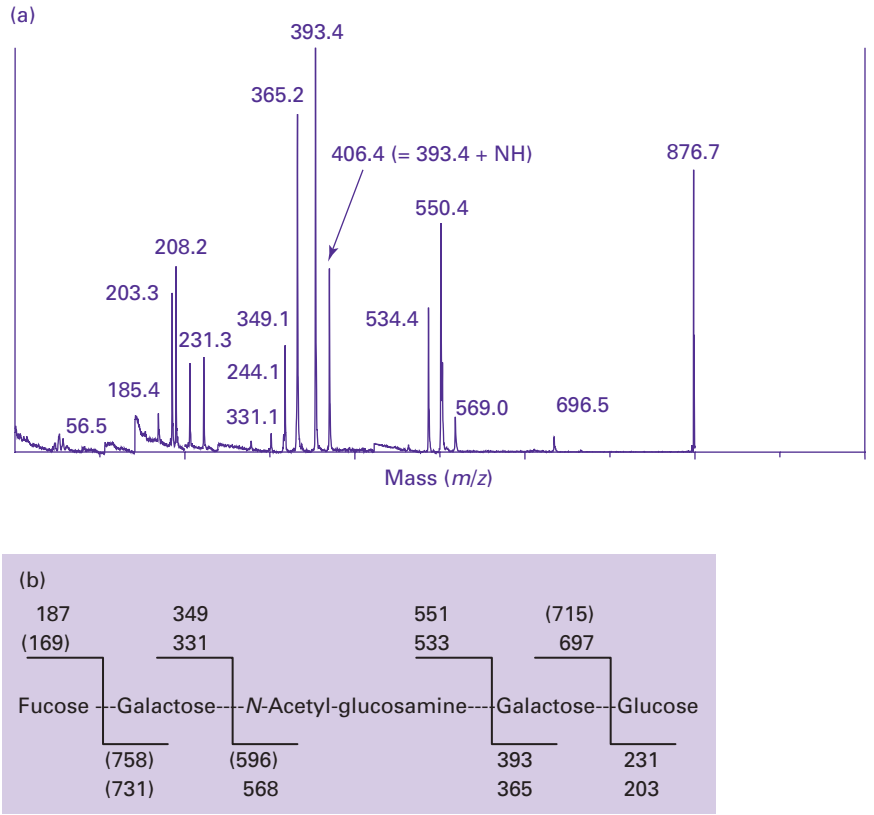


Fig. 9.24. MALDI–TOF PSD MS of carbohydrates. (a) PSD MS spectrum of the carbohydrate Fuc1–2Gal1–3GlucNAc1–3Gal1–4Glc using 2,5-dihydroxybenzoic acid as matrix. On careful inspection of the spectrum one can observe a number of abrupt changes in baseline corresponding to where the PSD spectra have been 'stitched' together. The peak at 876.7 Da is due to the mass of the intact molecule as a sodium adduct, i.e. the parent ion at 876.7 = $[M + Na]^+$. (b) Interpretation of the spectrum. Experimentally derived fragment masses are mainly within 1 Da of the theoretical. The masses in parentheses were not seen in this experiment.

Identification of disulphide linkages by mass spectrometry

MS is also used in the location of disulphide bonds in a protein. The identification of the position of the disulphide linkages involves the fragmentation of proteins into peptides under low pH conditions to minimise disulphide exchange. Proteases with active site thiols should be avoided (e.g. papain, bromelain). Pepsin and cyanogen bromide are particularly useful. The disulphide-linked peptide fragments are separated and identified under mild oxidising conditions by HPLC–MS. The separation is repeated after reduction with reagents such as mercaptoethanol and dithiothreitol (DTT) to cleave -S–S- bonds and the products reanalysed as before. Peptides that were disulphide linked disappear from the spectrum and reappear at the appropriate positions for the individual components.

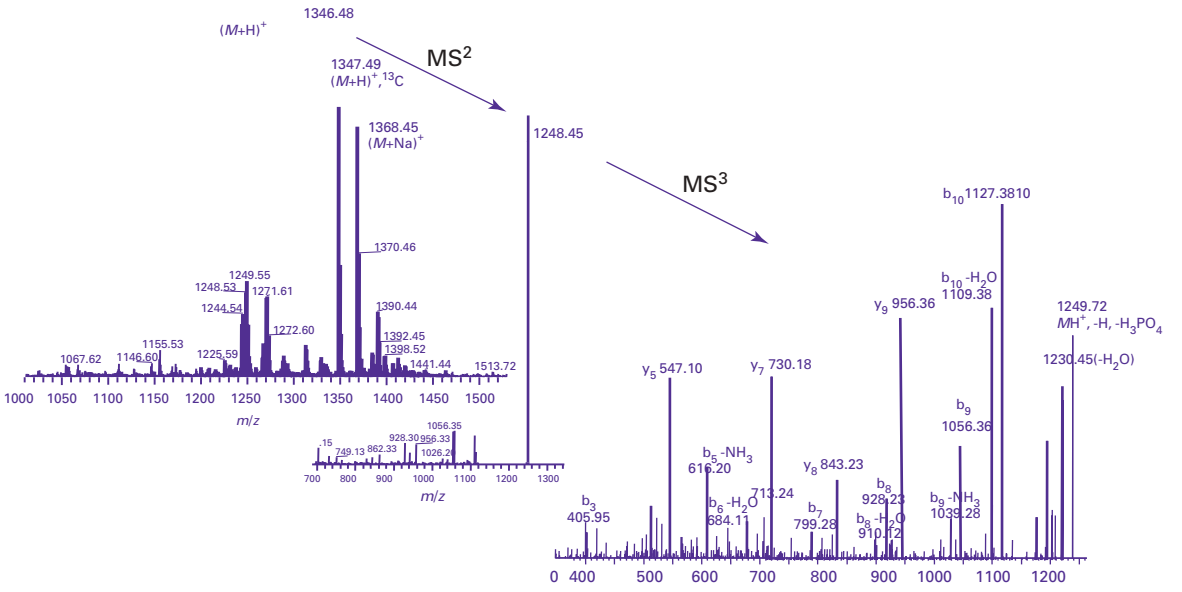


Fig. 9.25. MS identification of phosphopeptides. Sequence is YEILNS^{P}EKAC where S^{P} is phosphoserine. The MS^{2} and MS^{3} spectra are shown. The first tandem MS experiment results mainly in loss of H_3PO_4, 98 Da. Particular problems may also be associated with electrospray mass spectrometry of phosphopeptides, where a high level of Na^+ and K^+ adducts is seen regularly.

9.5.7 Selected ion monitoring

Selected ion monitoring (SIM) is typically used to look for ions that are characteristic of a target compound or family of compounds. This technique has particular application for on-line chromatography–MS, where the instruments can be set up to monitor selected ion masses as the components elute successively from the capillary LC or reverse-phase HPLC column for example (Sections 11.3.3 and 11.9.3). Detection programs or algorithms that are set up to carry out tandem MS on each component as it elutes from a chromatography column can be adapted to enable selective detection of many types of post-translationally modified peptides. This technique can selectively detect low mass fragment ions that are characteristic markers and identify the presence of post-translational modifications such as phosphorylation, glycosylation, sulphation and acylation in any particular peptide. For example, phosphopeptides can be identified by production of phosphate-specific fragment ions of 63 Da (PO_2^-) and 79 Da (PO_3^-) by collision-induced dissociation during negative ion HPLC-ES–MS. Glycopeptides can be identified by characteristic fragment ions including hexose$^+$ (163 Da) and *N*-acetylhexosamine$^+$ (204 Da). Phosphoserine- and phosphothreonine-containing peptides can also be identified by a process known as neutral loss scanning, where these peptides show loss of 98 Da by β-elimination of H_3PO_4 (Fig. 9. 25).

9.6 ANALYSING PROTEIN COMPLEXES

MS is frequently used to identify partner proteins that interact with a particular protein of interest. Interacting proteins can be isolated by a number of methods including immunoprecipitation of tagged proteins from cell transfection, affinity chromatography and surface plasmon resonance. Surface plasmon resonance (SPR; Section 16.3.2) technology has widespread application for biomolecular interaction analysis and, during characterization of protein–ligand and protein–protein interactions, direct analysis by MALDI–TOF MS of samples bound to the Biacore chips is now possible (where interaction kinetic data are also obtained; Section 16.3.2). Direct analysis of protein complexes by MS is also possible. As well as accurate molecular mass of large biopolymers such as proteins of mass greater than 400 kDa, intact virus particles of 40×10^6 Da (40 MDa) have been analysed using ESI–TOF. An icosahedral virus consisting of a single-stranded RNA surrounded by a homogeneous protein shell with a total mass of 6.5×10^6 Da and a rod-shaped RNA virus with a total mass of 40.5×10^6 Da were studied on this ESI–TOF hybrid mass spectrometer.

9.6.1 Sample preparation and handling

Mass analysis by ES–MS and MALDI–TOF is affected, seriously in some cases, by the presence of particular salts, buffers and detergents. Keratin contamination from flakes of skin and hair can be a major problem particularly when gels and slices are involved; therefore gloves and laboratory coats must be worn. Work on a clean surface in a hood with air filter if possible and use a dedicated box of clean polypropylene microcentrifuge tubes tested to confirm that it does not leach out polymers, mould-release agents, plasticisers, etc. Sample clean-up to remove or reduce levels of buffer salts, EDTA, DMSO, non-ionic and ionic detergents (e.g. SDS), etc. can be achieved by dilution, washing, drop dialysis, and ion-exchange resins. If one is analysing samples by MALDI–TOF, on-plate washing can remove buffers and salts. Sample clean-up can also be achieved by pipette tip chromatography (Section 11.2.5). This consists of a miniature C_{18} reverse-phase chromatography column, packed in a 10 nm^3 pipette tip. The sample, in low or zero organic solvent-containing buffer, is loaded into the tip with a few up and down movements of the pipette piston to ensure complete binding of the sample. Since most contaminants described above will not bind, the sample is trapped on the reverse-phase material and eluted with a solvent containing high organic solvent (typically 50–75% acetonitrile). This is particularly applicable for clean-up of samples after in-gel digestion of protein bands separated on SDS–PAGE. Coomassie Brilliant Blue dye is also removed by this procedure. The technique can be used to concentrate samples and fractionate a mixture. Purification can also be carried out to specifically bind one particular component in a mixture. Immobilised metal ion affinity columns are used to enrich phosphopeptides.

9.6.2 Quantitative analysis of complex protein mixtures by mass spectrometry

Proteome analysis (described in Section 8.5) involves the following basic steps:

- run a gel (one-dimensional (1-D) or two-dimensional (2-D)),
- stain,
- scan to identify spots of interest,
- excise gel spots,
- extract and digest proteins,
- mass analyse the resulting peptides,
- search database.

The initial separation of proteins currently relies on gel electrophoresis, which has a number of limitations including the difficulty in analysing all the proteins expressed owing to huge differences in expression levels. Although thousands of proteins can be reproducibly separated on one 2-D gel from approximately 1 mg of tissue/biopsy or biological fluid, the dynamic range of protein expression can be as high as nine orders of magnitude. One development that has helped to overcome some of the problems is the isotope-coded affinity tag (ICAT) strategy for quantifying differential protein expression. The heavy and light forms of the sulphydryl (thiol-)-specific ICAT reagent (whose structure is illustrated in Fig. 9.26) are used to derivatise proteins in respective samples isolated from cells or tissues in different states. The two samples are combined and proteolysed, normally with trypsin, for reasons explained above. The labelled peptides are purified by affinity chromatography utilising the biotin group on the ICAT reagent then analysed by MS on either LC–MS–MS (including ion trap) or MALDI–TOF instruments. The relative intensities of the ion from the two isotopically tagged forms of each specific peptide indicate their relative abundance. These pairs of peptides are easily detected because they coelute from reverse-phase microcapillary liquid chromatography (RP-μLC) and contain eight mass units of difference owing to the two forms of the ICAT tag. An initial MS scan identifies the peptides from proteins that show differential expression by measuring relative signal intensities of each ICAT-labelled peptide pair. Peptides of interest are then selected for sequencing by tandem MS and the particular protein from which a peptide originated can be identified by database searching the tandem MS spectral data.

Sequence tag methodology can permit the identification of a protein with only one peptide from that particular protein. This is especially useful if the protein is in a mixture. The data that are used in the search comprise the mass (or m/z) of the intact peptide and a small number of fragment ion masses. An example is illustrated in Fig. 9.27.

9.7 COMPUTING AND DATABASE ANALYSIS

9.7.1 Organic compound databases

MS organic compound databases are available to identify the compound(s) in the analyte. The spectra in the databases are obtained by electron impact ionisation.

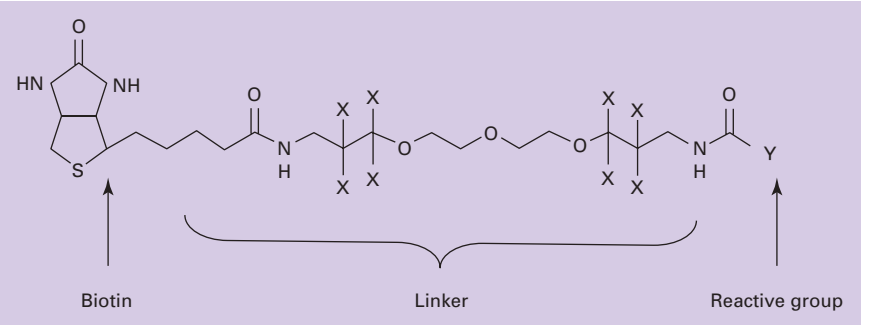


Fig. 9.26. Structure of the ICAT reagent. The ICAT reagent is in two forms, heavy (8 deuterium atoms) and light (no deuterium). The reagent has three elements: an affinity tag (biotin), to isolate ICAT-labelled peptides: a linker in two forms that has stable isotopes incorporated and a reactive group (Y) with specificity towards thiol groups (or other functional groups in proteins (e.g. SH, NH_2, COOH) (cysteines). The heavy reagent is D8-ICAT (where X is deuterium) and light reagent is D8-ICAT (where X is hydrogen). Two protein mixtures representing two different cell states are treated with the isotopically light and heavy ICAT reagents; an ICAT reagent is covalently attached to each cysteine residue in every protein. The protein mixtures are combined, proteolysed and ICAT-labelled peptides are isolated on an avidin column utilising the biotin tag. Peptides are separated by microbore HPLC. Since each pair of ICAT-labelled peptides is chemically identical they are easily visualised because they coelute, with an 8 Da mass difference. The ratios of the original amounts of proteins from the two cell states are strictly maintained in the peptide fragments. The relative quantification is determined by the ratio of the peptide pairs. The protein is identified by database searching with the sequence information from tandem MS analysis by selecting peptides that show differential expression between samples.

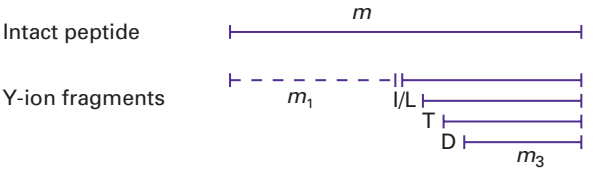


Fig. 9.27. Example of protein identification by sequence tag. In this example, the search data are based on the molecular mass of the intact peptide (m) and five fragmention masses. This defines the sequence tag of three residues positioned with respect to the N and C termini between masses m_1 and m_3.

