CHAPTER 5

Analytical Techniques

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CHAPTER OUTLINE

Analytic techniques and instrumentation provide the foundation for all measurements made in a modern clinical chemistry laboratory. The majority of techniques fall into one of four basic disciplines within the field of analytic chemistry: spectrometry (including **spectrophotometry**, **atomic absorption**, and **mass spectrometry** [MS]); luminescence (including fluorescence, **chemiluminescence**, and nephelometry); electroanalytic methods (including **electrophoresis**, potentiometry, and amperometry); and chromatography (including gas, liquid, and thin-layer). With the improvements in optics, electronics, and computerization, instrumentation has become miniaturized. This miniaturization has enabled the development of **point-of-care testing (POCT)** devices that produce results as accurate as those provided by large laboratory-based instrumentation.

SPECTROPHOTOMETRY AND PHOTOMETRY

The instruments that measure electromagnetic radiation have several concepts and components in common. Shared instrumental components are discussed in some detail in a later section. Photometric instruments measure light intensity without consideration of wavelength. Most instruments today use filters (photometers), prisms, or gratings (spectrometers) to select (isolate) a narrow range of the incident wavelength. Radiant energy that passes through an object will be partially reflected, absorbed, and transmitted.

Electromagnetic radiation is described as photons of energy traveling in waves. The relationship between wavelength and energy E is described by Planck's formula:

$$E = h\nu \qquad \textbf{(Eq. 5-1)}$$

where h is a constant (6.62×10^{-27} erg sec), known as Planck's constant, and v is frequency.

Because the frequency of a wave is inversely proportional to the wavelength, it follows that the energy of electromagnetic radiation is inversely proportional to wavelength. Figure 5-1*A* shows this relationship. Electromagnetic radiation includes a spectrum of energy from short-wavelength, highly energetic gamma rays and x-rays on the left in Figure 5-1*B* to long-wavelength radiofrequencies on the right. Visible light falls in between, with the color violet at 400-nm and red at 700-nm wavelengths being the approximate limits of the visible spectrum.

The instruments discussed in this section measure either absorption or emission of radiant energy to determine concentration of atoms or molecules. The two phenomena, absorption and emission, are closely related. For a ray of electromagnetic radiation to be absorbed, it must have the same frequency as a rotational or vibrational frequency in the atom or molecule that it strikes. Levels of energy that are absorbed move in discrete steps, and any particular type of molecule or atom will absorb only certain energies and not others. When energy is absorbed, valence electrons move to an orbital with a higher energy level. Following energy absorption, the excited electron will fall back to the ground state by emitting a discrete amount of energy in the form of a characteristic wavelength of radiant energy.

Absorption or emission of energy by atoms results in a line spectrum. Because of the relative complexity of molecules, they absorb or emit a bank of energy over a large region. Light emitted by incandescent solids (tungsten or deuterium) is in a continuum. The three types of spectra are shown in Figure 5-2.[1–3]

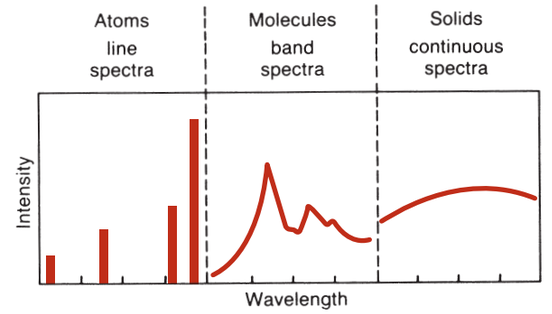


FIGURE 5-2. Characteristic absorption or emission spectra. (Reprinted with permission from Coiner D. Basic concepts in laboratory instrumentation. Bethesda, Md.: ASMT Education and Research Fund, 1975–1979.)

Beer's Law

The relationship between absorption of light by a solution and the concentration of that solution has been described by Beer and others. Beer's law states that the concentration of a substance is directly proportional to the amount of light absorbed or inversely proportional to the logarithm of the transmitted light. Percent transmittance (% T) and absorbance (A) are related photometric terms that are explained in this section.

Figure 5-3*A* shows a beam of monochromatic light entering a solution. Some of the light is absorbed. The remainder passes through, strikes a light detector, and is converted to an electric signal. Percent transmittance is the ratio of the radiant energy transmitted (T) divided by the radiant energy incident on the sample (I). All light absorbed or blocked results in 0% T. A level of 100% T is obtained if no light is absorbed. In practice, the solvent without the constituent of interest is placed in the light

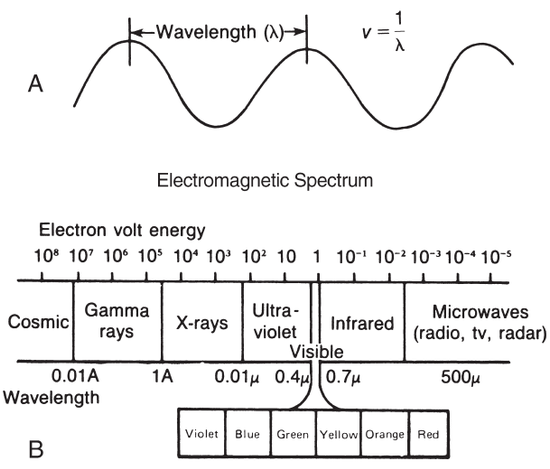


FIGURE 5-1. Electromagnetic radiation—relationship of energy and wavelength.

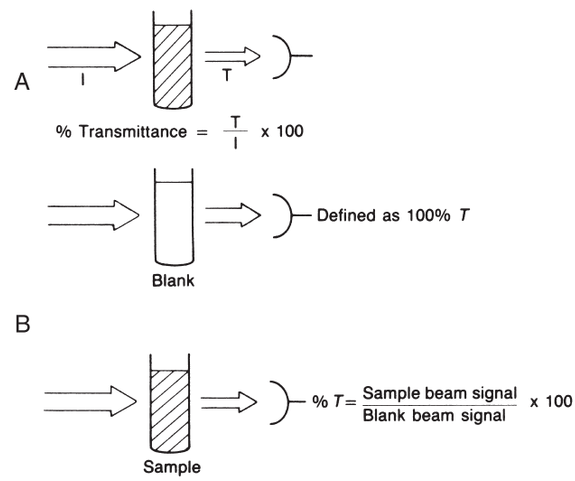


FIGURE 5-3. Percent transmittance (% T) defined.

path, as in Figure 5-3*B*. Most of the light is transmitted, but a small amount is absorbed by the solvent and cuvet or is reflected away from the detector. The electrical readout of the instrument is set arbitrarily at 100% T, while the light is passing through a "blank" or reference. The sample containing absorbing molecules to be measured is placed in the light path. The difference in amount of light transmitted by the blank and that transmitted by the sample is due only to the presence of the compound being measured. The % T measured by commercial spectrophotometers is the ratio of the sample transmitted beam divided by the blank transmitted beam.

Equal thicknesses of an absorbing material will absorb a constant fraction of the energy incident upon the layers. For example, in a tube containing layers of solution (Fig. 5-4*A*), the first layer transmits 70% of the light incident upon it. The second layer will, in turn, transmit 70% of the light incident upon it. Thus, 70% of 70% (49%) is transmitted by the second layer. The third layer transmits 70% of 49%, or 34% of the original light. Continuing on, successive layers transmit 24% and 17%, respectively. The % T values, when plotted on linear graph paper, yield the curve shown in Figure 5-4*B*. Considering each equal layer as many monomolecular layers, we can translate layers of material to concentration. If semilog graph paper is used to plot the same figures, a straight line is obtained (Fig. 5-4*C*), indicating that, as concentration increases, % T decreases in a logarithmic manner.

Absorbance A is the amount of light absorbed. It cannot be measured directly by a spectrophotometer but rather is mathematically derived from % T as follows:

$$\%T = \frac{I}{I_0} \times 100 \qquad \textbf{(Eq. 5-2)}$$

where I_0 is incident light and I is transmitted light.

Absorbance is defined as follows:

$$A = -\log(I/I_0) = \log(100\%) - \log \%T$$
$$= 2 - \log \%T \qquad \textbf{(Eq. 5-3)}$$

According to Beer's law, absorbance is directly proportional to concentration (Fig. 5-4*D*):

$$A = \varepsilon \times b \times c \qquad \textbf{(Eq. 5-4)}$$

where ε = molar absorptivity, the fraction of a specific wavelength of light absorbed by a given type of molecule; b is the length of light path through the solution; and c is the concentration of absorbing molecules.