Two such databases are:

- Integrated Spectral Data Base System for Organic Compounds (SDBS) from the National Institute of Advanced Industrial Science and Technology NIMC Spectral Database System. Data on specific compounds can be searched with compound name, molecular formula, number of atoms (CHNO) and molecular mass. The database contains 19 000 electron impact mass spectra and also includes 47 000 Fourier transform infrared (FT-IR) spectra; 11 500 ^{1}H-NMR spectra and 10 200 ^{13}C-NMR spectra. The URL is <http://www.aist.go.jp/RIODB/SDBS/menu-e.html>.

- NIST Chemistry WebBook. NIST is the National Institute for Standards and Technology. Data on specific compounds in the Chemistry WebBook can be searched by name, chemical formula, Chemical Abstracts Service registry number, molecular mass or selected ion energetics and spectral properties. This site comprises electron impact mass spectra for over 15 000 compounds as well as thermochemical data for over 7000 organic and small inorganic compounds and IR spectra for over 16 000 compounds. The URL is <http://webbook.nist.gov./chemistry/>.

9.7.2 Identification of proteins

Database searches to identify a particular protein that has been analysed by MS is particularly important. This section gives an overview of websites for proteomic identification.

The identification of proteins can be carried out by using many websites, for example 'Mascot' from Matrix Science <http://www.matrixscience.com/cgi/index.pl?page=/search_form_select.html> and 'Protein prospector' <http://128.40.158.151/mshome3.4.htm>. The search can be limited by searching particular species or genera e.g. 'mammalia' only, thus increasing the speed. However, when looking for a homologous sequence the species should not be defined. The modification of cysteine residues, if any, should be included otherwise the number of peptides matched to the theoretical list will be decreased, producing a worse hit. If no cysteine modification has been carried out, and if the protein originates from a gel sample, then much of this residue will have been converted to acrylamide-modified cysteine. Unmatched masses should be re-searched, since sometimes two or more proteins run together on electrophoresis. Note the delta p.p.m. (the difference between the theoretical and the experimental mass of a particular peptide), which should be low and consistent. This gives an indication of whether the result is genuine. If an internal calibration has been performed the mass accuracy parameter can be set to 20 p.p.m. For a close external calibration this should be set to 50 p.p.m. If a hit is not found with the first search, this parameter can be increased.

Different databases can be searched. NCBInr is the largest database whilst Swiss Prot is smaller. However Swiss Prot provides the most information with the protein hits. If it is known that the protein is not larger than, for example 100 kDa then the mass range should be limited to prevent false hits. Although the search will be refined by limiting to a particular mass range of the intact protein, the possibility of subunits or fragments must be considered. Some information on the isoelectric point of a protein will also be known for a 2-D gel sample but this should also be treated with caution. If a number of larger size peptides are seen in the digest then the missed cleavages parameter should be increased. Typically this is set to 1 or 2. If the possibility of post-translational or other modification is uncertain, then the top three options should be selected, i.e. acetylation of the N terminus, oxidation of Met, and conversion of Glu to pyro-Glu. If phosphorylation of S, T or Y is selected when not suspected this may lead to false hits. More than one

amino acid can usually be listed in the box (e.g. 'STY 80' to select any phosphorylation). The list of peptide masses should be input to four decimal places if possible. In the initial search, use masses from the higher signal intensity peaks and set the minimum number of peptides low as compared with the number of masses in the peptide list. To increase the specificity of the search this number can be increased. If no hits are found then this number can be decreased in subsequent passes. Be sure to select whether the fragment and precursor ions have been calculated from monoisotopic or average masses.

De-isotoping software is available to artificially remove the ^{13}C peaks arising from the presence of the ^{13}C isotopic form of carbon in otherwise chemically identical peptides. This simplifies the spectrum but more importantly this will ensure that the search algorithm will not be not confused and will attempt to find two or more distinct peptides that each differ by 1 Da. This is particularly valuable when one is analysing peptide mixtures, since overlapping isotope clusters are thus identified correctly and only the genuine ^{12}C peaks are reported. If the resolution of the mass spectrum is not sufficient to resolve individual isotope peaks then the average mass is often reported. This is still the case with larger polypeptides and proteins (see Fig. 9.14) but, in modern instruments, the all ^{12}C, one ^{13}C, two ^{13}C, etc. peptide forms can be resolved (see Fig. 9.13).

Various software packages (including commercial software packages such as *SEQUEST*) are available to use the information on the fragment ions obtained from a tandem MS experiment to search protein (and DNA translation) databases to identify the sequence and the protein from which it is derived. Once the protein has been identified, one can view the full protein summary and link to protein structure, Swiss 2-D PAGE, nucleic acid databases, etc.

9.8 SUGGESTIONS FOR FURTHER READING

AEBERSOLD, R. and MANN, M. (2003). Mass spectrometry-based proteomics. *Nature*, **422**, 198–207. (There are also a number of other, very informative proteomics reviews in this issue between pp. 193 and 225.)

HERNANDEZ, H. and ROBINSON, C. V. (2001). Dynamic protein complexes: insights from mass spectrometry. *Journal of Biological Chemistry*, **276**, 46685–46688.

LARSEN, M. R., SØRENSEN, G. L., FEY, S. J., LARSEN, P. M. and PROEPSTORFF, P. (2001). Phospho-proteomics: evaluation of the use of enzymatic de-phosphorylation and differential mass spectrometric peptide mass mapping for site specific phosphorylation assignment in proteins separated by gel electrophoresis. *Proteomics*, **1**, 223–238. (The paper reviews methods for identification of this important and widespread post-translational modification.)

MOSELEY, M. A. (2001). Current trends in differential expression proteomics: isotopically coded tags. *Trends in Biotechnology*, **19** (Suppl.), S10–S16. (A review of developments in isotopically coded tags, an innovation that allows quantitative analysis of differences in expression levels of protein between cells and tissues in different states, e.g. between diseased and normal.)

NELSON, R. W., NEDELKOV, D. and TABBS, K. A. (2000). Biosensor chip mass spectrometry: a chip-based proteomics approach. *Electrophoresis*, **21**, 1155–1163. (This is a review of biomolecular interaction analysis–mass spectrometry for the detailed characterization of proteins and protein–protein interactions and the development of biosensor chip mass spectrometry as a new chip-based proteomics approach.)

SIMPSON, R. J. (2003). *Proteins and Proteomics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. (This volume includes extensive coverage of protein mass spectrometry techniques).

Websites

The ExPASy (*Ex*pert *P*rotein *A*nalysis *Sy*stem) server of the Swiss Institute of Bioinformatics (SIB) contains a large suite of programs for the analysis of protein sequences, structures and proteomics as well as 2-D PAGE analysis (2-D gel documentation and 2-D gel image analysis programs). The ExPASy suite of programmes is at <http://www.expasy.ch/> and <http://us.expasy.org/tools/>. Glyocomod and GlycanMass are found at <http://us.expasy.org/tools/glycomod/> and <http://us.expasy.org/tools/glycomod/glycanmass.html>, respectively.

Also, Deltamass is a database of protein post-translational modifications at <http://www.abrf.org/index.cfm/dm.home>, which can be accessed to determine whether post-translational modifications are present. There are hyperlinks to references to the modifications.

There is also a prediction program 'findmod' for finding potential protein post-translational modifications in the ExPASy suite at <http://expasy.org/tools/findmod/>.

Information and protocols for sample clean-up are found at the URLs <www.millipore.com/ziptip> and <http://www.nestgrp.com/protocols/protocols.shtml#massspec>'Stylus™ Pipette Tips for Protein Sample Preparation'.

Products for Phosphorylated Peptide and Protein Enrichment and Detection IMAC columns and chromatography may be found at <http:// www.piercenet.com/files/phosphor.pdf> and GelCode Phosphoprotein Staining Kit at <http:// www. piercenet.com/>.

Chapter 10

Electrophoretic techniques

10.1 GENERAL PRINCIPLES

The term electrophoresis describes the migration of a charged particle under the influence of an electric field. Many important biological molecules, such as amino acids, peptides, proteins, nucleotides and nucleic acids, possess ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (−). Under the influence of an electric field these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge.

The equipment required for electrophoresis consists basically of two items, a power pack and an electrophoresis unit. Electrophoresis units are available for running either vertical or horizontal gel systems. Vertical slab gel units are commercially available and routinely used to separate proteins in acrylamide gels (Section 10.2). The gel is formed between two glass plates that are clamped together but held apart by plastic spacers. The most commonly used units are the so-called minigel apparatus (Fig. 10.1). Gel dimensions are typically 8.5 cm wide × 5 cm high, with a thickness of 0.5–1 mm. A plastic comb is placed in the gel solution and is removed after polymerisation to provide loading wells for up to 10 samples. When the apparatus is assembled, the lower electrophoresis tank buffer surrounds the gel plates and affords some cooling of the gel plates. A typical horizontal gel system is shown in Fig. 10.2. The gel is cast on a glass or plastic sheet and placed on a cooling plate (an insulated surface through which cooling water is passed to conduct away generated heat). Connection between the gel and electrode buffer is made using a thick wad of wetted filter paper (Fig. 10.2: note, however, that agarose gels for DNA electrophoresis are run submerged in the buffer (Section 10.4.1). The power pack supplies a direct current between the electrodes in the electrophoresis unit. All electrophoresis is carried out in an appropriate buffer, which is essential to maintain a constant state of ionisation of the molecules being separated. Any variation in pH would alter the overall charge and hence the mobilities (rate of migration in the applied field) of the molecules being separated.

In order to understand fully how charged species separate it is necessary to look at some simple equations relating to electrophoresis. When a potential difference (voltage) is applied across the electrodes, it generates a potential

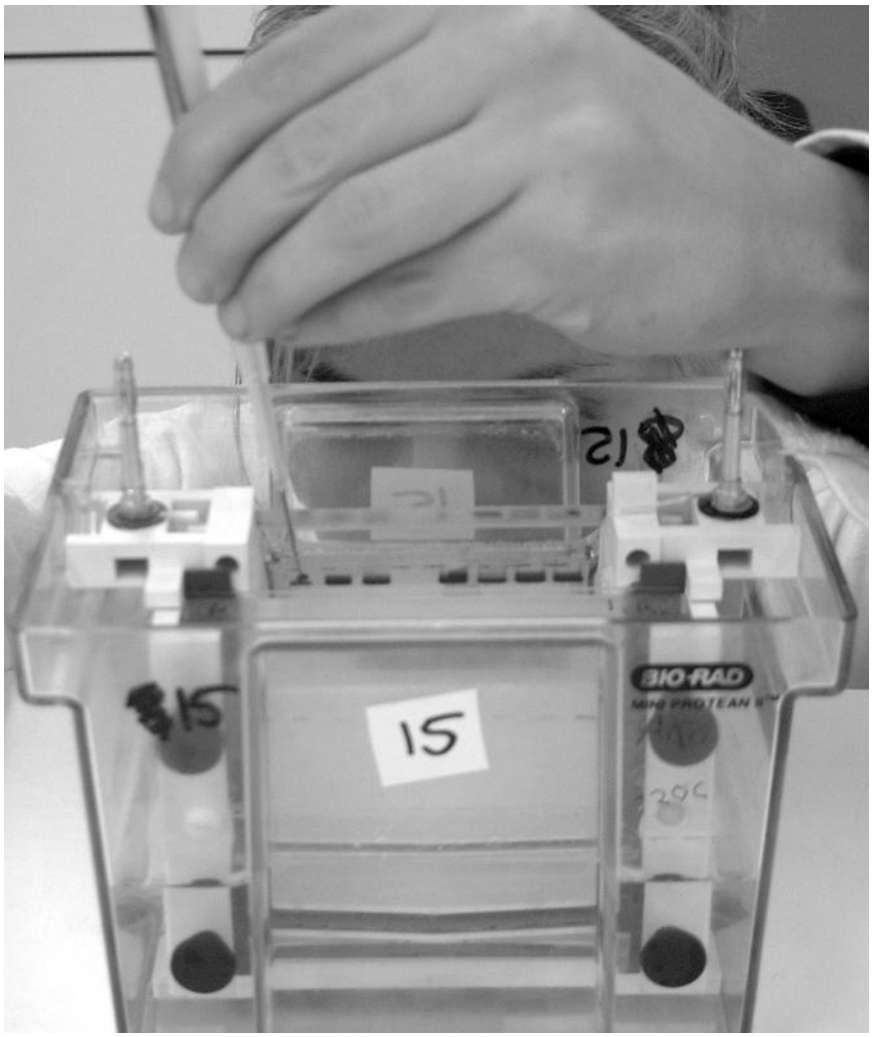


Fig. 10.1. Photograph showing samples being loaded into the wells of an SDS–PAGE minigel. Six wells that have been loaded can be identified by the blue dye (bromophenol blue) that is incorporated into the loading buffer.

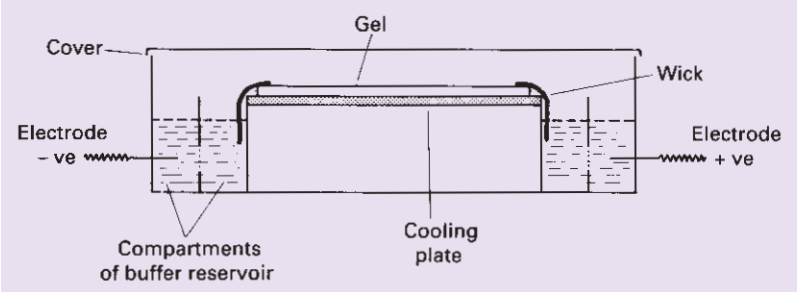


Fig. 10.2. A typical horizontal apparatus, such as that used for immunoelectrophoresis, isoelectric focusing and the electrophoresis of DNA and RNA in agarose gels.

gradient, E, which is the applied voltage, V, divided by the distance, d, between the electrodes. When this potential gradient E is applied, the force on a molecule bearing a charge of q coulombs is Eq newtons. It is this force that drives a charged molecule towards an electrode. However, there is also a frictional resistance that retards the movement of this charged molecule. This frictional force is a measure of the hydrodynamic size of the molecule, the shape of the molecule, the pore size of the medium in which electrophoresis is taking place and the viscosity of the buffer. The velocity, v, of a charged molecule in an electric field is therefore given by the equation:

$$v = \frac{Eq}{f} \tag{10.1}$$

where f is the frictional coefficient.

More commonly the term electrophoretic mobility (μ), of an ion is used, which is the ratio of the velocity of the ion to field strength (v/E). When a potential difference is applied, therefore, molecules with different overall charges will begin to separate owing to their different electrophoretic mobilities. Even molecules with similar charges will begin to separate if they have different molecular sizes, since they will experience different frictional forces. As will be seen below, some forms of electrophoresis rely almost totally on the different charges on molecules to effect separation, whilst other methods exploit differences in molecular size and therefore encourage frictional effects to bring about separation.

Provided the electric field is removed before the molecules in the sample reach the electrodes, the components will have been separated according to their electrophoretic mobility. Electrophoresis is thus an incomplete form of electrolysis. The separated samples are then located by staining with an appropriate dye or by autoradiography (Section 14.2.3) if the sample is radiolabelled.

The current in the solution between the electrodes is conducted mainly by the buffer ions, a small proportion being conducted by the sample ions. Ohm's law expresses the relationship between current (I), voltage (V) and resistance (R):

$$\frac{V}{I} = R \tag{10.2}$$

It therefore appears that it is possible to accelerate an electrophoretic separation by increasing the applied voltage, which would result in a corresponding increase in the current flowing. The distance migrated by the ions will be proportional to both current and time. However, this would ignore one of the major problems for most forms of electrophoresis, namely the generation of heat.

During electrophoresis the power (W, watts) generated in the supporting medium is given by

$$W = I^2R \tag{10.3}$$

Most of this power generated is dissipated as heat. Heating of the electrophoretic medium has the following effects:

- An increased rate of diffusion of sample and buffer ions leading to broadening of the separated samples.
- The formation of convection currents, which leads to mixing of separated samples.
- Thermal instability of samples that are rather sensitive to heat. This may include denaturation of proteins (e.g. thus the loss of enzyme activity).
- A decrease of buffer viscosity, and hence a reduction in the resistance of the medium.

If a constant voltage is applied, the current increases during electrophoresis owing to the decrease in resistance (see Ohm's law, equation 10.2) and the rise in current increases the heat output still further. For this reason, workers often use a stabilised power supply, which provides constant power and thus eliminates fluctuations in heating.

Constant heat generation is, however, a problem. The answer might appear to be to run the electrophoresis at very low power (low current) to overcome any heating problem, but this can lead to poor separations as a result of the increased amount of diffusion resulting from long separation times. Compromise conditions, therefore, have to be found with reasonable power settings, to give acceptable separation times, and an appropriate cooling system, to remove liberated heat. While such systems work fairly well, the effects of heating are not always totally eliminated. For example, for electrophoresis carried out in cylindrical tubes or in slab gels, although heat is generated uniformly through the medium, heat is removed only from the edges, resulting in a temperature gradient within the gel, the temperature at the centre of the gel being higher than that at the edges. Since the warmer fluid at the centre is less viscous, electrophoretic mobilities are therefore greater in the central region (electrophoretic mobilities increase by about 2% for each 1 °C rise in temperature), and electrophoretic zones develop a bowed shape, with the zone centre migrating faster than the edges.

A final factor that can effect electrophoretic separation is the phenomenon of electroendosmosis (also known as electroosmotic flow), which is due to the presence of charged groups on the surface of the support of the support medium. For example, paper has some carboxyl groups present, agarose (depending on the purity grade) contains sulphate groups and the surface of glass walls used in capillary electrophoresis (Section 10.5) contains silanol (Si—OH) groups. Figure 10.3 demonstrates how electroendosmosis occurs in a capillary tube, although the principle is the same for any support medium that has charged groups on it. In a fused-silica capillary tube, above a pH value of about 3, silanol groups on the silica capillary wall will ionise, generating negatively charged sites. It is these charges that generate electroendosmosis. The ionised silanol groups create an electrical double layer, or region of charge separation, at the capillary wall/electrolyte interface. When a voltage is applied, cations in the electrolyte near the capillary wall migrate towards the cathode, pulling electrolyte solution with them. This creates a net electroosmotic flow towards the cathode.

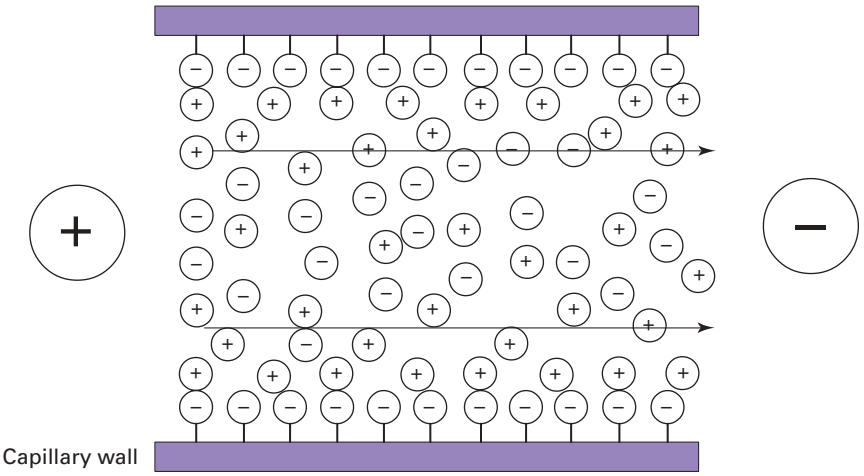


Fig. 10.3. Electroosmotic flow through a glass capillary. Electrolyte cations are attracted to the capillary walls, forming an electrical double layer. When a voltage is applied, the net movement of electrolyte solution towards the cathode is known as electro-endosmotic flow.

10.2 SUPPORT MEDIA

The pioneering work on electrophoresis by A. Tiselius and co-workers was performed in free solution. However, it was soon realised that many of the problems associated with this approach, particularly the adverse effects of diffusion and convection currents, could be minimised by stabilising the medium. This was achieved by carrying out electrophoresis on a porous mechanical support, which was wetted in electrophoresis buffer and in which electrophoresis of buffer ions and samples could occur. The support medium cuts down convection currents and diffusion so that the separated components remain as sharp zones. The earliest supports used were filter paper or cellulose acetate strips, wetted in electrophoresis buffer. Nowadays these media are infrequently used, although cellulose acetate still has its uses (see Section 10.3.6). In particular, for many years small molecules such as amino acids, peptides and carbohydrates were routinely separated and analysed by electrophoresis on supports such as paper or thin-layer plates of cellulose, silica or alumina. Although occasionally still used nowadays, such molecules are now more likely to be analysed by more modern and sensitive techniques such as high performance liquid chromatography (Section 11.3.2). While paper or thin-layer supports are fine for resolving small molecules, the separation of macromolecules such as proteins and nucleic acids on such supports is poor.

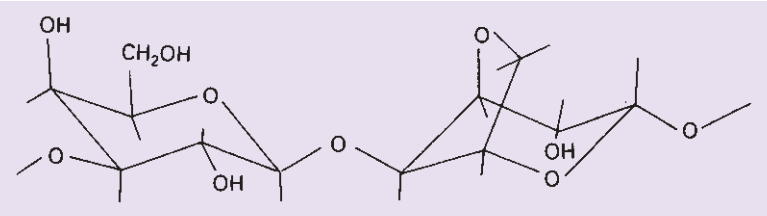


Fig. 10.4. Agarobiose, the repeating unit of agarose.

However, the introduction of the use of gels as a support medium led to a rapid improvement in methods for analysing macromolecules. The earliest gel system to be used was the starch gel and, although this still has some uses, the vast majority of electrophoretic techniques used nowadays involve either agarose gels or polyacrylamide gels.

10.2.1 Agarose gels

Agarose is a linear polysaccharide (average relative molecular mass about 12 000) made up of the basic repeat unit agarobiose, which comprises alternating units of galactose and 3,6-anhydrogalactose (Fig. 10.4). Agarose is one of the components of agar that is a mixture of polysaccharides isolated from certain seaweeds. Agarose is usually used at concentrations of between 1% and 3%. Agarose gels are formed by suspending dry agarose in aqueous buffer, then boiling the mixture until a clear solution forms. This is poured and allowed to cool to room temperature to form a rigid gel. The gelling properties are attributed to both inter- and intramolecular hydrogen bonding within and between the long agarose chains. This cross-linked structure gives the gel good anticonvectional properties. The pore size in the gel is controlled by the initial concentration of agarose; large pore sizes are formed from low concentrations and smaller pore sizes are formed from the higher concentrations. Although essentially free from charge, substitution of the alternating sugar residues with carboxyl, methyoxyl, pyruvate and especially sulphate groups occurs to varying degrees. This substitution can result in electroendosmosis during electrophoresis and ionic interactions between the gel and sample in all uses, both unwanted effects. Agarose is therefore sold in different purity grades, based on the sulphate concentration – the lower the sulphate content, the higher the purity.

Agarose gels are used for the electrophoresis of both proteins and nucleic acids. For proteins, the pore sizes of a 1% agarose gel are large relative to the sizes of proteins. Agarose gels are therefore used in techniques such as immunoelectrophoresis (Section 7.4.1) or flat-bed isoelectric focusing (Section 10.3.4), where the proteins are required to move unhindered in the gel matrix according to their native charge. Such large pore gels are also used to separate much larger molecules such as DNA or RNA, because the pore sizes in the gel are still large enough for DNA or RNA molecules to pass through the gel. Now, however, the pore size and

molecule size are more comparable and frictional effects begin to play a role in the separation of these molecules (Section 10.4). A further advantage of using agarose is the availability of low melting temperature agarose (62–65 °C). As the name suggests, these gels can be reliquified by heating to 65 °C and thus, for example, DNA samples separated in a gel can be cut out of the gel, returned to solution and recovered.

Owing to the poor elasticity of agarose gels and the consequent problems of removing them from small tubes, the gel rod system sometimes used for acrylamide gels is not used. Horizontal slab gels are invariably used for isoelectric focusing or immunoelectrophoresis in agarose. Horizontal gels are also used routinely for DNA and RNA gels (Section 10.4), although vertical systems have been used by some workers.

10.2.2 Polyacrylamide gels

Electrophoresis in acrylamide gels is frequently referred to as PAGE, being an abbreviation for *p*oly*a*crylamide *g*el *e*lectrophoresis.

Cross-linked polyacrylamide gels are formed from the polymerisation of acrylamide monomer in the presence of smaller amounts of *N*,*N*′-methylenebisacrylamide (normally referred to as 'bis'-acrylamide) (Fig. 10.5). Note that bisacrylamide is essentially two acrylamide molecules linked by a methylene group, and is used as a cross-linking agent. Acrylamide monomer is polymerised in a head-to-tail fashion into long chains and occasionally a bis-acrylamide molecule is built into the growing chain, thus introducing a second site for chain extension. Proceeding in this way a cross-linked matrix of fairly well-defined structure is formed (Fig. 10.5). The polymerisation of acrylamide is an example of free-radical catalysis, and is initiated by the addition of ammonium persulphate and the base *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED). TEMED catalyses the decomposition of the persulphate ion to give a free radical (i.e. a molecule with an unpaired electron):

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^{-\bullet}$$

If this free radical is represented as R$^\bullet$ (where the dot represents an unpaired electron) and M as an acrylamide monomer molecule, then the polymerisation can be represented as follows:

$$R^\bullet + M \rightarrow RM^\bullet$$

$$RM^\bullet + M \rightarrow RMM^\bullet$$

$$RMM^\bullet + M \rightarrow RMMM^\bullet \text{ etc.}$$

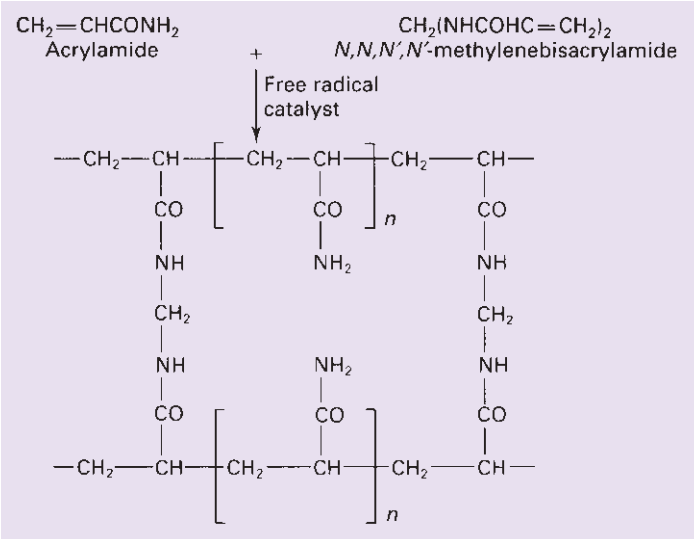


Fig. 10.5. The formation of a polyacrylamide gel from acrylamide and bis-acrylamide.

Free radicals are highly reactive species due to the presence of an unpaired electron that needs to be paired with another electron to stabilise the molecule. R• therefore reacts with M, forming a single bond by sharing its unpaired electron with one from the outer shell of the monomer molecule. This therefore produces a new free radical molecule $R-M^{\bullet}$, which is equally reactive and will attack a further monomer molecule. In this way long chains of acrylamide are built up, being cross-linked by the introduction of the occasional bis-acrylamide molecule into the growing chain. Oxygen mops up free radicals and therefore all gel solutions are normally degassed (the solutions are briefly placed under vacuum to remove loosely dissolved air) prior to use. The degassing of the gel solution also serves a second purpose. The polymerisation of acrylamide is an exothermic reaction (i.e. heat is liberated) and the warming up of the gel solution as it sets can liberate air bubbles that become trapped in the polymerised gel. The degassing step prevents this possibility.

Photopolymerisation is an alternative method that can be used to polymerise acrylamide gels. The ammonium persulphate and TEMED are replaced by riboflavin and when the gel is poured it is placed in front of a bright light for 2–3 h. Photodecomposition of riboflavin generates a free radical that initiates polymerisation.

Acrylamide gels are defined in terms of the total percentage of acrylamide present, and the pore size in the gel can be varied by changing the concentrations of both the acrylamide and bis-acrylamide. Acrylamide gels can be made with a content of between 3% and 30% acrylamide. Thus low percentage gels (e.g. 4%) have large pore sizes and are used, for example, in the electrophoresis of proteins, where free movement of the proteins by electrophoresis is required without any noticeable frictional effect, for example in flat-bed isoelectric focusing (Section 10.3.4) or the stacking gel system of an SDS–polyacrylamide gel (Section 10.3.1).

Low percentage acrylamide gels are also used to separate DNA (Section 10.4). Gels of between 10% and 20% acrylamide are used in techniques such as SDS–gel electrophoresis, where the smaller pore size now introduces a sieving effect that contributes to the separation of proteins according to their size (Section 10.3.1).

Proteins were originally separated on polyacrylamide gels that were polymerised in glass tubes, approximately 7 mm in diameter and about 10 cm in length. The tubes were easy to load and run, with minimum apparatus requirements. However, only one sample could be run per tube and, because conditions of separation could vary from tube to tube, comparison between different samples was not always accurate. The later introduction of vertical gel slabs allowed running of up to 20 samples under identical conditions in a single run. Vertical slabs are now used routinely both for the analysis of proteins (Section 10.3) and for the separation of DNA fragments during DNA sequence analysis (Section 10.4). Although some workers prepare their own acrylamide gels, others purchase commercially available ready-made gels for techniques such as SDS–PAGE, native gels and isoelectric focusing (IEF) (see below).