Absorptivity depends on molecular structure and the way in which the absorbing molecules react with different energies. For any particular molecular type, absorptivity changes as wavelength of radiation changes. The amount of light absorbed at a particular wavelength

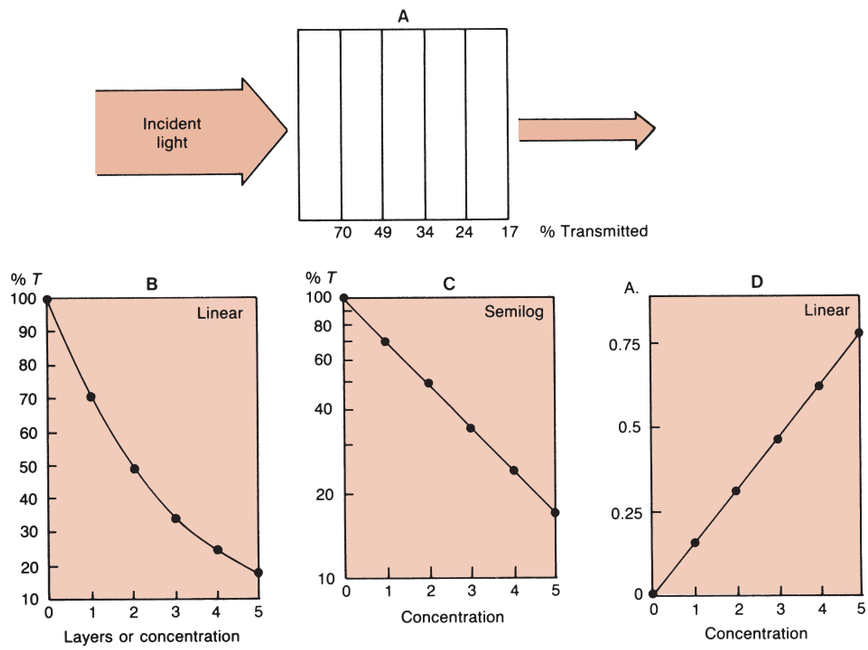


FIGURE 5-4. (A) Percent of original incident light transmitted by equal layers of light-absorbing solution. **(B)** Percent T versus concentration on linear graph paper. **(C)** Percent T versus concentration on semilog graph paper. **(D)** A versus concentration on linear graph paper.

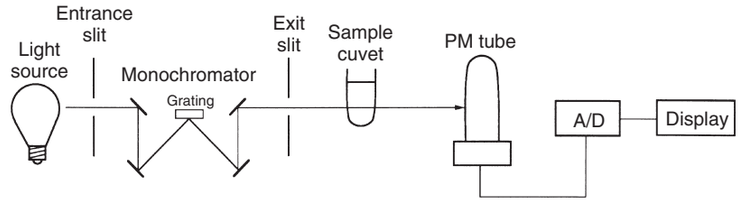


FIGURE 5-5. Single-beam spectrophotometer.

depends on the molecular and ion types present and may vary with concentration, pH, or temperature.

Because the path length and molar absorptivity are constant for a given wavelength,

$$A \sim C \qquad \text{(Eq. 5-5)}$$

Unknown concentrations are determined from a calibration curve that plots absorbance at a specific wavelength versus concentration for standards of known concentration. For calibration curves that are linear and have a zero *y*-intercept, unknown concentrations can be determined from a single calibrator. Not all calibration curves result in straight lines. Deviations from linearity are typically observed at high absorbances. The stray light within an instrument will ultimately limit the maximum absorbance that a spectrophotometer can achieve, typically 2.0 absorbance units.

Spectrophotometric Instruments

A spectrophotometer is used to measure the light transmitted by a solution to determine the concentration of the light-absorbing substance in the solution. Figure 5-5 illustrates the basic components of a single-beam spectrophotometer, which are described in subsequent sections.

Components of a Spectrophotometer

Light Source

The most common source of light for work in the visible and near-infrared region is the incandescent tungsten or tungsten-iodide lamp. Only about 15% of radiant energy emitted falls in the visible region, with most emitted as near-infrared.[1–3] Often, a heat-absorbing filter is inserted between the lamp and sample to absorb the infrared radiation.

The lamps most commonly used for ultraviolet (UV) work are the deuterium-discharge lamp and the mercury-arc lamp. Deuterium provides continuous emission down to 165 nm. Low-pressure mercury lamps emit a sharp-line spectrum, with both UV and visible lines. Medium and high-pressure mercury lamps emit a continuum from UV to the mid visible region. The most important factors for a light source are range, spectral distribution within the range, the source of radiant production, stability of the radiant energy, and temperature.

Monochromators

Isolation of individual wavelengths of light is an important and necessary function of a monochromator. The degree of wavelength isolation is a function of the type of device used and the width of entrance and exit slits. The bandpass of a monochromator defines the range of wavelengths transmitted and is calculated as width at more than half the maximum transmittance (Fig. 5-6).

Numerous devices are used for obtaining monochromatic light. The least expensive are colored-glass filters. These filters usually pass a relatively wide band of radiant energy and have a low transmittance of the selected wavelength. Although not precise, they are simple, inexpensive, and useful.

Interference filters produce monochromatic light based on the principle of constructive interference of waves. Two pieces of glass, each mirrored on one side, are separated by a transparent spacer that is precisely one-half the desired wavelength. Light waves enter one side of the filter and are reflected at the second surface. Wavelengths that are twice the space between the two glass surfaces will reflect back and forth, reinforcing others of the same wavelengths, and finally passing on through. Other wavelengths will cancel out because of phase differences (destructive interference). Because interference filters also transmit multiples of the desired

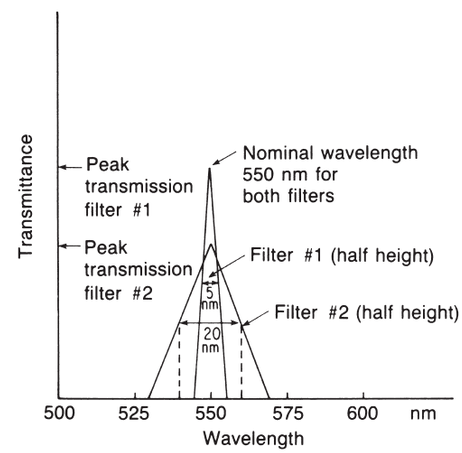


FIGURE 5-6. Spectral transmittance of two monochromators with band pass at half height of 5 nm and 20 nm.

wavelengths, they require accessory filters to eliminate these harmonic wavelengths. Interference filters can be constructed to pass a very narrow range of wavelengths with good efficiency.

The prism is another type of monochromator. A narrow beam of light focused on a prism is refracted as it enters the more dense glass. Short wavelengths are refracted more than long wavelengths, resulting in dispersion of white light into a continuous spectrum. The prism can be rotated, allowing only the desired wavelength to pass through an exit slit.

Diffraction gratings are most commonly used as monochromators. A diffraction grating consists of many parallel grooves (15,000 or 30,000 per inch) etched onto a polished surface. Diffraction, the separation of light into component wavelengths, is based on the principle that wavelengths bend as they pass a sharp corner. The degree of bending depends on the wavelength. As the wavelengths move past the corners, wave fronts are formed. Those that are in phase reinforce one another, whereas those not in phase cancel out and disappear. This results in complete spectra. Gratings with very fine line rulings produce a widely dispersed spectrum. They produce linear spectra, called orders, in both directions from the entrance slit. Because the multiple spectra have a tendency to cause stray light problems, accessory filters are used.

Sample Cell

The next component of the basic spectrophotometer is the sample cell or cuvet, which may be round or square. The light path must be kept constant to have absorbance proportional to concentration. This is easily checked by preparing a colored solution to read midscale when using the wavelength of maximum absorption. Fill each cuvet to be tested, take readings, and save those that match within an acceptable tolerance (e.g., ±0.25% T). Because it is difficult to manufacture round tubes with uniform diameters, they should be etched to indicate the position for use. Cuvets are sold in matched sets. Square cuvets have plane-parallel optical surfaces and a constant light path. They have an advantage over round cuvets in that there is less error from the lens effect, orientation in the spectrophotometer, and refraction. Cuvets with scratched optical surfaces scatter light and should be discarded. Inexpensive glass cuvets can be used for applications in the visible range, but they absorb light in the UV region. Quartz cuvets must, therefore, be used for applications requiring UV radiation.

Photodetectors

The purpose of the detector is to convert the transmitted radiant energy into an equivalent amount of electrical energy. The least expensive of the devices is known as a barrier-layer cell, or photocell. The photocell is composed of a film of light-sensitive material, frequently selenium, on a plate of iron. Over the light-sensitive material is a thin, transparent layer of silver. When exposed to light, electrons in the light-sensitive material are excited and released to flow to the highly conductive silver. In comparison with the silver, a moderate resistance opposes the electron flow toward the iron, forming a hypothetical barrier to flow in that direction. Consequently, this cell generates its own electromotive force, which can be measured. The produced current is proportional to incident radiation. Photocells require no external voltage source but rely on internal electron transfer to produce a current in an external circuit. Because of their low internal resistance, the output of electrical energy is not easily amplified. Consequently, this type of detector is used mainly in filter photometers with a wide bandpass, producing a fairly high level of illumination so that there is no need to amplify the signal. The photocell is inexpensive and durable; however, it is temperature sensitive and nonlinear at very low and very high levels of illumination.