10.3 ELECTROPHORESIS OF PROTEINS

10.3.1 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) is the most widely used method for analysing protein mixtures qualitatively. It is particularly useful for monitoring protein purification and, because the method is based on the separation of proteins according to size, it can also be used to determine the relative molecular mass of proteins. SDS ($CH_3-(CH_2)_{10}-CH_2OSO_3^-Na^+$) is an anionic detergent. Samples to be run on SDS–PAGE are firstly boiled for 5 min in sample buffer containing β-mercaptoethanol and SDS. The mercaptoethanol reduces any disulphide bridges present that are holding together the protein tertiary structure, and the SDS binds strongly to, and denatures, the protein. Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain. On average, one SDS molecule binds for every two amino acid residues. The original native charge on the molecule is therefore completely swamped by the negatively charged SDS molecules. The rod-like structure remains, as any rotation that tends to fold up the protein chain would result in repulsion between negative charges on different parts of the protein chain, returning the conformation back to the rod shape. The sample buffer also contains an ionisable tracking dye, usually bromophenol blue, that allows the electrophoretic run to be monitored, and sucrose or glycerol, which gives the sample solution density thus allowing the sample to settle easily through the electrophoresis buffer to the bottom when injected into the loading well (see Fig. 10.1). Once the samples are all loaded, a current is passed through the gel. The samples to be separated are not in fact loaded directly into the main separating gel. When the main separating gel (normally about 5 cm long) has been poured between the glass plates and allowed to set, a shorter (approximately 0.8 cm) stacking gel is poured

on top of the separating gel and it is into this gel that the wells are formed and the proteins loaded. The purpose of this stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel. This is achieved by utilising differences in ionic strength and pH between the electrophoresis buffer and the stacking gel buffer and involves a phenomenon known as isotachophoresis. The stacking gel has a very large pore size (4% acrylamide), which allows the proteins to move freely and concentrate, or stack, under the effect of the electric field. The band-sharpening effect relies on the fact that negatively charged glycinate ions (in the electrophoresis buffer) have a lower electrophoretic mobility than do the protein–SDS complexes, which, in turn, have lower mobility than the chloride ions (Cl^-) of the loading buffer and the stacking gel buffer. When the current is switched on, all the ionic species have to migrate at the same speed otherwise there would be a break in the electrical circuit. The glycinate ions can move at the same speed as Cl^- only if they are in a region of higher field strength. Field strength is inversely proportional to conductivity, which is proportional to concentration. The result is that the three species of interest adjust their concentrations so that $[Cl^-] > [\text{protein–SDS}] > [\text{glycinate}]$. There is only a small quantity of protein–SDS complexes, so they concentrate in a very tight band between glycinate and Cl^- boundaries. Once the glycinate reaches the separating gel it becomes more fully ionised in the higher pH environment and its mobility increases. (The pH of the stacking gel is 6.8, that of the separating gel is 8.8.) Thus, the interface between glycinate and Cl^- leaves behind the protein–SDS complexes, which are left to electrophorese at their own rates. The negatively charged protein–SDS complexes now continue to move towards the anode, and, because they have the same charge per unit length, they travel into the separating gel under the applied electric field with the same mobility. However, as they pass through the separating gel the proteins separate, owing to the molecular sieving properties of the gel. Quite simply, the smaller the protein the more easily it can pass through the pores of the gel, whereas large proteins are successively retarded by frictional resistance due to the sieving effect of the gels. Being a small molecule, the bromophenol blue dye is totally unretarded and therefore indicates the electrophoresis front. When the dye reaches the bottom of the gel, the current is turned off, and the gel is removed from between the glass plates and shaken in an appropriate stain solution (usually Coomassie Brilliant Blue, see Section 10.3.7) and then washed in destain solution. The destain solution removes unbound background dye from the gel, leaving stained proteins visible as blue bands on a clear background. A typical minigel would take about 1 h to prepare and set, 40 min to run at 200 V and have a 1 h staining time with Coomassie Brilliant Blue. Upon destaining, strong protein bands would be seen in the gel within 10–20 min, but overnight destaining is needed to completely remove all background stain. Vertical slab gels are invariably run, since this allows up to 10 different samples to be loaded onto a single gel. A typical SDS–polyacrylamide gel is shown in Fig. 10.6.

Typically, the separating gel used is a 15% polyacrylamide gel. This gives a gel of a certain pore size in which proteins of relative molecular mass (M_r) 10 000 move through the gel relatively unhindered, whereas proteins of M_r 100 000 can only

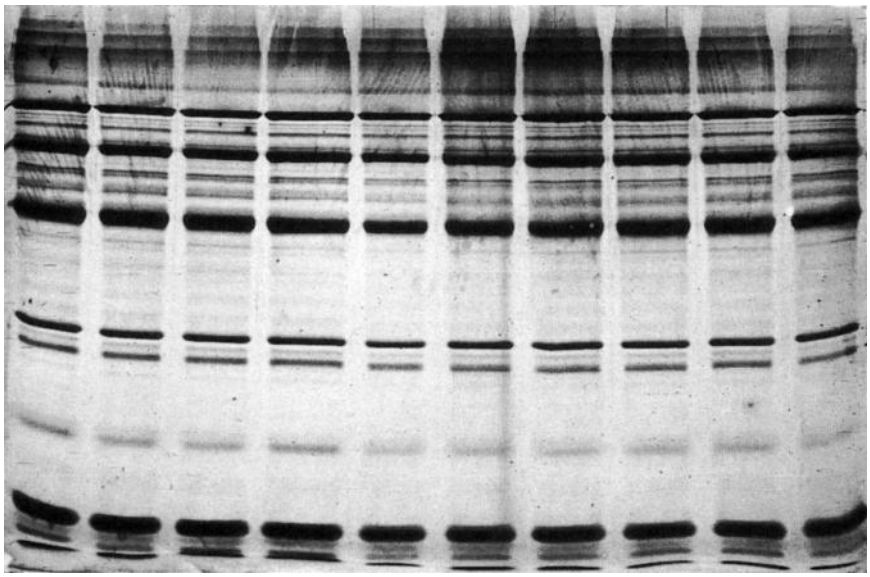


Fig. 10.6. A typical SDS–polyacrylamide gel. All 10 wells in the gel have been loaded with the same complex mixture of proteins. (Courtesy of Bio-Rad Laboratories.)

just enter the pores of this gel. Gels of 15% polyacrylamide are therefore useful for separating proteins in the range M_r 100 000 to 10 000. However, a protein of M_r 150 000, for example, would be unable to enter a 15% gel. In this case a larger-pored gel (e.g. a 10% or even 7.5% gel) would be used so that the protein could now enter the gel and be stained and identified. It is obvious, therefore, that the choice of gel to be used depends on the size of the protein being studied. The fractionation range of different percentage acrylamide gels is shown in Table 10.1. This shows, for example, that in a 10% polyacrylamide gel proteins greater than 200 kDa in mass cannot enter the gel, whereas proteins with relative molecular mass ($M_{r)}$ in the range 200 000 to 15 000 will separate. Proteins of M_r 15 000 or less are too small to experience the sieving effect of the gel matrix, and all run together as a single band at the electrophoresis front.

The M_r of a protein can be determined by comparing its mobility with those of a number of standard proteins of known M_r that are run on the same gel. By plotting a graph of distance moved against log M_r for each of the standard proteins, a calibration curve can be constructed. The distance moved by the protein of unknown M_r is then measured, and then its log M_r and hence M_r can be determined from the calibration curve.

SDS–gel electrophoresis is often used after each step of a purification protocol to assess the purity or otherwise of the sample. A pure protein should give a single band on an SDS–polyacrylamide gel, unless the molecule is made up of two unequal subunits. In the latter case two bands, corresponding to the two subunits, will be seen. Since only submicrogram amounts of protein are needed for the gel, very little material is used in this form of purity assessment and at the same time a

Table 10.1 The relationship between acrylamide gel concentration and protein fractionation range

Acrylamide concentration (%)	Protein fractionation range ($M_r \times 10^{-3}$)
5	60–350
10	15–200
15	10–100

value for the relative molecular mass of the protein can be determined on the same gel run (as described above), with no more material being used.

Example 1 MOLECULAR MASS DETERMINATION BY ELECTROPHORESIS

Question

The following table shows the distance moved in an SDS–polyacrylamide gel by a series of marker proteins of known relative molecular mass (M_r). A newly purified protein (X) run on the same gel showed a single band that had moved a distance of 45 mm. What was the M_r of protein X?

Protein	M_r	Distance moved (mm)
Transferrin	78 000	6.0
Bovine serum albumin	66 000	12.5
Ovalbumin (egg albumin)	45 000	32.0
Glyceraldehyde-3-phosphate dehydrogenase	36 000	38.0
Carbonic anhydrase	29 000	50.0
Trypsinogen	24 000	54.0
Soyabean trypsin inhibitor	20 100	61.0
β-Lactoglobulin	18 400[a]	69.0
Myoglobin	17 800	69.0
Lysozyme	14 300	79.0
Cytochrome *c*	12 400	86.5

[a] Note: β-lactoglobulin has a relative molecular mass of 36 800 but is a dimer of two identical subunits of 18 400 relative molecular mass. Under the reducing conditions of the sample buffer the disulphide bridges linking the subunits are reduced and thus the monomer chains are seen on the gel.

Answer

Construct a calibration graph by plotting log M_r versus distance moved for each of the marker proteins. From a graph of log M_r versus the distance moved by each protein you can determine a relative molecular mass for protein X of approximately 31 000. Note that this method is accurate to ± 10%, so your answer is 31 000 ± 3100.

10.3.2 Native (buffer) gels

While SDS–PAGE is the most frequently used gel system for studying proteins, the method is of no use if one is aiming to detect a particular protein (often an enzyme)

on the basis of its biological activity, because the protein (enzyme) is denatured by the SDS–PAGE procedure. In this case it is necessary to use non-denaturing conditions. In native or buffer gels, polyacrylamide gels are again used (normally a 7.5% gel) but the SDS is absent and the proteins are *not* denatured prior to loading. Since all the proteins in the sample being analysed carry their native charge at the pH of the gel (normally pH 8.7), proteins separate according to their different electrophoretic mobilities *and* the sieving effects of the gel. It is therefore not possible to predict the behaviour of a given protein in a buffer gel but, because of the range of different charges and sizes of proteins in a given protein mixture, good resolution is achieved. The enzyme of interest can be identified by incubating the gel in an appropriate substrate solution such that a coloured product is produced at the site of the enzyme. An alternative method for enzyme detection is to include the substrate in an agarose gel that is poured over the acrylamide gel and allowed to set. Diffusion and interaction of enzyme and substrate between the two gels results in colour formation at the site of the enzyme. Often, duplicate samples will be run on a gel, the gel cut in half and one half stained for activity, the other for total protein. In this way the total protein content of the sample can be analysed and the particular band corresponding to the enzyme identified by reference to the activity stain gel.

10.3.3 Gradient gels

This is again a polyacrylamide gel system, but instead of running a slab gel of uniform pore size throughout (e.g. a 15% gel) a gradient gel is formed, where the acrylamide concentration varies uniformly from, typically, 5% at the top of the gel to 25% acrylamide at the bottom of the gel. The gradient is formed via a gradient mixer (Section 11.3.1) and run down between the glass plates of a slab gel. The higher percentage acrylamide (e.g. 25%) is poured between the glass plates first and a continuous gradient of decreasing acrylamide concentration follows. Therefore at the top of the gel there is a large pore size (5% acrylamide) but as the sample moves down through the gel the acrylamide concentration slowly increases and the pore size correspondingly decreases. Gradient gels are normally run as SDS gels with a stacking gel. There are two advantages to running gradient gels. First, a much greater range of protein M_r values can be separated than on a fixed-percentage gel. In a complex mixture, very low molecular weight proteins travel freely through the gel to begin with, and start to resolve when they reach the smaller pore sizes towards the lower part of the gel. Much larger proteins, on the other hand, can still enter the gel but start to separate immediately due to the sieving effect of the gel. The second advantage of gradient gels is that proteins with very similar M_r values may be resolved, although they cannot otherwise be resolved in fixed percentage gels. As each protein moves through the gel the pore sizes become smaller until the protein reaches its pore size limit. The pore size in the gel is now too small to allow passage of the protein, and the protein sample stacks up at this point as a sharp band. A similar-sized protein, but with slightly lower M_r will be able to travel a little further through the gel before reaching its

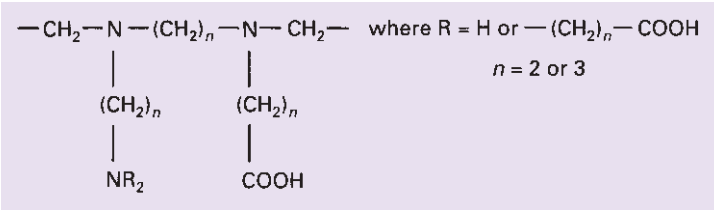


Fig. 10.7. The general formula for ampholytes.

pore size limit, at which point it will form a sharp band. These two proteins, of slightly different M_r values, therefore separate as two, close, sharp bands.

10.3.4 Isoelectric focusing gels

This method is ideal for the separation of amphoteric substances such as proteins because it is based on the separation of molecules according to their different isoelectric points (Section 8.1). The method has high resolution, being able to separate proteins that differ in their isoelectric points by as little as 0.01 of a pH unit. The most widely used system for IEF utilises horizontal gels on glass plates or plastic sheets. Separation is achieved by applying a potential difference across a gel that contains a pH gradient. The pH gradient is formed by the introduction into the gel of compounds known as ampholytes, which are complex mixtures of synthetic polyamino-polycarboxylic acids (Fig. 10.7). Ampholytes can be purchased in different pH ranges covering either a wide band (e.g. pH 3–10) or various narrow bands (e.g. pH 7–8), and a pH range is chosen such that the samples being separated will have their isoelectric points (pI values) within this range. Commercially available ampholytes include Bio-Lyte and Pharmalyte.

Traditionally 1–2 mm thick IEF gels have been used by research workers, but the relatively high cost of ampholytes makes this a fairly expensive procedure if a number of gels are to be run. However, the introduction of thin-layer IEF gels, which are only 0.15 mm thick and which are prepared using a layer of electrical insulation tape as the spacer between the gel plates, has considerably reduced the cost of preparing IEF gels, and such gels are now commonly used. Since this method requires the proteins to move freely according to their charge under the electric field, IEF is carried out in low percentage gels to avoid any sieving effect within the gel. Polyacrylamide gels (4%) are commonly used, but agarose is also used, especially for the study of high M_r proteins that may undergo some sieving even in a low percentage acrylamide gel.

To prepare a thin-layer IEF gel, carrier ampholytes, covering a suitable pH range, and riboflavin are mixed with the acrylamide solution, and the mixture is then poured over a glass plate (typically 25 cm × 10 cm), which contains the spacer. The second glass plate is then placed on top of the first to form the gel cassette, and the gel polymerised by photopolymerisation by placing the gel in front of a bright light. The photodecomposition of the riboflavin generates a free radical, which initiates polymerisation (Section 10.2.2). This takes 2–3 h. Once the gel has set, the

glass plates are prised apart to reveal the gel stuck to one of the glass sheets. Electrode wicks, which are thick (3 mm) strips of wetted filter paper (the anode is phosphoric acid, the cathode sodium hydroxide) are laid along the long length of each side of the gel and a potential difference applied. Under the effect of this potential difference, the ampholytes form a pH gradient between the anode and cathode. The power is then turned off and samples applied by laying on the gel small squares of filter paper soaked in the sample. A voltage is again applied for about 30 min to allow the sample to electrophorese off the paper and into the gel, at which time the paper squares can be removed from the gel. Depending on which point on the pH gradient the sample has been loaded, proteins that are initially at a pH region below their isoelectric point will be positively charged and will initially migrate towards the cathode. As they proceed, however, the surrounding pH will be steadily increasing, and therefore the positive charge on the protein will decrease correspondingly until eventually the protein arrives at a point where the pH is equal to its isoelectric point. The protein will now be in the zwitterion form with no net charge, so further movement will cease. Likewise, substances that are initially at pH regions above their isoelectric points will be negatively charged and will migrate towards the anode until they reach their isoelectric points and become stationary. It can be seen that as the samples will always move towards their isoelectric points it is not critical where on the gel they are applied. To achieve rapid separations (2–3 h) relatively high voltages (up to 2500 V) are used. As considerable heat is produced, gels are run on cooling plates (10 °C) and power packs used to stabilise the power output and thus to minimise thermal fluctuations. Following electrophoresis, the gel must be stained to detect the proteins. However, this cannot be done directly, because the ampholytes will stain too, giving a totally blue gel. The gel is therefore first washed with fixing solution (e.g. 10% (v/v) trichloroacetic acid). This precipitates the proteins in the gel and allows the much smaller ampholytes to be washed out. The gel is stained with Coomassie Brilliant Blue and then destained (Section 10.3.7). A typical IEF gel is shown in Fig. 10.8. The technique is very similar to the technique of chromatofocusing (Section 11.6.3).