A phototube (Fig. 5-7) is similar to a barrier-layer cell in that it has photosensitive material that gives off electrons when light energy strikes it. It differs in that an outside voltage is required for operation. Phototubes contain a negatively charged cathode and a positively charged anode enclosed in a glass case. The cathode is composed of a material (e.g., rubidium or lithium) that acts as a resistor in the dark but emits electrons when exposed to light. The emitted electrons jump over to the positively charged anode, where they are collected and return through an external, measurable circuit. The cathode usually has a large surface area. Varying the cathode material changes the wavelength at which the phototube gives its highest response. The photocurrent is linear, with the intensity of the light striking the cathode as long as voltage between the cathode and anode

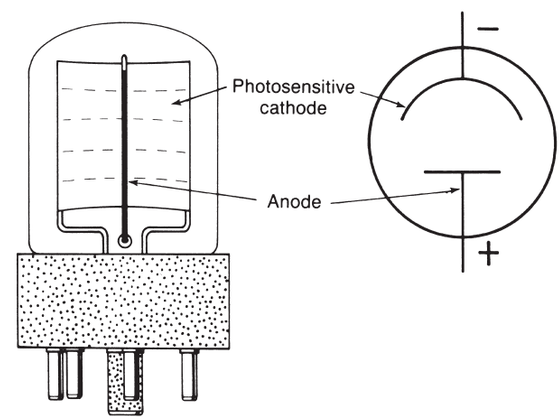


FIGURE 5-7. Phototube drawing and schematic.

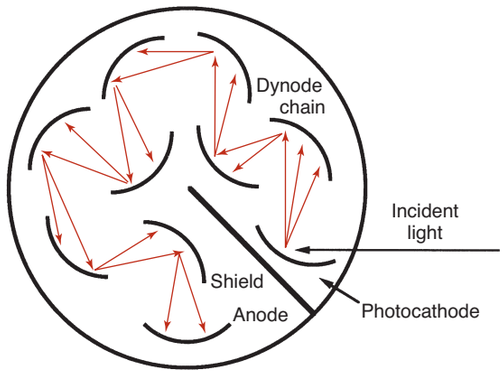


FIGURE 5-8. Dynode chain in a photomultiplier.

remains constant. A vacuum within the tubes avoids scattering of the photoelectrons by collision with gas molecules.

The third major type of light detector is the photomultiplier (PM) tube, which detects and amplifies radiant energy. As shown in Figure 5-8, incident light strikes the coated cathode, emitting electrons. The electrons are attracted to a series of anodes, known as dynodes, each having a successively higher positive voltage. These dynodes are of a material that gives off many secondary electrons when hit by single electrons. Initial electron emission at the cathode triggers a multiple cascade of electrons within the PM tube itself. Because of this amplification, the PM tube is 200 times more sensitive than the phototube. PM tubes are used in instruments designed to be extremely sensitive to very low light levels and light flashes of very short duration. The accumulation of electrons striking the anode produces a current signal, measured in amperes, that is proportional to the initial intensity of the light. The analog signal is converted first to a voltage and then to a digital signal through the use of an analog-to-digital (A/D) converter. Digital signals are processed electronically to produce absorbance readings.

In a photodiode, absorption of radiant energy by a reverse-biased pn-junction diode (*pn*, positive-negative) produces a photocurrent that is proportional to the incident radiant power. Although photodiodes are not as

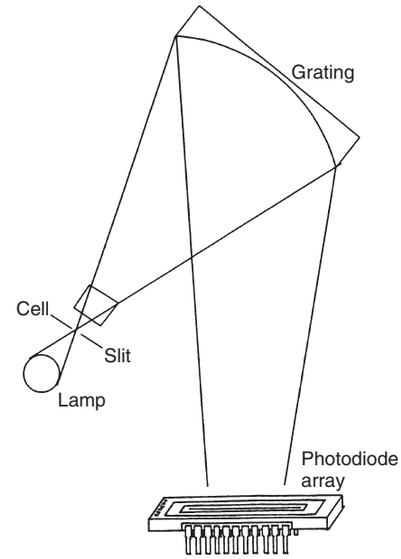


FIGURE 5-9. Photodiode array spectrophotometer illustrating the placement of the sample cuvet before the monochromator.

sensitive as PM tubes because of the lack of internal amplification, their excellent linearity (6–7 decades of radiant power), speed, and small size make them useful in applications where light levels are adequate.[4] Photodiode array (PDA) detectors are available in integrated circuits containing 256 to 2,048 photodiodes in a linear arrangement. A linear array is shown in Figure 5-9. Each photodiode responds to a specific wavelength, and as a result, a complete UV/visible spectrum can be obtained in less than 1 second. Resolution is 1 to 2 nm and depends on the number of discrete elements. In spectrophotometers using PDA detectors, the grating is positioned after the sample cuvet and disperses the transmitted radiation onto the PDA detector (Fig. 5-9).

For single-beam spectrophotometers, the absorbance reading from the sample must be blanked using an appropriate reference solution that does not contain the compound of interest. Double-beam spectrophotometers permit automatic correction of sample and reference absorbance, as shown in Figure 5-10. Because the

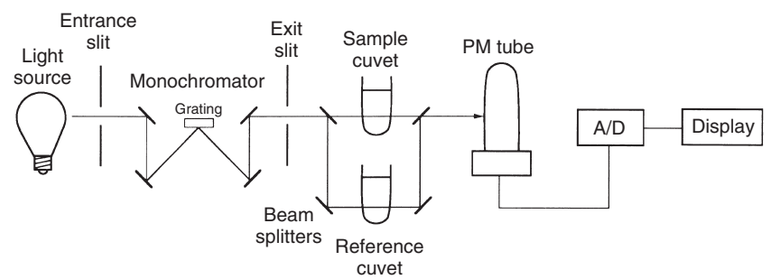


FIGURE 5-10. Double-beam spectrophotometer.

intensities of light sources vary as a function of wavelength, double-beam spectrophotometers are necessary when the absorption spectrum for a sample is to be obtained. Computerized, continuous zeroing, single-beam spectrophotometers have replaced most double-beam spectrophotometers.

Spectrophotometer Quality Assurance

Performing at least the following checks should validate instrument function: wavelength accuracy, stray light, and linearity. Wavelength accuracy means that the wavelength indicated on the control dial is the actual wavelength of light passed by the monochromator. It is most commonly checked using standard absorbing solutions or filters with absorbance maxima of known wavelength. Didymium or holmium oxide in glass is stable and frequently used as filters. The filter is placed in the light path and the wavelength control is set at the wavelength at which maximal absorbance is expected. The wavelength control is then rotated in either direction to locate the actual wavelength that has maximal absorbance. If these two wavelengths do not match, the optics must be adjusted to calibrate the monochromator correctly.

Some instruments with narrow bandpass use a mercury-vapor lamp to verify wavelength accuracy. The mercury lamp is substituted for the usual light source, and the spectrum is scanned to locate mercury emission lines. The wavelength indicated on the control is compared with known mercury emission peaks to determine the accuracy of the wavelength indicator control.

Stray light refers to any wavelengths outside the band transmitted by the monochromator. The most common causes of stray light are reflection of light from scratches on optical surfaces or from dust particles anywhere in the light path and higher-order spectra produced by diffraction gratings. The major effect is absorbance error, especially in the high-absorbance range. Stray light is detected by using cutoff filters, which eliminate all radiation at wavelengths beyond the one of interest. To check for stray light in the near-UV region, for example, insert a filter that does not transmit in the region of 200 nm to 400 nm. If the instrument reading is greater than 0% T, stray light is present. Certain liquids, such as $NiSO_4$, $NaNO_2$, and acetone, absorb strongly at short wavelengths and can be used in the same way to detect stray light in the UV range.

Linearity is demonstrated when a change in concentration results in a straight-line calibration curve, as discussed under Beer's law. Colored solutions may be carefully diluted and used to check linearity, using the wavelength of maximal absorbance for that color. Sealed sets of different colors and concentrations are available commercially. They should be labeled with expected absorbance for a given bandpass instrument. Less than expected absorbance is an indication of stray light or of a bandpass that is wider than specified. Sets of neutral-density filters to check linearity over a range of wavelengths are also commercially available.

A routine system should be devised for each instrument to check and record each parameter. The probable cause of a problem and the maintenance required to eliminate it are generally described in the instrument's manual.