The pI of a particular protein may be determined conveniently by running a mixture of proteins of known isoelectric point on the same gel. A number of mixtures of proteins with differing pI values are commercially available, covering the pH range 3.5–10. After staining, the distance of each band from one electrode is measured and a graph of distance for each protein against its pI (effectively the pH at that point) plotted. By means of this calibration line, the pI of an unknown protein can be determined from its position on the gel.

IEF is a highly sensitive analytical technique and is particularly useful for studying microheterogeneity in a protein. For example, a protein may show a single band on an SDS gel, but may show three bands on an IEF gel. This may occur, for example, when a protein exists in mono-, di- and tri-phosphorylated forms. The difference of a couple of phosphate groups has no significant effect on the overall relative molecular mass of the protein, hence a single band on SDS gels, but the small charge difference introduced on each molecule can be detected by IEF.

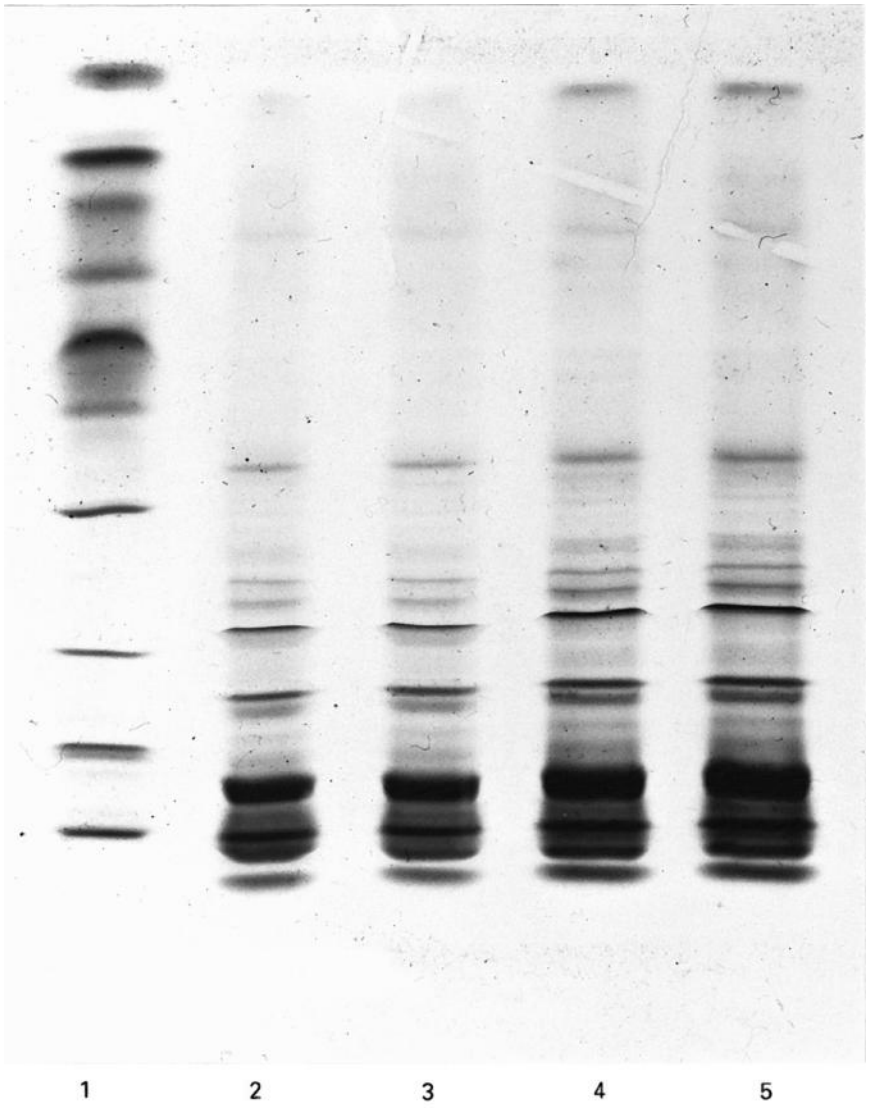


Fig. 10.8. A typical isoelectric focusing gel. Track 1 contains a mixture of standard proteins of known isoelectric points. Tracks 2–5 show increasing loadings of venom from the Japanese water moccasin snake. (Courtesy of Bio-Rad Laboratories Ltd.)

The method is particularly useful for separating isoenzymes (Section 8.2), which are different forms of the same enzyme often differing by only one or two amino acid residues. Since the proteins are in their native form, enzymes can be detected in the gel either by washing the unfixed and unstained gel in an appropriate substrate or by overlayering with agarose containing the substrate. The approach has found particular use in forensic science, where traces of blood or other biological fluids can be analysed and compared according to the composition of certain isoenzymes.

Although IEF is used mainly for analytical separations, it can also be used for preparative purposes. In vertical column IEF, a water-cooled vertical glass column is used, filled with a mixture of ampholytes dissolved in a sucrose solution containing a density gradient to prevent diffusion. When the separation is complete, the current is switched off and the sample components run out through a valve in the base of the column. Alternatively, preparative IEF can be carried out in beds of granulated gel, such as Sephadex G-75 (Section 11.8.2).

10.3.5 Two-dimensional polyacrylamide gel electrophoresis

This technique combines the technique of IEF (first dimension), which separates proteins in a mixture according to charge (pI), with the size separation technique of SDS–PAGE (second dimension). The combination of these two techniques to give two-dimensional (2-D) PAGE provides a highly sophisticated analytical method for analysing protein mixtures. To maximise separation, most workers use large format 2-D gels (20 cm × 20 cm), although the minigel system can be used to provide useful separation in some cases. For large-format gels, the first dimension (isoelectric focusing) is carried out in an acrylamide gel that has been cast on a plastic strip (18 cm × 3 mm wide). The gel contains ampholytes (for forming the pH gradient) together with 8 M urea and a non-ionic detergent, both of which denature and maintain the solubility of the proteins being analysed. The denatured proteins therefore separate in this gel according to their isoelectric points. The IEF strip is then incubated in a sample buffer containing SDS (thus binding SDS to the denatured proteins) and then placed between the glass plates of, and on top of, a previously prepared 10% SDS–PAGE gel. Electrophoresis is commenced and the SDS-bound proteins run into the gel and separate according to size, as described in Section 10.3.1. The IEF gels are provided as dried strips and need rehydrating overnight. The first dimension IEF run then takes 6–8 h, the equilibration step with SDS sample buffer takes about 15 min, and then the SDS–PAGE step takes about 5 h. A typical 2-D gel is shown in Fig. 10.9. Using this method one can routinely resolve between 1000 and 3000 proteins from a cell or tissue extract and in some cases workers have reported the separation of between 5000 and 10 000 proteins. The applications of 2-D PAGE, and a description of the method's central role in proteomics is described in Section 8.5.1.

10.3.6 Cellulose acetate electrophoresis

Although one of the older methods, cellulose acetate electrophoresis still has a number of applications. In particular it has retained a use in the clinical analysis of serum samples. Cellulose acetate has the advantage over paper in that it is a much more homogeneous medium, with uniform pore size, and does not adsorb proteins in the way that paper does. There is therefore much less trailing of protein bands and resolution is better, although nothing like as good as that achieved with polyacrylamide gels. The method is, however, far simpler to set up and run. Single

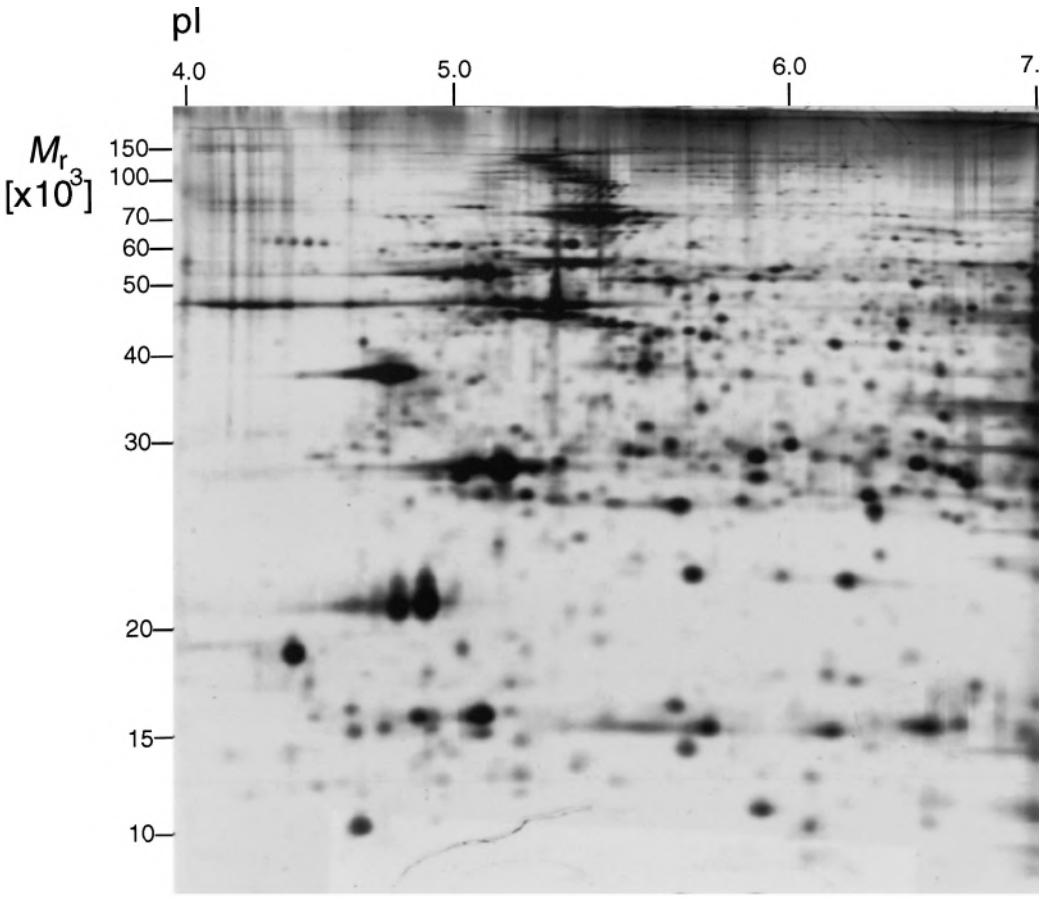


Fig. 10.9. A typical two-dimensional gel. The sample applied was 100 μg of total protein extracted from a normal dog heart ventricle. The first dimension was carried out using a pH 4–7 isoelectric-focusing gel. The second dimension was a 12% SDS–PAGE vertical slab gel. The pattern was visualised by silver staining. (Courtesy of Monique Heinke and Dr Mike Dunn, Division of Cardiothoracic Surgery, Imperial College School of Medicine, Heart Science Centre, Harefield, UK.)

samples are normally run on cellulose acetate strips (2.5 cm × 12 cm), although multiple samples are frequently run on wider sheets. The cellulose acetate is first wetted in electrophoresis buffer (pH 8.6 for serum samples) and the sample (1–2 mm^3) loaded as a 1 cm wide strip about one-third of the way along the strip. The ends of the strip make contact with the electrophoresis buffer tanks via a filter paper wick that overlaps the end of the cellulose acetate strip, and electrophoresis conducted at 6–8 Vcm^{-1} for about 3 h. Following electrophoresis, the strip is stained for protein (see Section 10.3.7), destained, and the bands visualised. A typical serum protein separation shows about six major bands. However, in many disease states, this serum protein profile changes and a clinician can obtain information concerning the disease state of a patient from the altered pattern. Although

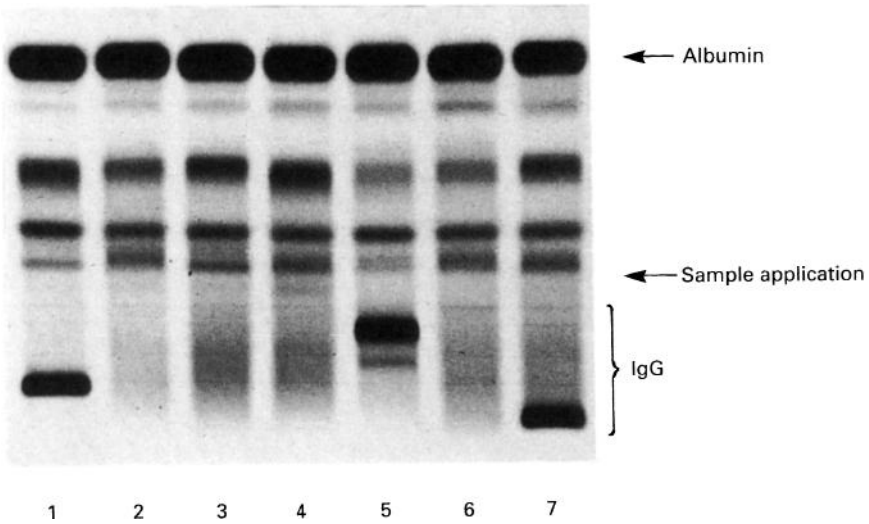


Fig. 10.10. Electrophoresis of human serum samples on an agarose gel. Tracks 2, 3, 4 and 6 show normal serum protein profiles. Tracks 1, 5 and 7 show myeloma patients, who are identified by the excessive production of a particular monoclonal antibody seen in the IgG fraction. (Courtesy of Charles Andrews and Nicholas Cundy, Edgware General Hospital, London.)

still frequently used for serum analysis, electrophoresis on cellulose acetate is being replaced by the use of agarose gels, which give similar but somewhat better resolution. A typical example of the analysis of serum on an agarose gel is shown in Fig. 10.10. Similar patterns are obtained when cellulose acetate is used.

Enzymes can easily be detected, in samples electrophoresed on cellulose acetate, by using the zymogram technique. The cellulose strip is laid on a strip of filter paper soaked in buffer and substrate. After an appropriate incubation period, the strips are peeled apart and the paper zymogram treated accordingly to detect enzyme product; hence, it is possible to identify the position of the enzyme activity on the original strip. An alternative approach to detecting and semiquantifying *any* particular protein on a strip is to treat the strip as the equivalent of a protein blot and to probe for the given protein using primary antibody and then enzyme-linked secondary antibody (Section 10.3.8). Substrate colour development indicates the presence of the particular protein and the amount of colour developed in a given time is a semiquantitative measure of the amount of protein. Thus, for example, large numbers of serum samples can be run on a wide sheet, the sheet probed using antibodies, and elevated levels of a particular protein identified in certain samples by increased levels of colour development in these samples.

10.3.7 Detection, estimation and recovery of proteins in gels

The most commonly used general protein stain for detecting protein on gels is the sulphated trimethylamine dye Coomassie Brilliant Blue R-250 (CBB). Staining is

usually carried out using 0.1% (w/v) CBB in methanol:water:glacial acetic acid (45:45:10, by vol.). This acid–methanol mixture acts as a denaturant to precipitate or fix the protein in the gel, which prevents the protein from being washed out whilst it is being stained. Staining of most gels is accomplished in about 2 h and destaining, usually overnight, is achieved by gentle agitation in the same acid–methanol solution but in the absence of the dye. The Coomassie stain is highly sensitive; a very weakly staining band on a polyacrylamide gel would correspond to about 0.1 μg (100 ng) of protein. The CBB stain is not used for staining cellulose acetate (or indeed protein blots) because it binds quite strongly to the paper. In this case, proteins are first denatured by brief immersion of the strip in 10% (v/v) trichloroacetic acid, and then immersed in a solution of a dye that does not stain the support material, for example Procion blue, Amido black or Procion S.

Although the Coomassie stain is highly sensitive, many workers require greater sensitivity such as that provided by silver staining. Silver stains are based either on techniques developed for histology or on methods based on the photographic process. In either case, silver ions (Ag^+) are reduced to metallic silver on the protein, where the silver is deposited to give a black or brown band. Silver stains can be used immediately after electrophoresis, or, alternatively, after staining with CBB. With the latter approach, the major bands on the gel can be identified with CBB and then minor bands, not detected with CBB, resolved using the silver stain. The silver stain is at least 100 times more sensitive than CBB, detecting proteins down to 1 ng amounts. Other stains with similar sensitivity include the fluorescent stains Sypro Orange (30 ng) and Sypro Ruby (10 ng).

Glycoproteins have traditionally been detected on protein gels by use of the periodic acid–Schiff (PAS) stain. This allows components of a mixture of glycoproteins to be distinguished. However, the PAS stain is not very sensitive and often gives very weak, red-pink bands, difficult to observe on a gel. A far more sensitive method used nowadays is to blot the gel (Section 10.3.8) and use lectins to detect the glycoproteins. Lectins are protein molecules that bind carbohydrates, and different lectins have been found that have different specificities for different types of carbohydrate. For example, certain lectins recognise mannose, fucose, or terminal glucosamine of the carbohydrate side-chains of glycoproteins. The sample to be analysed is run on a number of tracks of an SDS–polyacrylamide gel. Coloured bands appear at the point where the lectins bind if each blotted track is incubated with a different lectin, washed, incubated with a horseradish peroxidase-linked antibody to the lectin, and then peroxidase substrate added. In this way, by testing a protein sample against a series of lectins, it is possible to determine not only that a protein is a *glyco*protein, but to obtain information about the type of glycosylation.

Quantitative analysis (i.e. measurements of the relative amounts of different proteins in a sample) can be achieved by scanning densitometry. A number of commercial scanning densitometers are available, and work by passing the stained gel track over a beam of light (laser) and measuring the transmitted light. A graphic presentation of protein zones (peaks of absorbance) against migration

distance is produced, and peak areas can be calculated to obtain quantitative data. However, such data must be interpreted with caution because there is only a limited range of protein concentrations over which there is a linear relationship between absorbance and concentration. Also, equal amounts of different proteins do not always stain equally with a given stain, so any data comparing the relative amounts of protein can only be semiquantitative. An alternative and much cheaper way of obtaining such data is to cut out the stained bands of interest, elute the dye by shaking overnight in a known volume of 50% pyridine, and then to measure spectrophotometrically the amount of colour released. More recently gel documentation systems have been developed, which are replacing scanning densitometers. Such benchtop systems comprise a video imaging unit (computer linked) attached to a small 'darkroom' unit that is fitted with a choice of white or ultraviolet light (transilluminator). Gel images can be stored on the computer, enhanced accordingly and printed as required on a thermal printer, thus eliminating the need for wet developing in a purpose built darkroom, as is the case for traditional photography.

Although gel electrophoresis is used generally as an analytical tool, it can be utilised to separate proteins in a gel to achieve protein purification. Protein bands can be cut out of protein blots and sequence data obtained by placing the blot in a protein sequencer (see Section 8.4.3). Stained protein bands can be cut out of protein gels and the protein recovered by electrophoresis of the protein out of the gel piece (electroelution). A number of different designs of electroelution cells are commercially available, but perhaps the easiest method is to seal the gel piece in buffer in a dialysis sac and place the sac in buffer between two electrodes. Protein will electrophorese out of the gel piece towards the appropriate electrode but will be retained by the dialysis sac. After electroelution, the current is reversed for a few seconds to drive off any protein that has adsorbed to the wall of the dialysis sac and then the protein solution within the sac is recovered.

10.3.8 Protein (western) blotting

Although essentially an analytical technique, PAGE does of course achieve fractionation of a protein mixture during the electrophoresis process. It is possible to make use of this fractionation to examine further individual separated proteins. The first step is to transfer or blot the pattern of separated proteins from the gel onto a sheet of nitrocellulose paper. The method is known as protein blotting, or western blotting by analogy with Southern blotting (Section 5.9.2), the equivalent method used to recover DNA samples from an agarose gel. Transfer of the proteins from the gel to nitrocellulose is achieved by a technique known as electroblotting. In this method a sandwich of gel and nitrocellulose is compressed in a cassette and immersed, in buffer, between two parallel electrodes (Fig. 10.11). A current is passed at right angles to the gel, which causes the separated proteins to electrophorese out of the gel and into the nitrocellulose sheet. The nitrocellulose with its transferred protein is referred to as a blot. Once transferred onto nitrocellulose, the separated proteins can be examined further. This

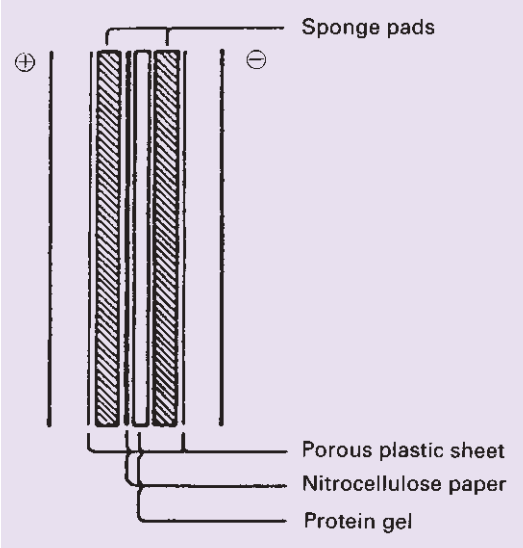


Fig. 10.11. Diagrammatic representation of electroblotting. The gel to be blotted is placed on top of a sponge pad saturated in buffer. The nitrocellulose sheet is then placed on top of the gel, followed by a second sponge pad. This sandwich is supported between two rigid porous plastic sheets and held together with two elastic bands. The sandwich is then placed between parallel electrodes in a buffer reservoir and an electric current passed. The sandwich must be placed such that the immobilising medium is between the gel and the anode for SDS–polyacrylamide gels, because all the proteins carry a negative charge.

involves probing the blot, usually using an antibody to detect a specific protein. The blot is first incubated in a protein solution, for example 10% (w/v) bovine serum albumin, or 5% (w/v) non-fat dried milk (the so-called blotto technique), which will block all remaining hydrophobic binding sites on the nitrocellulose sheet. The blot is then incubated in a dilution of an antiserum (primary antibody) directed against the protein of interest. This IgG molecule will bind to the blot if it detects its antigen, thus identifying the protein of interest. In order to visualise this interaction the blot is incubated further in a solution of a secondary antibody, which is directed against the IgG of the species that provided the primary antibody. For example, if the primary antibody was raised in a rabbit then the secondary antibody would be anti-rabbit IgG. This secondary antibody is appropriately labelled so that the interaction of the secondary antibody with the primary antibody can be visualised on the blot. Anti-species IgG molecules are readily available commercially, with a choice of a different labels attached. One of the most common detection methods is to use an enzyme-linked secondary antibody (Fig. 10.12). In this case, following treatment with enzyme-labelled secondary antibody, the blot is incubated in enzyme–substrate solution, when the enzyme converts the substrate into an insoluble coloured product that is precipitated onto the nitrocellulose. The presence of a coloured band therefore indicates the position of the protein of interest. By careful comparisons of the blot with a

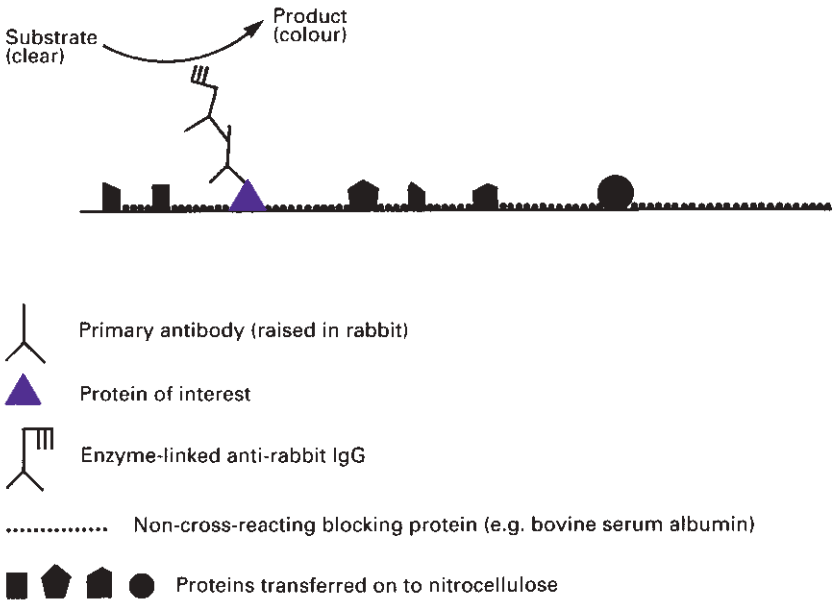


Fig. 10.12. The use of enzyme-linked second antibodies in immunodetection of protein blots. First, the primary antibody (e.g. raised in a rabbit) detects the protein of interest on the blot. Secondly, enzyme-linked anti-rabbit IgG detects the primary antibody. Thirdly, addition of enzyme substrate results in coloured product deposited at the site of protein of interest on the blot.

stained gel of the same sample, the protein of interest can be identified. The enzyme used in enzyme-linked antibodies is usually either alkaline phosphatase, which converts colourless 5-bromo-4-chloro-indolylphosphate (BCIP) substrate into a blue product, or horseradish peroxidase, which, with H_2O_2 as a substrate, oxidises either 3-amino-9-ethylcarbazole into an insoluble brown product, or 4-chloro-1-naphthol into an insoluble blue product. An alternative approach to the detection of horseradish peroxidase is to use the method of enhanced chemiluminescence (ECL). In the presence of hydrogen peroxide and the chemiluminescent substrate luminol (Fig. 10.13) horseradish peroxidase oxidises the luminol with concomitant production of light, the intensity of which is increased 1000-fold by the presence of a chemical enhancer. The light emission can be detected by exposing the blot to a photographic film. Corresponding ECL substrates are available for use with alkaline-phosphatase-labelled antibodies. The principle behind the use of enzyme-linked antibodies to detect antigens in blots is highly analogous to that used in enzyme-linked immunosorbent assays (Section 7.7.3).

Although enzymes are commonly used as markers for second antibodies, other markers can also be used. These include:

- *^{125}I-labelled secondary antibody:* Binding to the blot is detected by autoradiography (Section 14.2.3).

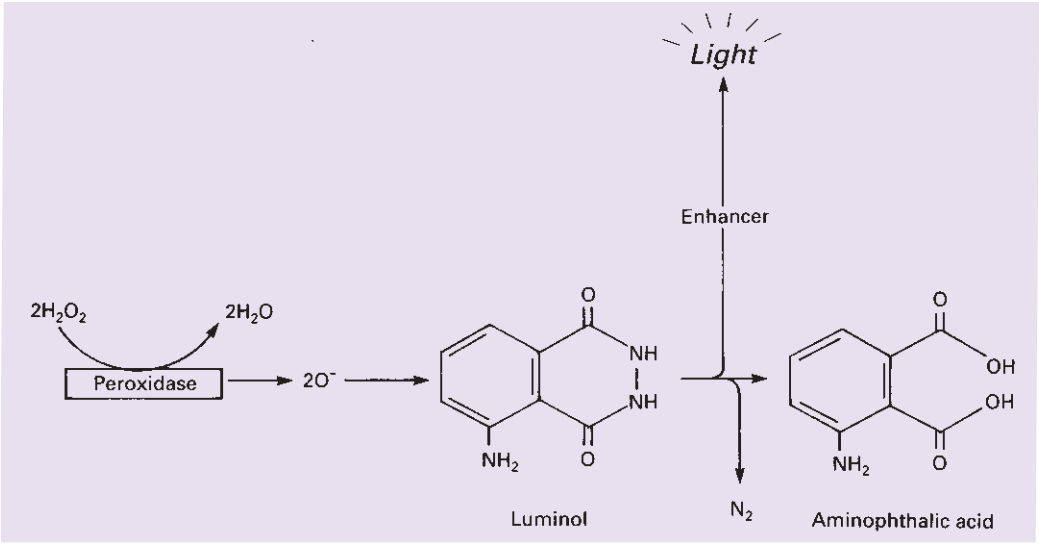


Fig. 10.13. The use of enhanced chemiluminescence to detect horseradish peroxidase.

- *Fluorescein isothiocyanate-labelled secondary antibody:* This fluorescent label is detected by exposing the blot to ultraviolet light.
- *^{125}I-labelled protein A:* Protein A is purified from *Staphylococcus aureus* and specifically binds to the Fc region of IgG molecules. ^{125}I-labelled protein A is therefore used instead of a second antibody, and binding to the blot is detected by autoradiography.
- *Gold-labelled secondary antibodies:* Second antibodies (anti-species IgG) coated with minute gold particles are commercially available. These are directly visible as a red colour when they bind to the primary antibody on the blot.
- *Biotinylated secondary antibodies:* Biotin is a small molecular weight vitamin that binds strongly to the egg protein avidin ($K_d = 10^{-15}$ M). The blot is incubated with biotinylated second antibody, then incubated further with enzyme-conjugated avidin. Since multiple biotin molecules can be linked to a single antibody molecule, many enzyme-linked avidin molecules can bind to a single biotinylated antibody molecule, thus providing an enhancement of the signal. The enzyme used is usually alkaline phosphatase or horseradish peroxidase.

In addition to the use of labelled antibodies or proteins, other probes are sometimes used. For example, radioactively labelled DNA can be used to detect DNA-binding proteins on a blot. The blot is first incubated in a solution of radiolabelled DNA, then washed, and an autoradiograph of the blot made. The presence of radioactive bands, detected on the autoradiograph, identifies the positions of the DNA-binding proteins on the blot.