Atomic Absorption Spectrophotometer

The atomic absorption spectrophotometer is used to measure concentration by detecting absorption of electromagnetic radiation by atoms rather than by molecules. The basic components are shown in Figure 5-11. The

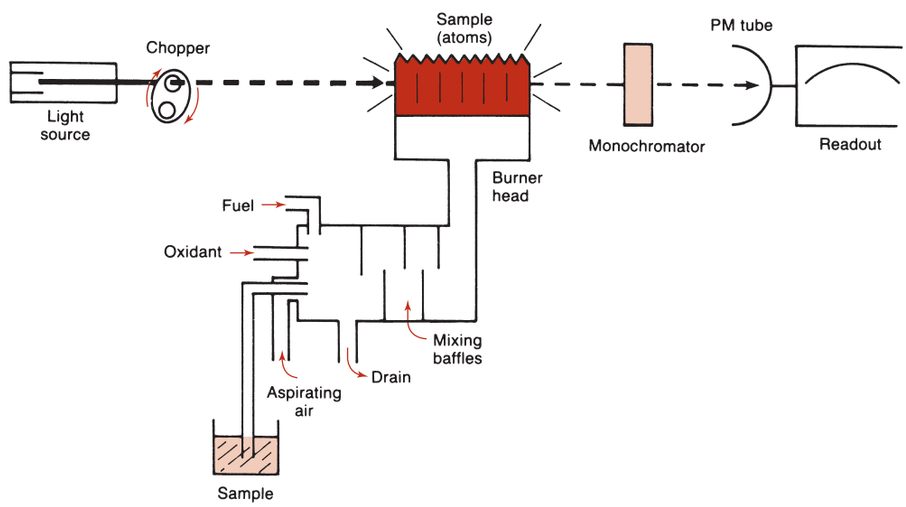


FIGURE 5-11. Single-beam atomic absorption spectrophotometer—basic components.

usual light source, known as a hollow-cathode lamp, consists of an evacuated gas-tight chamber containing an anode, a cylindrical cathode, and an inert gas, such as helium or argon. When voltage is applied, the filler gas is ionized. Ions attracted to the cathode collide with the metal, knock atoms off, and cause the metal atoms to be excited. When they return to the ground state, light energy is emitted that is characteristic of the metal in the cathode. Generally, a separate lamp is required for each metal (e.g., a copper hollow-cathode lamp is used to measure Cu).

Electrodeless discharge lamps are a relatively new light source for atomic absorption spectrophotometers. A bulb is filled with argon and the element to be tested. A radiofrequency generator around the bulb supplies the energy to excite the element, causing a characteristic emission spectrum of the element.

The analyzed sample must contain the reduced metal in the atomic vaporized state. Commonly, this is done by using the heat of a flame to break the chemical bonds and form free, unexcited atoms. The flame is the sample cell in this instrument, rather than a cuvet. There are various designs; however, the most common burner is the premix long-path burner. The sample, in solution, is aspirated as a spray into a chamber, where it is mixed with air and fuel. This mixture passes through baffles, where large drops fall and are drained off. Only fine droplets reach the flame. The burner is a long, narrow slit, to permit a longer path length for absorption of incident radiation. Light from the hollow-cathode lamp passes through the sample of ground-state atoms in the flame. The amount of light absorbed is proportional to the concentration. When a ground-state atom absorbs light energy, an excited atom is produced. The excited atom then returns to the ground state, emitting light of the same energy as it absorbed. The flame sample thus contains a dynamic population of ground-state and excited atoms, both absorbing and emitting radiant energy. The emitted energy from the flame will go in all directions, and it will be a steady emission. Because the purpose of the instrument is to measure the amount of light absorbed, the light detector must be able to distinguish between the light beam emitted by the hollow-cathode lamp and that emitted by excited atoms in the flame. To do this, the hollow-cathode light beam is modulated by inserting a mechanical rotating chopper between the light and the flame or by pulsing the electric supply to the lamp. Because the light beam being absorbed enters the sample in pulses, the transmitted light also will be in pulses. There will be less light in the transmitted pulses because part of it will be absorbed. There are, therefore, two light signals from the flame—an alternating signal from the hollow-cathode lamp and a direct signal from the flame emission. The measuring circuit is tuned to the modulated frequency. Interference from the constant flame emission is electronically eliminated by accepting only the pulsed signal from the hollow cathode.

The monochromator is used to isolate the desired emission line from other lamp emission lines. In addition, it serves to protect the photodetector from excessive light emanating from flame emissions. A PM tube is the usual light detector.

Flameless atomic absorption requires an instrument modification that uses an electric furnace to break chemical bonds (electrothermal atomization). A tiny graphite cylinder holds the sample, either liquid or solid. An electric current passes through the cylinder walls, evaporates the solvent, ashes the sample and, finally, heats the unit to incandescence to atomize the sample. This instrument, like the spectrophotometer, is used to determine the amount of light absorbed. Again, Beer's law is used for calculating concentration. A major problem is that background correction is considerably more necessary and critical for electrothermal techniques than for flame-based atomic absorption methods. Currently, the most common approach uses a deuterium lamp as a secondary source and measures the difference between the two absorbance signals. However, there has also been extensive development of background correction techniques based on the Zeeman effect.[1] The presence of an intense static magnetic field will cause the wavelength of the emitted radiation to split into several components. This shift in wavelength is the Zeeman effect.

Atomic absorption spectrophotometry is sensitive and precise. It is routinely used to measure concentration of trace metals that are not easily excited. It is generally more sensitive than flame emission because the vast majority of atoms produced in the usual propane or air-acetylene flame remain in the ground state available for light absorption. It is accurate, precise, and specific. One disadvantage, however, is the inability of the flame to dissociate samples into free atoms. For example, phosphate may interfere with calcium analysis by formation of calcium phosphate. This may be overcome by adding cations that compete with calcium for phosphate. Routinely, lanthanum or strontium is added to samples to form stable complexes with phosphate. Another possible problem is the ionization of atoms following dissociation by the flame, which can be decreased by reducing the flame temperature. Matrix interference, due to the enhancement of light absorption by atoms in organic solvents or formation of solid droplets as the solvent evaporates in the flame, can be another source of error. This interference may be overcome by pretreatment of the sample by extraction.[5]

Recently, inductively coupled plasma (ICP) has been used to increase sensitivity for atomic emission. The

torch, an argon plasma maintained by the interaction of a radiofrequency field and an ionized argon gas, is reported to have used temperatures between 5,500 K and 8,000 K. Complete atomization of elements is thought to occur at these temperatures. Use of inductively coupled plasma as a source is recommended for determinations involving refractory elements such as uranium, zirconium, and boron. ICP with MS detection is the most sensitive and specific assay technique for all elements on the periodic chart. Atomic absorption spectrophotometry is used less frequently because of this newer technology.

Flame Photometry

The flame-emission photometer, which measures light emitted by excited atoms, was widely used to determine concentration of Na^+, K^+, or Li^+. With the development of ion selective electrodes for these analytes, flame photometers are no longer routinely used in clinical chemistry laboratories. Discussion of this technique, therefore, is no longer included in this edition; the reader should refer to previous editions of this book.

Fluorometry

As seen with the spectrophotometer, light entering a solution may pass mainly on through or may be absorbed partly or entirely, depending on the concentration and the wavelength entering that particular solution. Whenever absorption occurs, there is a transfer of energy to the medium. Each molecular type possesses a series of electronic energy levels and can pass from a lower energy level to a higher level only by absorbing an integral unit (quantum) of light that is equal in energy to the difference between the two energy states. There are additional energy levels owing to rotation or vibration of molecular parts. The excited state lasts about 10^{-5} seconds before the electron loses energy and returns to the ground state. Energy is lost by collision, heat loss, transfer to other molecules, and emission of radiant energy. Because the molecules are excited by absorption of radiant energy and lose energy by multiple interactions, the radiant energy emitted is less than the absorbed energy. The difference between the maximum wavelengths, excitation, and emitted fluorescence is called Stokes shift. Both excitation (absorption) and fluorescence (emission) energies are characteristic for a given molecular type; for example, Figure 5-12 shows the absorption and fluorescence spectra of quinine in 0.1 N sulfuric acid. The dashed line on the left shows the short-wavelength excitation energy that is maximally absorbed, whereas the solid line on the right is the longer-wavelength (less energy) fluorescent spectrum.

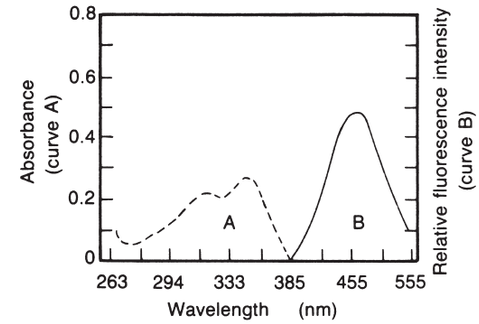


FIGURE 5-12. Absorption and fluorescence spectra of quinine in 0.1 N sulfuric acid. (Reprinted with permission from Coiner D. Basic concepts in laboratory instrumentation. Bethesda, Md.: ASMT Education and Research Fund, 1975–1979.)

Basic Instrumentation

Filter fluorometers measure the concentrations of solutions that contain fluorescing molecules. A basic instrument is shown in Figure 5-13. The source emits short-wavelength high-energy excitation light. A mechanical attenuator controls light intensity. The primary filter, placed between the radiation source and the sample, selects the wavelength that is best absorbed by the solution to be measured. The fluorescing sample in the cuvet emits radiant energy in all directions. The detector (placed at right angles to the sample cell) and a secondary filter that passes the longer wavelengths of fluorescent light prevent incident light from striking the photodetector. The electrical output of the photodetector is proportional to the intensity of fluorescent energy. In spectrofluorometers, the filters are replaced by prisms or grating monochromators.