10.4 ELECTROPHORESIS OF NUCLEIC ACIDS

10.4.1 Agarose gel electrophoresis of DNA

For the majority of DNA samples, electrophoretic separation is carried out in agarose gels. This is because most DNA molecules and their fragments that are analysed routinely are considerably larger than proteins and therefore, because most DNA fragments would be unable to enter a polyacrylamide gel, the larger pore size of an agarose gel is required. For example, the commonly used plasmid pBR322 has an M_r of 2.4×10^6. However, rather than use such large numbers it is more convenient to refer to DNA size in terms of the number of base-pairs. Although, originally, DNA size was referred to in terms of base-pairs (bp) or kilobase-pairs (kbp), it has now become the accepted nomenclature to abbreviate kbp to simply kb when referring to double-stranded DNA. pBR322 is therefore 4.36 kb. Even a small restriction fragment of 1 kb has an M_r of 620 000. When talking about single-stranded DNA it is common to refer to size in terms of nucleotides (nt). Since the charge per unit length (owing to the phosphate groups) in any given fragment of DNA is the same, all DNA samples should move towards the anode with the same mobility under an applied electrical field. However, separation in agarose gels is achieved because of resistance to their movement caused by the gel matrix. The largest molecules will have the most difficulty passing through the gel pores (very large molecules may even be blocked completely), whereas the smallest molecules will be relatively unhindered. Consequently the mobility of DNA molecules during gel electrophoresis will depend on size, the smallest molecules moving fastest. This is analogous to the separation of proteins in SDS–polyacrylamide gels (Section 10.3.1), although the analogy is not perfect, as double-stranded DNA molecules form relatively stiff rods and it is not completely understood how they pass through the gel, it is probable that long DNA molecules pass through the gel pores end-on. While passing through the pores, a DNA molecule will experience drag; so the longer the molecule, the more it will be retarded by each pore. Sideways movement may become more important for very small double-stranded DNA and for the more flexible single-stranded DNA. It will be obvious from the above that gel concentrations must be chosen to suit the size range of the molecules to be separated. Gels containing 0.3% agarose will separate double-stranded DNA molecules of between 5 and 60 kb size, whereas 2% gels are used for samples of between 0.1 and 3 kb. Many laboratories routinely use 0.8% gels, which are suitable for separating DNA molecules in the range 0.5–10 kb. Since agarose gels separate DNA according to size, the M_r of a DNA fragment may be determined from its electrophoretic mobility by running a number of standard DNA markers of known M_r on the same gel. This is most conveniently achieved by running a sample of bacteriophage λ DNA (49 kb) that has been cleaved with a restriction enzyme such as *Eco*RI. Since the base sequence of λ DNA is known, and the cleavage sites for *Eco*RI are known, this generates fragments of accurately known size (Fig. 10.14).

DNA gels are invariably run as horizontal, submarine or submerged gels; so named because such a gel is totally immersed in buffer. Agarose, dissolved in gel

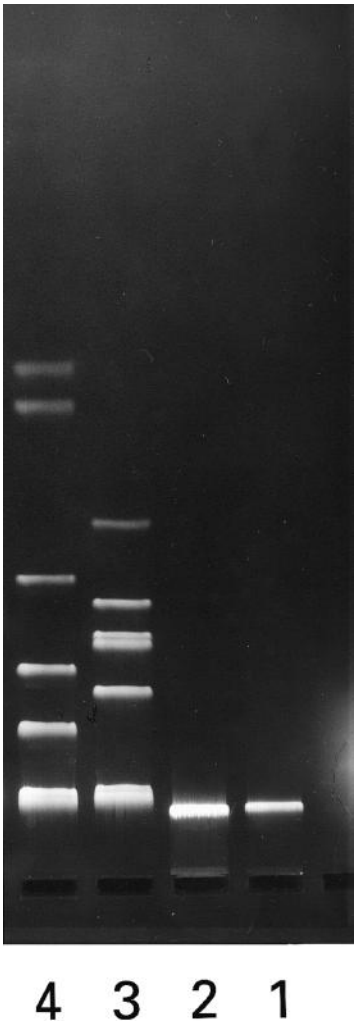


Fig. 10.14. Photograph showing four tracks from a 0.8% agarose submarine gel. The gel was run at 40 V in Tris/borate/EDTA buffer for 16 h, stained with ethidium bromide and viewed under ultraviolet light. Sample loadings were about 0.5 μg of DNA per track. Tracks 1 and 2, λ DNA (49 kb). Track 3, λ DNA cleaved with the enzyme *Eco*RI to generate fragments of the following size (in order from the origin): 21.80 kb, 7.52 kb, 5.93 kb, 5.54 kb, 4.80 kb, 3.41 kb. Track 4, λ DNA cleaved with the enzyme *Hin*dIII to generate fragments of the following size (in order from the origin): 23.70 kb, 9.46 kb, 6.75 kb, 4.26 kb, 2.26 kb, 1.98 kb. (Courtesy of Stephen Boffey, University of Hertfordshire.)

buffer by boiling, is poured onto a glass or plastic plate, surrounded by a wall of adhesive tape or a plastic frame to provide a gel about 3 mm in depth. Loading wells are formed by placing a plastic well-forming template or comb in the poured gel solution, and removing this comb once the gel has set. The gel is placed in the electrophoresis tank, covered with buffer, and samples loaded by directly injecting the sample into the wells. Samples are prepared by dissolving them in a buffer solution that contains sucrose, glycerol or Ficoll, which makes the solution dense and allows

it to sink to the bottom of the well. A dye such as bromophenol blue is also included in the sample solvent; it makes it easier to see the sample that is being loaded and also acts as a marker of the electrophoresis front. No stacking gel (Section 10.3.1) is needed for the electrophoresis of DNA because the mobilities of DNA molecules are much greater in the well than in the gel, and therefore all the molecules in the well pile up against the gel within a few minutes of the current being turned on, forming a tight band at the start of the run. General purpose gels are approximately 25 cm long and 12 cm wide, and are run at a voltage gradient of about 1.5 V cm^{-1} overnight. A higher voltage would cause excessive heating. For rapid analyses that do not need extensive separation of DNA molecules, it is common to use mini-gels that are less than 10 cm long. In this way information can be obtained in 2–3 h.

Once the system has been run, the DNA in the gel needs to be stained and visualised. The reagent most widely used is the fluorescent dye ethidium bromide. The gel is rinsed gently in a solution of ethidium bromide (0.5 $\mu g\, cm^{-3}$) and then viewed under ultraviolet light (300 nm wavelength). Ethidium bromide is a cyclic planar molecule that binds between the stacked base-pairs of DNA (i.e. it intercalates) (Section 5.7.4). The ethidium bromide concentration therefore builds up at the site of the DNA bands and under ultraviolet light the DNA bands fluoresce orange-red. As little as 10 ng of DNA can be visualised as a 1 cm wide band. It should be noted that extensive viewing of the DNA with ultraviolet light can result in damage of the DNA by nicking and base-pair dimerisation. This is of no consequence if a gel is only to be viewed, but obviously viewing of the gel should be kept to a minimum if the DNA is to be recovered (see below). It is essential to protect one's eyes by wearing goggles when ultraviolet light is used. If viewing of gels under ultraviolet is carried out for long periods, a plastic mask that covers the whole face should be used to avoid 'sunburn'.

10.4.2 DNA sequencing gels

Although agarose gel electrophoresis of DNA is a 'workhorse' technique for the molecular biologist, a different form of electrophoresis has to be used when DNA sequences are to be determined. Whichever DNA sequencing method is used (Section 5.11), the final analysis usually involves separating single-stranded DNA molecules shorter than about 1000 nt and differing in size by only 1 nt. To achieve this it is necessary to have a small-pored gel and so acrylamide gels are used instead of agarose. For example, 3.5% polyacrylamide gels are used to separate DNA in the range 80–1000 nt and 12% gels to resolve fragments of between 20 and 100 nt. If a wide range of sizes is being analysed it is often convenient to run a gradient gel, for example from 3.5% to 7.5%. Sequencing gels are run in the presence of denaturing agents, urea and formamide. Since it is necessary to separate DNA molecules that are very similar in size, DNA sequencing gels tend to be very long (100 cm) to maximise the separation achieved. A typical DNA sequencing gel is shown in Fig. 5.38.

As mentioned above, electrophoresis in agarose can be used as a preparative method for DNA. The DNA bands of interest can be cut out of the gel and the DNA

recovered by: (a) electroelution, (b) macerating the gel piece in buffer, centrifuging and collecting the supernatant; or (c), if low melting point agarose is used, melting the gel piece and diluting with buffer. In each case, the DNA is finally recovered by precipitation of the supernatant with ethanol.

10.4.3 Pulsed-field gel electrophoresis

The agarose gel methods for DNA described above can fractionate DNA of 60 kb or less. The introduction of pulsed-field gel electrophoresis (PFGE) and the further development of variations on the basic technique now means that DNA fragments up to 2×10^3 kb can be separated. This therefore allows the separation of whole chromosomes by electrophoresis. The method basically involves electrophoresis in agarose where two electric fields are applied alternately at different angles for defined time periods (e.g. 60 s). Activation of the first electric field causes the coiled molecules to be stretched in the horizontal plane and start to move through the gel. Interruption of this field and application of the second field force the molecule to move in the new direction. Since there is a length-dependent relaxation behaviour when a long-chain molecule undergoes conformational change in an electric field, the smaller a molecule, the quicker it realigns itself with the new field and is able to continue moving through the gel. Larger molecules take longer to realign. In this way, with continual reversing of the field, smaller molecules draw ahead of larger molecules and separate according to size. Fig. 10.15 shows the separation of yeast chromosomes that vary in size from 260 to 850 kb. Needless to say the physics of designing a PFGE system is complex and in recent years a number of different developments on the same basic theme have resulted in a bewildering array of related techniques. Detailed description of these techniques is beyond the scope of this chapter but the names of a few of these techniques indicate the principles involved, for example orthogonal field alternating gel electrophoresis (OFAGE), field inversion gel electrophoresis (FIGE), transverse alternating field gel electrophoresis (TAFE), contour-clamped homogeneous electric field electrophoresis (CHEF), and rotating field electrophoresis (RFE).

10.4.4 Electrophoresis of RNA

Like that of DNA, electrophoresis of RNA is usually carried out in agarose gels, and the principle of the separation, based on size, is the same. Often one requires a rapid method for checking the integrity of RNA immediately following extraction but before deciding whether to process it further. This can be achieved easily by electrophoresis in a 2% agarose gel in about 1 h. Ribosomal RNAs (18 S and 28 S) are clearly resolved and any degradation (seen as a smear) or DNA contamination is seen easily. However, if greater resolution is required, a smaller-pored acrylamide gel is used to enhance resolution, for example to resolve transfer RNAs (4 S) from 5 S ribosomal RNA. This can be achieved on a 2.5–5% acrylamide

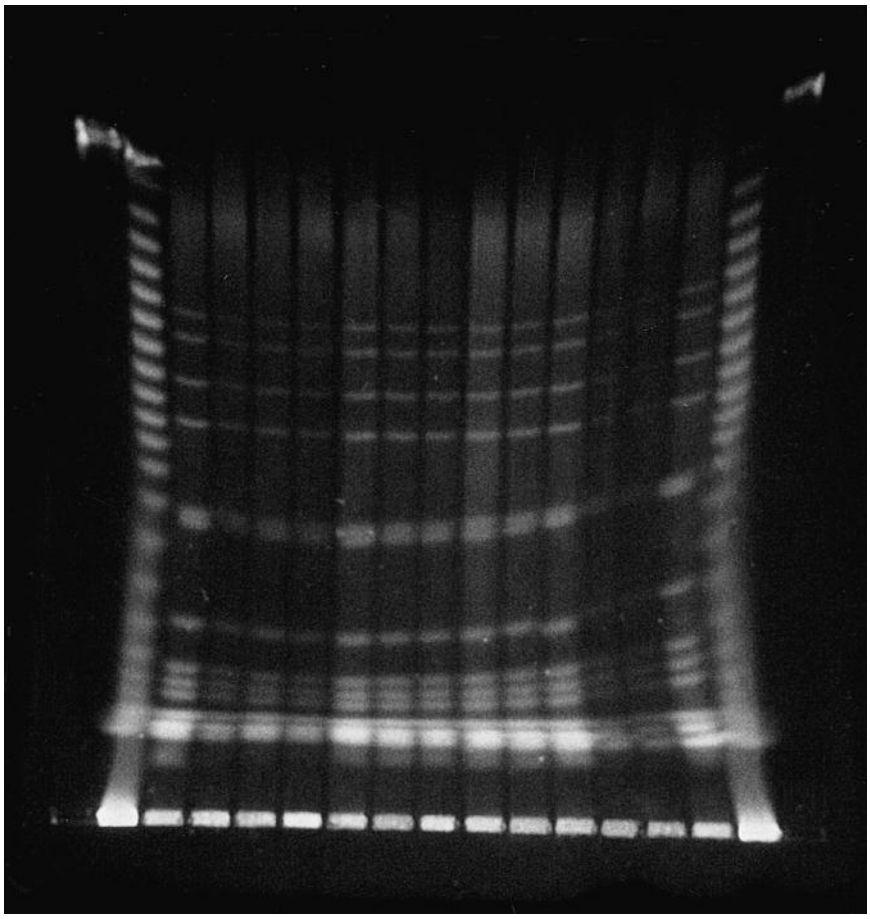


Fig. 10.15. CHEF gel electrophoresis of yeast (repeat samples run in central 13 tracks) and bacteriophage λ multimers (the 'ladders' on the two outside lanes). Every step of the ladder in the two outer lanes is about 43.5 kb and 20 steps are resolved up to 850 kb. The yeast chromosomes are of sizes 260, 290, 370, 460, 580/600, 700, 780, 820 and 850 kb. (Courtesy of Margit Burmeister, University of Michigan.)

gradient gel with an overnight run. Both these methods involve running native RNA. There will almost certainly be some secondary structure within the RNA molecule owing to intramolecular hydrogen bonding (see e.g. the clover leaf structure of tRNA, Fig. 5.6). For this reason native RNA run on gels can be stained and visualised with ethidium bromide. However, if the study objective is to determine RNA size by gel electrophoresis, then full denaturation of the RNA is needed to prevent hydrogen bond formation within or even between polynucleotides that will otherwise affect the electrophoretic mobility. There are three denaturing agents (formaldehyde, glyoxal and methylmercuric hydroxide) that are compatible with both RNA and agarose. Either one of these may be incorporated into the agarose gel and electrophoresis buffer, and the sample is heat denatured in the presence of the denaturant prior to electrophoresis. After heat

denaturation, each of these agents forms adducts with the amino groups of guanine and uracil, thereby preventing hydrogen bond reformation at room temperature during electrophoresis. It is also necessary to run denaturing gels if the RNA is to be blotted (northern blots, Section 5.9.2) and probed, to ensure that the base sequence is available to the probe. Denatured RNA stains only very weakly with ethidium bromide, so acridine orange is commonly used to visualise RNA on denaturing gels. However, it should be noted that many workers will be using radiolabelled RNA and will therefore identify bands by autoradiography. An example of the electrophoresis of RNA is shown in Fig. 10.16.