Gas-discharge lamps (mercury and xenon-arc) are the most frequently used sources of excitation radiant energy. Incandescent tungsten lamps are seldom used because they release little energy in the UV region. Mercury-vapor lamps are commonly used in filter fluorometers. Mercury emits a characteristic line spectrum. Resonance lines at 365 nm to 366 nm are commonly used. Energy at wavelengths other than the resonance lines is provided by coating the inner surface of the lamp with a material that absorbs the 254-nm mercury radiation and emits a broad band of longer wavelengths. Most spectrofluorometers use a high-pressure xenon lamp. Xenon has a good continuum, which is necessary for determining excitation spectra.

Monochromator fluorometers use grating, prisms, or filters for isolation of incident radiation. Light detectors are almost exclusively PM tubes because of their higher sensitivity to low light intensities. Double-beam instruments are used to compensate for instability due to electric-power fluctuation.

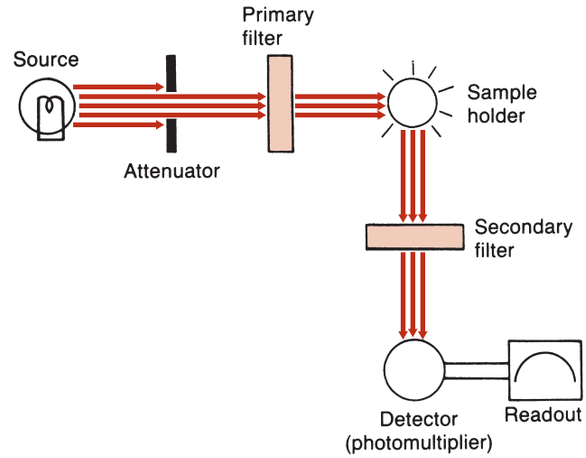


FIGURE 5-13. Basic filter fluorometer. (Reprinted with permission from Coiner D. Basic concepts in laboratory instrumentation. Bethesda, Md.: ASMT Education and Research Fund, 1975–1979.)

Fluorescence concentration measurements are related to molar absorptivity of the compound, intensity of the incident radiation, quantum efficiency of the energy emitted per quantum absorbed, and length of the light path. In dilute solutions with instrument parameters held constant, fluorescence is directly proportional to concentration. Generally, a linear response will be obtained until the concentration of the fluorescent species is so high that the sample begins to absorb significant amounts of excitation light. A curve demonstrating nonlinearity as concentration increases is shown in Figure 5-14. The solution must absorb less than 5% of the exciting radiation for a linear response to occur.[6] As with all quantitative measurements, a standard curve must be prepared to demonstrate that the concentration used falls in a linear range.

In fluorescence polarization, radiant energy is polarized in a single plane. When the sample (fluorophor) is excited, it emits polarized light along the same plane as the incident light if the fluorophor is attached to a large molecule. In contrast, a small molecule emits depolarized light because it will rotate out of the plane of polarization during its excitation lifetime. This technique is widely used for the detection of therapeutic and abused drugs. In the procedure, the sample analyte is allowed to compete with a fluorophor-labeled analyte for a limited antibody to the analyte. The lower the concentration of the sample analyte, the higher is the macromolecular

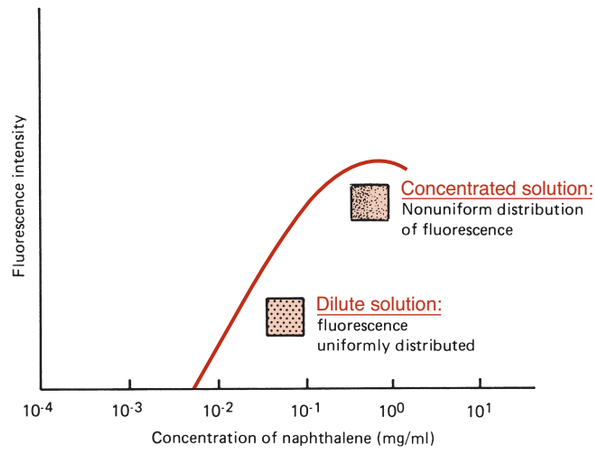


FIGURE 5-14. Dependence of fluorescence on the concentration of fluorophor. (Reprinted with permission from Guilbault GG. Practical fluorescence, theory, methods and techniques. New York, N.Y.: Marcel Dekker, 1973.)

antibody-analyte-fluorophor formed and the lower is the depolarization of the radiant light.

Advantages and Disadvantages of Fluorometry

Fluorometry has two advantages over conventional spectrophotometry: specificity and sensitivity. Fluorometry increases specificity by selecting the optimal wavelength for both absorption and fluorescence, rather than just the absorption wavelength seen with spectrophotometry.

Fluorometry is approximately 1,000 times more sensitive than most spectrophotometric methods.[6] One reason is because emitted radiation is measured directly; it can be increased simply by increasing the intensity of the exciting radiant energy. In addition, fluorescence measures the amount of light intensity present over a zero background. In absorbance, however, the quantity of absorbed light is measured indirectly as the difference between the transmitted beams. At low concentrations, the small difference between 100% T and the transmitted beam is difficult to measure accurately and precisely, limiting the sensitivity.

The biggest disadvantage is that fluorescence is very sensitive to environmental changes. Changes in pH affect availability of electrons, and temperature changes the probability of loss of energy by collision rather than fluorescence. Contaminating chemicals or a change of solvents may change the structure. UV light used for excitation can cause photochemical changes. Any decrease in fluorescence resulting from any of these possibilities is known as quenching. Because so many factors may change the intensity or spectra of fluorescence, extreme care is mandatory in analytic technique and instrument maintenance.

Chemiluminescence

In chemiluminescence reactions, part of the chemical energy generated produces excited intermediates that decay to a ground state with the emission of photons.[7] The emitted radiation is measured with a PM tube, and the signal is related to analyte concentration. Chemiluminescence is different than fluorescence in that no excitation radiation is required and no monochromators are needed because the chemiluminescence arises from one species. Most important, chemiluminescence reactions are oxidation reactions of luminol, acridinium esters, and dioxetanes characterized by a rapid increase in intensity of emitted light followed by a gradual decay. Usually, the signal is taken as the integral of the entire peak. Enhanced chemiluminescence techniques increase the chemiluminescence efficiency by including an enhancer system in the reaction of a chemiluminescent agent with an enzyme. The time course for the light intensity is much longer (60 minutes) than that for conventional chemiluminescent reactions, which last for about 30 seconds (Fig. 5-15).

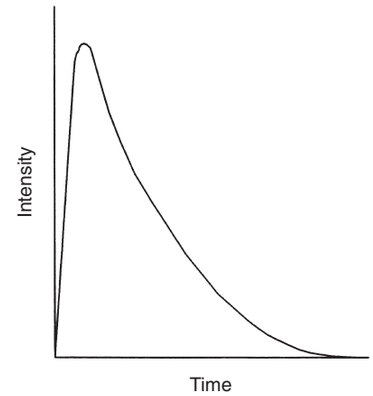


FIGURE 5-15. Representative intensity-versus-time curve for a transient chemiluminescence signal.

Advantages of chemiluminescence assays include subpicomolar detection limits, speed (with flash-type reactions, light is only measured for 10 seconds), ease of use (most assays are one-step procedures), and simple instrumentation.[7] The main disadvantage is that impurities can cause a background signal that degrades sensitivity and specificity.

Turbidity and Nephelometry

Turbidimetric measurements are made with a spectrophotometer to determine concentration of particulate matter in a sample. The amount of light blocked by a suspension of particles depends not only on concentration but also on size. Because particles tend to aggregate and settle out of suspension, sample handling becomes critical. Instrument operation is the same as for any spectrophotometer.

Nephelometry is similar, except that light scattered by the small particles is measured at an angle to the beam incident on the cuvet. Figure 5-16 demonstrates two possible optical arrangements for a nephelometer. Light scattering depends on wavelength and particle size. For macromolecules with a size close to or larger than the

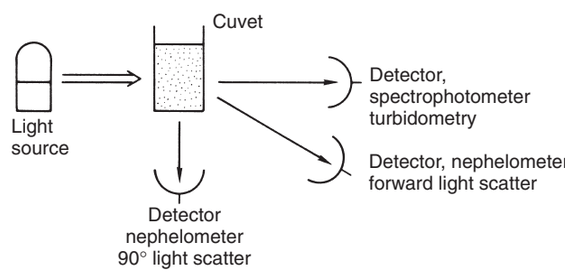


FIGURE 5-16. Nephelometer versus spectrophotometer—optical arrangements.

wavelength of incident light, sensitivity is increased by measuring forward light scatter.[8] Instruments are available with detectors placed at various forward angles, as well as at 90 degrees to the incident light. Monochromatic light obtains uniform scatter and minimizes sample heating. Certain instruments use lasers as a source of monochromatic light; however, any monochromator may be used.

Measuring light scatter at an angle other than at 180 degrees in turbidimetry minimizes error from colored solutions and increases sensitivity. Because both methods depend on particle size, some instruments quantitate initial change in light scatter rather than total scatter. Reagents must be free of any particles, and cuvets must be free of any scratches.

Laser Applications

Light amplification by stimulated emission of radiation (LASER) is based on the interaction of radiant energy and suitably excited atoms or molecules. The interaction leads to stimulated emission of radiation. The wavelength, direction of propagation, phase, and plane of polarization of the emitted light are the same as those of the incident radiation. Laser light is polarized and coherent and has narrow spectral width and small cross-sectional area with low divergence. The radiant emission can be very powerful and either continuous or pulsating.

Laser light can serve as the source of incident energy in a spectrometer or nephelometer. Some lasers produce bandwidths of a few kilohertz in both the visible and infrared regions, making these applications about three to six orders more sensitive than conventional spectrometers.[9]

Laser spectrometry also can be used for the determination of structure and identification of samples, as well as for diagnosis. Quantitation of samples depends on the spectrometer used. An example of the clinical application of the laser is the Coulter counter, which is used for differential analysis of white blood cells.[10]

ELECTROCHEMISTRY

Many types of electrochemical analyses are used in the clinical laboratory, including potentiometry, amperometry, coulometry, and polarography. The two basic electrochemical cells involved in these analyses are galvanic and electrolytic cells.

Galvanic and Electrolytic Cells

An electrochemical cell can be set up as shown in Figure 5-17. It consists of two half-cells and a salt bridge, which can be a piece of filter paper saturated with electrolytes. Instead of two as shown, the electrodes can be immersed in a single, large beaker containing a salt solution. In such a setup, the solution serves as the salt bridge.

In a galvanic cell, as the electrodes are connected, there is spontaneous flow of electrons from the electrode with the lower electron affinity (oxidation; e.g., silver). These electrons pass through the external meter to the cathode (reduction), where OH^- ions are liberated. This reaction continues until one of the chemical components is depleted, at which point, the cell is "dead" and cannot produce electrical energy to the external meter.

Current may be forced to flow through the dead cell only by applying an external electromotive force E. This is called an electrolytic cell. In short, a galvanic cell can be built from an electrolytic cell. When the external E is turned off, accumulated products at the electrodes will spontaneously produce current in the opposite direction of the electrolytic cell.

Half-Cells

It is impossible to measure the electrochemical activity of one half-cell; two reactions must be coupled and one reaction compared with the other. To rate half-cell reactions, a specific electrode reaction is arbitrarily assigned 0.00 V. Every other reaction coupled with this arbitrary zero reaction is either positive or negative, depending on the relative affinity for electrons. The electrode defined as 0.00 V is the standard hydrogen electrode: H_2

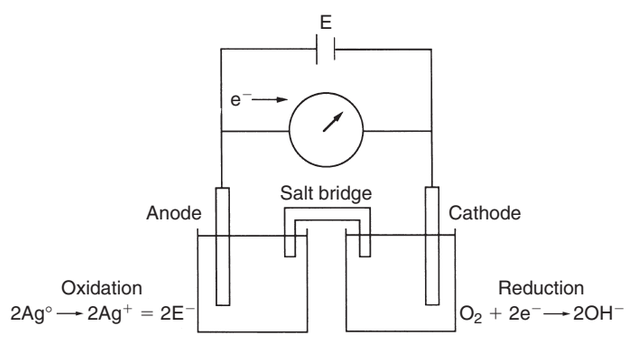


FIGURE 5-17. Electrochemical cell.

TABLE 5-1 STANDARD REDUCTION POTENTIALS

	POTENTIAL, V
$Zn^{2+} + 2e \leftrightarrow Z$	−0.7628
$Cr^{2+} + 2e \leftrightarrow Cr$	−0.913
$Ni^{2+} + 2e \leftrightarrow Ni$	−0.257
$2H^{+} + 2e \leftrightarrow H_2$	0.000
$Cu^{2+} + 2e \leftrightarrow Cu$	0.3419
$Ag^{+} + e \leftrightarrow Ag$	0.7996

Data presented are examples from Lide DR. CRC handbook of chemistry and physics. 83rd ed. Boca Raton, Fla.: CRC Press, 2003–2004.

gas at 1 atmosphere (atm). The hydrogen gas in contact with H^+ in solution develops a potential. The hydrogen electrode coupled with a zinc half-cell is cathodic, with the reaction $2H^+ + 2e^- \rightarrow H_2$, because H_2 has a greater affinity than Zn for electrons. Cu, however, has a greater affinity than H_2 for electrons, and thus the anodic reaction $H_2 \rightarrow 2H^+ + 2e^-$ occurs when coupled to the Cu-electrode half-cell.

The potential generated by the hydrogen-gas electrode is used to rate the electrode potential of metals in 1 mol/L solution. Reduction potentials for certain metals are shown in Table 5-1.[11] A hydrogen electrode is used to determine the accuracy of reference and indicator electrodes, the stability of standard solutions, and the potentials of liquid junctions.

Ion-Selective Electrodes

Potentiometric methods of analysis involve the direct measurement of electrical potential due to the activity of free ions. Ion-selective electrodes (ISEs) are designed to be sensitive toward individual ions.

pH Electrodes

An ISE universally used in the clinical laboratory is the pH electrode. The basic components of a pH meter are presented in Figure 5-18.

Indicator Electrode

The pH electrode consists of a silver wire coated with AgCl, immersed into an internal solution of 0.1 mmol/L HCl, and placed into a tube containing a special glass membrane tip. This membrane is only sensitive to hydrogen ions (H^+). Glass membranes that are selectively sensitive to H^+ consist of specific quantities of lithium, cesium, lanthanum, barium, or aluminum oxides in silicate. When the pH electrode is placed into the test solution, movement of H^+ near the tip of the electrode produces a potential difference between the internal solution and the test solution, which is measured as pH and read by a voltmeter. The combination pH electrode

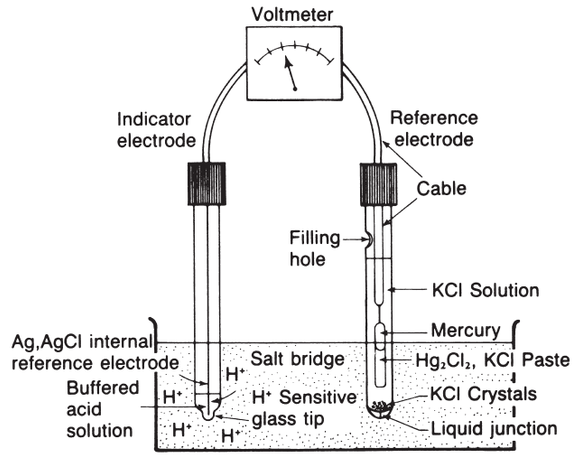


FIGURE 5-18. Necessary components of a pH meter.

also contains a built-in reference electrode, either Ag/AgCl or calomel (Hg/Hg_2Cl_2) immersed in a solution of saturated KCl.

The specially formulated glass continually dissolves from the surface. The present concept of the selective mechanism that causes formation of electromotive force at the glass surface is that an ion-exchange process is involved. Cationic exchange occurs only in the gel layer—there is no penetration of H^+ through the glass. Although the glass is constantly dissolving, the process is slow, and the glass tip generally lasts for several years. pH electrodes are highly selective for H^+; however, other cations in high concentration interfere, the most common of which is sodium. Electrode manufacturers should list the concentration of interfering cations that may cause error in pH determinations.

Reference Electrode

The reference electrode commonly used is the calomel electrode. Calomel, a paste of predominantly mercurous chloride, is in direct contact with metallic mercury in an electrolyte solution of potassium chloride. As long as the electrolyte concentration and the temperature remain constant, a stable voltage is generated at the interface of the mercury and its salt. A cable connected to the mercury leads to the voltmeter. The filling hole is needed for adding potassium chloride solution. A tiny opening at the bottom is required for completion of electric contact between the reference and indicator electrodes. The liquid junction consists of a fiber or ceramic plug that allows a small flow of electrolyte filling solution.

Construction varies, but all reference electrodes must generate a stable electrical potential. Reference electrodes generally consist of a metal and its salt in contact with a solution containing the same anion. Mercury/mercurous chloride, as in this example, is a frequently used

reference electrode; the disadvantage is that it is slow to reach a new stable voltage following temperature change and it is unstable above 80°C.[1,2] Ag/AgCl is another common reference electrode. It can be used at high temperatures, up to 275°C, and the AgCl-coated Ag wire makes a more compact electrode than that of mercury. In measurements in which chloride contamination must be avoided, a mercury sulfate and potassium sulfate reference electrode may be used.

Liquid Junctions

Electrical connection between the indicator and reference electrodes is achieved by allowing a slow flow of electrolyte from the tip of the reference electrode. A junction potential is always set up at the boundary between two dissimilar solutions because of positive and negative ions diffusing across the boundary at unequal rates. The resultant junction potential may increase or decrease the potential of the reference electrode. Therefore, it is important that the junction potential be kept to a minimum reproducible value when the reference electrode is in solution.

KCl is a commonly used filling solution because K^+ and Cl^- have nearly the same mobilities. When KCl is used as the filling solution for Ag/AgCl electrodes, the addition of AgCl is required to prevent dissolution of the AgCl salt. One way of producing a lower junction potential is to mix K^+, Na^+, NO_3^-, and Cl^- in appropriate ratios.