10.5 CAPILLARY ELECTROPHORESIS

The technique has variously been referred to as high performance capillary electrophoresis (HPCE), capillary zone electrophoresis (CZE), free solution capillary electrophoresis (FSCE) and capillary electrophoresis (CE), but the term CE is the one most common nowadays. The microscale nature of the capillary used, where only microlitres of reagent are consumed by analysis and only nanolitres of sample needed for analysis, together with the ability for on-line detection down to femtomole (10^{-15} moles) sensitivity in some cases has for many years made capillary electrophoresis the method of choice for many biomedical and clinical analyses. Capillary electrophoresis can be used to separate a wide spectrum of biological molecules including amino acids, peptides, proteins, DNA fragments (e.g. synthetic oligonucleotides) and nucleic acids, as well as any number of small organic molecules such as drugs or even metal ions (see below). The method has also been applied successfully to the problem of chiral separations (Section 11.5.5).

As the name suggests, capillary electrophoresis involves electrophoresis of samples in very narrow-bore tubes (typically 50 μm internal diameter, 300 μm external diameter). One advantage of using capillaries is that they reduce problems resulting from heating effects. Because of the small diameter of the tubing there is a large surface-to-volume ratio, which gives enhanced heat dissipation. This helps to eliminate both convection currents and zone broadening owing to increased diffusion caused by heating. It is therefore not necessary to include a stabilising medium in the tube and allows free-flow electrophoresis.

Theoretical considerations of CE generate two important equations:

$$t = \frac{L^2}{\mu V} \tag{10.4}$$

where t is the migration time for a solute, L is the tube length, μ is the electrophoretic mobility of the solute, and V is the applied voltage.

The separation efficiency, in terms of the total number of theoretical plates, N, is given by

$$N = \frac{\mu V}{2D} \tag{10.5}$$

where D is the solute's diffusion coefficient.

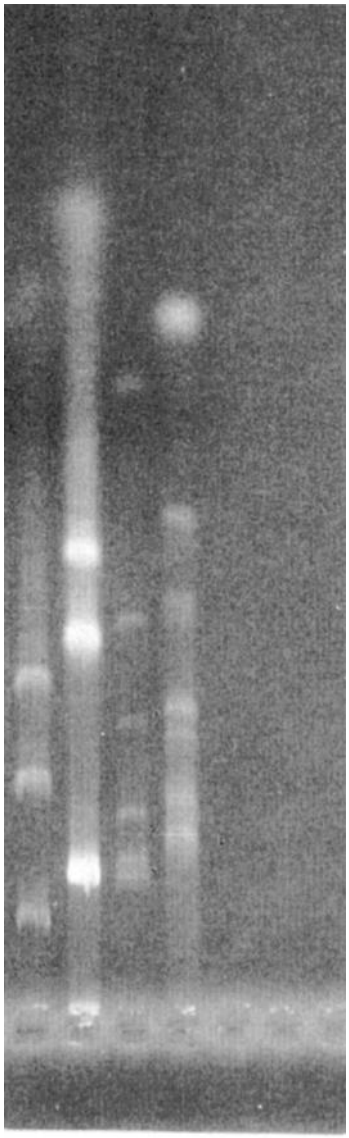


Fig. 10.16. Gel electrophoresis of RNA in a 1.4% agarose gel. Track 1 is total RNA from the tobacco plant denatured with glyoxal prior to running. Track 2 is the same sample, *not* denatured. The two faster-running major bands are 18 S and 25 S ribosomal RNA. The slower running major band is nuclear DNA. Tracks 3 and 4 show a mixture of RNA marker fragments, with (track 3) and without (track 4) glyoxal treatment. The sizes of the marker RNA fragments are 0.24, 1.4, 2.4, 4.4, 7.5 and 9.5 kb. Note that, with each sample, denaturation results in lower mobilities for the components of each sample. (Courtesy of Debbie Cook and Robert Slater, Department of Life Sciences, University of Hertfordshire.)

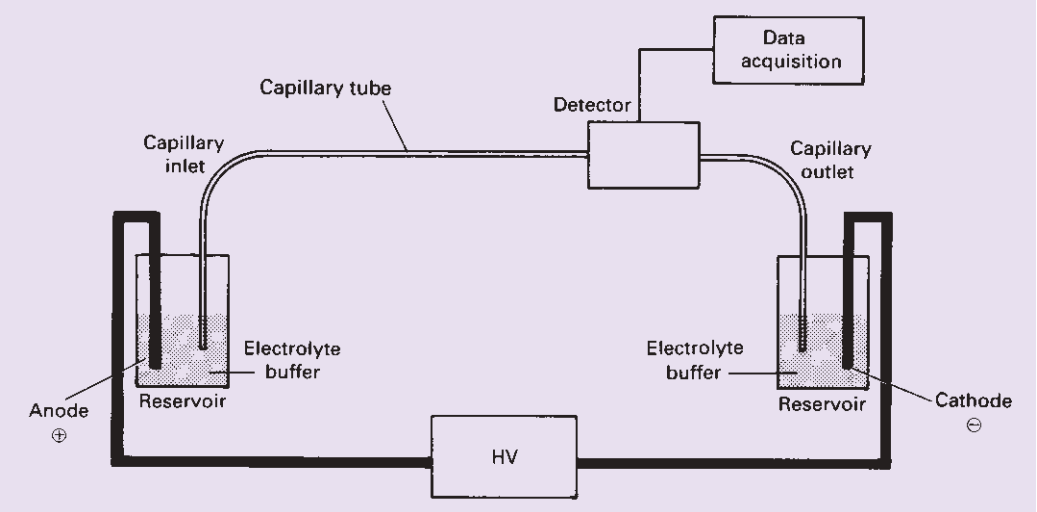


Fig. 10.17. Diagrammatic representation of a typical capillary electrophoresis apparatus.

From these equations it can be seen, first, that the column length plays no role in separation efficiency, but that it has an important influence on migration time and hence analysis time, and, secondly, high separation efficiencies are best achieved through the use of high voltages (μ and D are dictated by the solute and are not easily manipulated).

It therefore appears that the ideal situation is to apply as high a voltage as possible to as short a capillary as possible. However, there are practical limits to this approach. As the capillary length is reduced, the amount of heat that must be dissipated increases owing to the decreasing electrical resistance of the capillary. At the same time the surface area available for heat dissipations is decreasing. Therefore at some point significant thermal effect will occur, placing a practical limit on how short a tube can be used. Also the higher the voltage that is applied, the greater the current, and therefore the heat generated. In practical terms a compromise between voltage used and capillary length is required. Voltages of 10–50 kV with capillaries of 50–100 cm are commonly used.

The basic apparatus for CE is shown diagrammatically in Fig. 10.17. A small plug of sample solution (typically 5–30 μm^3) is introduced into the anode end of a fused silica capillary tube containing an appropriate buffer. Sample application is carried out in one of two ways: by high voltage injection or by pressure injection.

- *High voltage injection.* With the high voltage switched off, the buffer reservoir at the positive electrode is replaced by a reservoir containing the sample, and a plug of sample (e.g. 5–30 μm^3 of a 1 mg cm^{-3} solution) is introduced into the capillary by briefly applying high voltage. The sample reservoir is then removed, the buffer reservoir replaced, voltage again applied and the separation is then commenced.

- *Pressure injection.* The capillary is removed from the anodic buffer reservoir and inserted through an air-tight seal into the sample solution. A second tube provides pressure to the sample solution, which forces the sample into the capillary. The capillary is then removed, replaced in the anodic buffer and a voltage applied to initiate electrophoresis.

A high voltage (up to 50 kV) is then put across the capillary tube and component molecules in the injected sample migrate at different rates along the length of the capillary tube. Electrophoretic migration causes the movement of charged molecules in solution towards an electrode of opposite charge. Owing to this electrophoretic migration, positive and negative sample molecules migrate at different rates. However, although analytes are separated by electrophoretic migration, they are all drawn towards the cathode by electroendosmosis (Section 10.1). Since this flow is quite strong, the rate of electroendosmotic flow usually being much greater than the electrophoretic velocity of the analytes, all ions, regardless of charge sign, and neutral species are carried towards the cathode. Positively charged molecules reach the cathode first because the combination of electrophoretic migration and electroosmotic flow cause them to move fastest. As the separated molecules approach the cathode, they pass through a viewing window where they are detected by an ultraviolet monitor that transmits a signal to a recorder, integrator or computer. Typical run times are between 10 and 30 min. A typical capillary electrophoretograph is shown in Fig. 10.18.

This free solution method is the simplest and most widely practised mode of capillary electrophoresis. However, while the generation of ionised groups on the capillary wall is advantageous via the introduction of electroendosmotic flow, it can also sometimes be a disadvantage. For example, protein adsorption to the capillary wall can occur with cationic groups on protein surfaces binding to the ionised silanols. This can lead to smearing of the protein as it passes through the capillary (recognised as peak broadening) or, worse, complete loss of protein due to total adsorption on the walls. Some workers therefore use coated tubes where a neutral coating group has been used to block the silanol groups. This of course eliminates electroendosmotic flow. Therefore, during electrophoresis in coated capillaries, neutral species are immobile while acid species migrate to the anode and basic species to the cathode. Since detection normally takes place at only one end of the capillary, only one class of species can be detected at a time in an analysis using a coated capillary.

A range of variations on this basic technique also exist. For example, as seen above, in normal CE neutral molecules do not separate but rather travel as a single band. However, separation of neutral molecules can be achieved by including a surfactant such as SDS with the buffer. Above a certain concentration some surfactant molecules agglomerate and form micelles, which, under the influence of an applied electric field, will migrate towards the appropriate electrode. Solutes will interact and partition with the moving micelles. If a solute interacts strongly it will reach the detector later than one which partitions to a lesser degree. This method is known as micellular electrokinetic

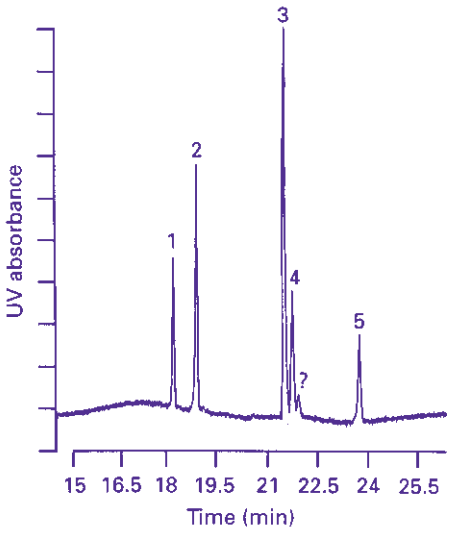


Fig. 10.18. Capillary electrophoresis of five structurally related peptides. Column length was 100 cm and the separation voltage 50 kV. Peptides were detected by their ultraviolet absorbance at 200 nm.

Peptide	
1	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
2	Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
3	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
4	Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
5	Ile-Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

(Courtesy of Patrick Camilleri and George Okafo, GSK Ltd.)

capillary electrophoresis (MECC). Since ionic solutes will also migrate under the applied field, separation by MECC is due to a combination of both electrophoresis and chromatography.

Original developments in CE concentrated on the separation of peptides and proteins, but in recent years CE has been successfully applied to the separation of a range of other biological molecules. The following provides a few examples.

- In the past, peptide analysis has been performed routinely using reversed-phase HPLC, achieving separation based on hydrophobicity differences between peptides. Peptide separation by CE is now also routinely carried out, and is particularly useful, for example as a means of quality (purity) control for peptides and proteins produced by preparative HPLC. Fig. 10.18 shows the impressive separation that can be achieved for peptides with very similar structures.
- High purity synthetic oligodeoxyribonucleotides are necessary for a range of applications including use as hybridisation probes in diagnostic and gene

cloning experiments, use as primers for DNA sequencing and the polymerase chain reaction (PCR), use in site-directed mutagenesis and use as antisense therapeutics. CE can provide a rapid method for analysing the purity of such samples. For example, analysis of an 18-mer antisense oligonucleotide containing contaminant fragments (8-mer to 17-mer) can be achieved in only 5 min.

- Point mutations in DNA, such as occur in a range of human diseases, can be identified by CE.
- CE can be used to quantify DNA. For example, CE analysis of PCR products from HIV-I allowed the identification of between 200 000 and 500 000 viral particles per cubic centimetre of serum.
- Chiral compounds can be resolved using CE. Most work has been carried in free solution using cyclodextrins as chiral selectors.
- A range of small molecules, drug and metabolites can be measured in physiological solutions such as urine and serum. These include amino acids (over 50 are found in urine), nucleotides, nucleosides, bases, anions such as chloride and sulphate (NO_2^- and NO_3^- can be separated in human plasma) and cations such as Ca^{2+} and Fe^{3+}.

10.6 MICROCHIP ELECTROPHORESIS

The further miniaturisation of electrophoretic systems has led to the development of microchip electrophoresis, which has many advantages over conventional electrophoresis methods, allowing very high speed analyses at very low sample sizes. For example, microchip analysis can often be completed in tens of seconds whereas capillary electrophoresis (CE) can take 20 min and conventional gel electrophoresis at least 2 h. Using new detection systems, such as laser-induced fluorescence, picomole to attomole (10^{-18} moles) sensitivity can be achieved, which is at least two orders of magnitude greater than for conventional CE. Detection systems for molecules that do not fluoresce include electrochemical detectors (Section 11.3.3), pulsed amperometric detection (PAD), and sinusoidal voltometry. All these detection techniques offer high sensitivity, are ideally suited to miniaturisation, are very low cost, and all are highly compatible with advanced micromachining and microfabrication (see below) technologies. Finally, the applied voltage required is only a few volts, which eliminates the need for the high voltages used by CE.

The manufacturing process that produces microchips is called microfabrication. The process etches precise and reproducible capillary-like channels (typically, 50 μm wide and 10 μm deep; slightly smaller than a strand of human hair) on the surface of sheets of quartz, glass or plastic. A second sheet is then fused on top of the first sheet, turning the etched channels into closed microfluidic channels. The end of each channel connects to a reservoir through which fluids are introduced/removed. Typically, the size of chips can be as small as 2 cm^2. Basically the microchip provides an electrophoretic system similar to CE but with more flexibility.

Current developments of this technology are based on integrating functions other than just separation into the chip. For example, sample extraction, preconcentration of samples prior to separation, PCR amplification of DNA samples using infrared-mediated thermocycling for rapid on-chip amplification, and the extraction of separated molecules using microchamber-bound solid phases are all examples of where further functions have been built into a microchip electrophoresis system. An interface has also been developed for microchip electrophoresis–mass spectrometry (MCE–MS) where drugs have been separated by MCE and then identified by MS.

10.7 SUGGESTIONS FOR FURTHER READING

ALTRIA, K. D. (1996). *Capillary Electrophoresis Guide Book*. Humana Press, Totowa, NJ. (Detailed theory and practical procedures for the analysis of proteins, nucleic acids and metabolites.)

HAMES, B. D. and RICKWOOD, D. (2002). *Gel Electrophoresis of Proteins: A Practical Approach*, 3rd edn. Oxford University Press, Oxford. (Detailed theory and practical procedures for the electrophoresis of proteins.)

WALKER, J. M. (2002). *The Protein Protocols Handbook*, 2nd edn. Humana Press, Totowa, NJ. (Detailed theory and laboratory protocols for a range of electrophoretic techniques and blotting procedures.)