Readout Meter

Electromotive force produced by the reference and indicator electrodes is in the millivolt range. Zero potential for the cell indicates that each electrode half-cell is generating the same voltage, assuming there is no liquid junction potential. The isopotential is that potential at which a temperature change has no effect on the response of the electrical cell. Manufacturers generally achieve this by making midscale (pH 7.0) correspond to 0 V at all temperatures. They use an internal buffer whose pH changes due to temperature compensate for the changes in the internal and external reference electrodes.

Nernst Equation

The electromotive force generated because of H^+ at the glass tip is described by the Nernst equation, which is shown in a simplified form:

$$\varepsilon = \Delta\text{pH} \times \frac{RT \ln 10}{F} = \Delta\text{pH} \times 0.059 \text{ V} \quad \textbf{(Eq. 5-6)}$$

where ε is the electromotive force of the cell, F is the Faraday constant (96,500 C/mol), R is the molar gas constant, and T is temperature, in Kelvin.

As the temperature increases, H^+ activity increases and the potential generated increases. Most pH meters have a temperature-compensation knob that amplifies the millivolt response when the meter is on pH function. pH units on the meter scale are usually printed for use at room temperature. On the voltmeter, 59.16 is read as 1 pH unit change. The temperature compensation changes millivolt response to compensate for changes due to temperature from 54.2 at 0°C to 66.10 at 60°C. However, most pH meters are manufactured for greatest accuracy in the 10°C to 60°C range.

Calibration

The steps necessary to standardize a pH meter are fairly straightforward. First, balance the system with the electrodes in a buffer with a 7.0 pH. The balance or intercept control shifts the entire slope, as shown in Figure 5-19. Next, replace the buffer with one of a different pH. If the meter does not register the correct pH, amplification of the response changes the slope to match that predicted by the Nernst equation. If the instrument does not have a slope control, the temperature compensator performs the same function.

pH Combination Electrode

The most commonly used pH electrode has both the indicator and reference electrodes combined in one small probe, which is convenient when small samples are tested. It consists of an Ag/AgCl internal reference electrode sealed in a narrow glass cylinder with a pH-sensitive glass tip. The reference electrode is an Ag/AgCl wire

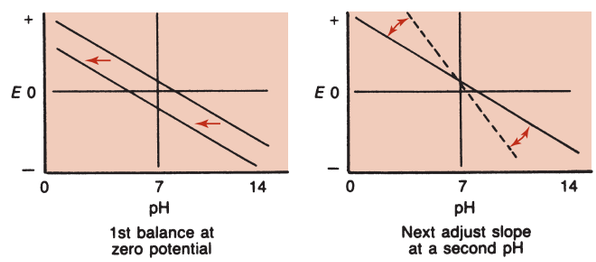


FIGURE 5-19. pH meter calibration. (Reprinted with permission from Willard HH, Merritt LL, Dean JA, Settle FA. Instrumental methods of analysis. Belmont, Calif.: Wadsworth, 1981.)

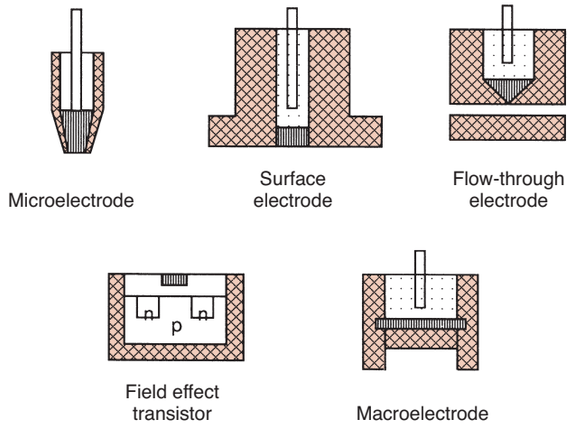


FIGURE 5-20. Other examples of ion-selective electrodes.

wrapped around the indicator electrode. The outer glass envelope is filled with KCl and has a tiny pore near the tip of the liquid junction. The solution to be measured must completely cover the glass tip. Examples of other ISEs are shown in Figure 5-20. The reference electrode, electrometer, and calibration system described for pH measurements are applicable to all ISEs.

There are three major ISE types: inert-metal electrodes in contact with a redox couple, metal electrodes that participate in a redox reaction, and membrane electrodes. The membrane can be solid material (e.g., glass), liquid (e.g., ion-exchange electrodes), or special membrane (e.g., compound electrodes), such as gas-sensing and enzyme electrodes.

The standard hydrogen electrode is an example of an inert-metal electrode. The Ag/AgCl electrode is an example of the second type. The electrode process $AgCl + e^- \rightarrow Ag^+ + Cl^-$ produces an electrical potential proportional to chloride ion (Cl^-) activity. When Cl^- is held constant, the electrode is used as a reference electrode. The electrode in contact with varying Cl^- concentrations is used as an indicator electrode to measure Cl^- concentration.

The H^+-sensitive gel layer of the glass pH electrode is considered a membrane. A change in the glass formulation makes the membrane more sensitive to sodium ions (Na^+) than to H^+, creating a sodium ISE. Other solid-state membranes consist of either a single crystal or fine crystals immobilized in an inert matrix such as silicone rubber. Conduction depends on a vacancy defect mechanism, and the crystals are formulated to be selective for a particular size, shape, and change—for example, F^--selective electrodes of LaF, Cl^--sensitive electrodes with AgCl crystals, and AgBr electrodes for the detection of Br^-.

The calcium ISE is a liquid-membrane electrode. An ion-selective carrier, such as dioctyphenyl phosphate dissolved in an inert water-insoluble solvent, diffuses through a porous membrane. Because the solvent is insoluble in water, the test sample cannot cross the membrane, but calcium ions (Ca^{2+}) are exchanged. The Ag/AgCl internal reference in a filling solution of $CaCl_2$ is in contact with the carrier by means of the membrane.

Potassium-selective liquid membranes use the antibiotic valinomycin as the ion-selective carrier. Valinomycin membranes show great selectivity for K^+. Liquid-membrane electrodes are recharged every few months to replace the liquid ion exchanger and the porous membrane.

Gas-Sensing Electrodes

Gas electrodes are similar to pH glass electrodes but are designed to detect specific gases (e.g., CO_2 and NH_3) in solutions and are usually separated from the solution by a thin, gas-permeable hydrophobic membrane. Figure 5-21 shows a schematic illustration of the pCO_2 electrode. The membrane in contact with the solution is permeable only to CO_2, which diffuses into a thin film of sodium bicarbonate solution. The pH of the bicarbonate solution is changed as follows:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \quad \textbf{(Eq. 5-7)}$$

The change in pH of the HCO_3^- is detected by a pH electrode. The pCO_2 electrode is widely used in clinical laboratories as a component of instruments for measuring serum electrolytes and blood gases.

In the NH_3 gas electrode, the bicarbonate solution is replaced by ammonium chloride solution, and the membrane is permeable only to NH_3 gas. As in the pCO_2 electrode, NH_3 changes the pH of NH_4Cl as follows:

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^- \quad \textbf{(Eq. 5-8)}$$

The amount of OH^- produced varies linearly with the log of the partial pressure of NH_3 in the sample.

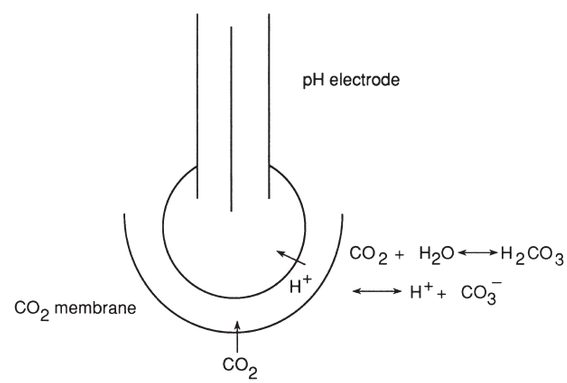


FIGURE 5-21. The pCO_2 electrode.

Other gas-sensing electrodes function on the basis of an amperometric principle—that is, measurement of the current flowing through an electrochemical cell at a constant applied electrical potential to the electrodes. Examples are the determination of pO_2, glucose, and peroxidase.

The chemical reactions of the pO_2 electrode (Clark electrode), an electrochemical cell with a platinum cathode and an Ag/AgCl anode, are illustrated in Figure 5-17. The electrical potential at the cathode is set to −0.65 V and will not conduct current without oxygen in the sample. The membrane is permeable to oxygen, which diffuses through to the platinum cathode. Current passes through the cell and is proportional to the pO_2 in the test sample.

Glucose determination is based on the reduction in pO_2 during glucose oxidase reaction with glucose and oxygen. Unlike the pCO_2 electrode, the peroxidase electrode has a polarized platinum anode and its potential is set to +0.6 V. Current flows through the system when peroxide is oxidized at the anode as follows:

$$H_2O_2 \rightarrow 2H^+ + 2e^- + O_2 \qquad \text{(Eq. 5-9)}$$

Enzyme Electrodes

The various ISEs may be covered by immobilized enzymes that can catalyze a specific chemical reaction. Selection of the ISE is determined by the reaction product of the immobilized enzyme. Examples include urease, which is used for the detection of urea, and glucose oxidase, which is used for glucose detection. A urea electrode must have an ISE that is selective for NH_4^+ or NH_3, whereas glucose oxidase is used in combination with a pH electrode.

Coulometric Chloridometers and Anodic Stripping Voltammetry

Chloride ISEs have largely replaced coulometric titrations for determination of chloride in body fluids. Anodic stripping voltammetry was widely used for analysis of lead and is best measured by electrothermal (graphite furnace) atomic absorption spectroscopy or, preferably, ICP-MS.

ELECTROPHORESIS

Electrophoresis is the migration of charged solutes or particles in an electrical field. Iontophoresis refers to the migration of small ions, whereas zone electrophoresis is the migration of charged macromolecules in a porous support medium such as paper, cellulose acetate, or agarose gel film. An electrophoretogram is the result of zone electrophoresis and consists of sharply separated zones of a macromolecule. In a clinical laboratory, the macromolecules of interest are proteins in serum, urine, cerebrospinal fluid (CSF), and other biologic body fluids and erythrocytes and tissue.

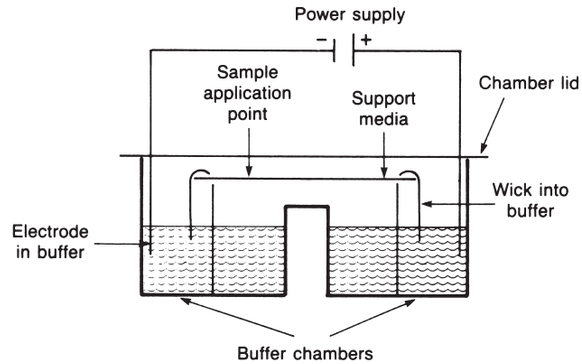


FIGURE 5-22. Electrophoresis apparatus—basic components.

Electrophoresis consists of five components: the driving force (electrical power), the support medium, the buffer, the sample, and the detecting system. A typical electrophoretic apparatus is illustrated in Figure 5-22.

Charged particles migrate toward the opposite charged electrode. The velocity of migration is controlled by the net charge of the particle, the size and shape of the particle, the strength of the electric field, chemical and physical properties of the supporting medium, and the electrophoretic temperature. The rate of mobility[12] of the molecule (μ) is given by

$$\mu = \frac{Q}{K} \leftrightarrow r \leftrightarrow n \qquad \text{(Eq. 5-10)}$$

where Q is net charge of particle, k is constant, r is ionic radius of the particle, and n is viscosity of the buffer.

From the equation, the rate of migration is directly proportional to the net charge of the particle and inversely proportional to its size and the viscosity of the buffer.

Procedure

The sample is soaked in hydrated support for approximately 5 minutes. The support is put into the electrophoresis chamber, which was previously filled with the buffer. Sufficient buffer must be added to the chamber to maintain contact with the support. Electrophoresis is carried out by applying a constant voltage or constant current for a specific time. The support is then removed and placed in a fixative or rapidly dried to prevent diffusion of the sample. This is followed by staining the zones with appropriate dye. The uptake of dye by the sample is proportional to sample concentration. After excess dye is washed away, the supporting medium may need to be placed in a clearing agent. Otherwise, it is completely dried.

Power Supply

Power supplies operating at either constant current or constant voltage are available commercially. In electrophoresis, heat is produced when current flows through a medium that has resistance, resulting in an increase in thermal agitation of the dissolved solute (ions) and leading to a decrease in resistance and an increase in current. The increase leads to increases in heat and evaporation of water from the buffer, increasing the ionic concentration of the buffer and subsequent further increases in the current. The migration rate can be kept constant by using a power supply with constant current. This is true because, as electrophoresis progresses, a decrease in resistance as a result of heat produced also decreases the voltage.

Buffers

Two buffer properties that affect the charge of ampholytes are pH and ionic strength. The ions carry the applied electric current and allow the buffer to maintain constant pH during electrophoresis. An ampholyte is a molecule, such as protein, whose net charge can be either positive or negative. If the buffer is more acidic than the isoelectric point (pI) of the ampholyte, it binds H^+, becomes positively charged, and migrates toward the cathode. If the buffer is more basic than the pI, the ampholyte loses H^+, becomes negatively charged, and migrates toward the anode. A particle without a net charge will not migrate, remaining at the point of application. During electrophoresis, ions cluster around a migrating particle. The higher the ionic concentration, the higher the size of the ionic cloud and the lower the mobility of the particle. Greater ionic strength produces sharper protein-band separation but leads to increased heat production. This may cause denaturation of heat-labile proteins. Consequently, the optimal buffer concentration should be determined for any electrophoretic system. Generally, the most widely used buffers are made of monovalent ions because their ionic strength and molality are equal.

Support Materials

Cellulose Acetate

Paper electrophoresis use has been replaced by cellulose acetate or agarose gel in clinical laboratories. Cellulose is acetylated to form cellulose acetate by treating it with acetic anhydride. Cellulose acetate, a dry, brittle film composed of about 80% air space, is produced commercially. When the film is soaked in buffer, the air spaces fill with electrolyte and the film becomes pliable. After electrophoresis and staining, cellulose acetate can be made transparent for densitometer quantitation. The dried transparent film can be stored for long periods. Cellulose acetate prepared to reduce electroendosmosis is available commercially. Cellulose acetate is also used in isoelectric focusing.

Agarose Gel

Agarose gel is another widely used supporting medium. Used as a purified fraction of agar, it is neutral and, therefore, does not produce electroendosmosis. After electrophoresis and staining, it is detained (cleared), dried, and scanned with a densitometer. The dried gel can be stored indefinitely. Agarose gel electrophoresis requires small amounts of sample (approximately 2 mL); it does not bind protein and, therefore, migration is not affected.

Polyacrylamide Gel

Polyacrylamide gel electrophoresis involves separation of protein on the basis of charge and molecular size. Layers of gel with different pore sizes are used. The gel is prepared before electrophoresis in a tube-shaped electrophoresis cell. The small-pore separation gel is at the bottom, followed by a large-pore spacer gel and, finally, another large-pore gel containing the sample. Each layer of gel is allowed to form a gelatin before the next gel is poured over it. At the start of electrophoresis, the protein molecules move freely through the spacer gel to its boundary with the separation gel, which slows their movement. This allows for concentration of the sample before separation by the small-pore gel. Polyacrylamide gel electrophoresis separates serum proteins into 20 or more fractions rather than the usual five fractions separated by cellulose acetate or agarose. It is widely used to study individual proteins (e.g., isoenzymes).

Starch Gel

Starch gel electrophoresis separates proteins on the basis of surface charge and molecular size, as does polyacrylamide gel. The procedure is not widely used because of technical difficulty in preparing the gel.

Treatment and Application of Sample

Serum contains a high concentration of protein, especially albumin and, therefore, serum specimens are routinely diluted with buffer before electrophoresis. In contrast, urine and CSF are usually concentrated. Hemoglobin hemolysate is used without further concentration. Generally, preparation of a sample is done according to the suggestion of the manufacturer of the electrophoretic supplies.

Cellulose acetate and agarose gel electrophoresis require approximately 2 to 5 mL of sample. These are the most common routine electrophoreses performed in clinical laboratories. Because most commercially manufactured plates come with a thin plastic template that has small slots through which samples are applied, overloading of agarose gel with sample is not a frequent problem. After serum is allowed to diffuse into the gel for approximately 5 minutes, the template is blotted to remove

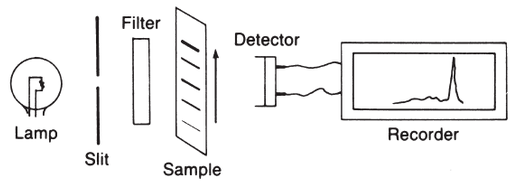


FIGURE 5-23. Densitometer—basic components.

excess serum before being removed from the gel surface. Sample is applied to cellulose acetate with a twin-wire applicator designed to transfer a small amount.

Detection and Quantitation

Separated protein fractions are stained to reveal their locations. Different stains come with different plates from different manufacturers. The simplest way to accomplish detection is visualization under UV light, whereas densitometry is the most common and reliable way for quantitation. Most densitometers will automatically integrate the area under a peak, and the result is printed as percentage of the total. A schematic illustration of a densitometer is shown in Figure 5-23.

Electroendosmosis

The movement of buffer ions and solvent relative to the fixed support is called endosmosis or electroendosmosis. Support media, such as paper, cellulose acetate, and agar gel, take on a negative charge from adsorption of hydroxyl ions. When current is applied to the electrophoresis system, the hydroxyl ions remain fixed while the free positive ions move toward the cathode. The ions are highly hydrated, resulting in net cathodic movement of solvent. Molecules that are nearly neutral are swept toward the cathode with the solvent. Support media such as agarose and acrylamide gel are essentially neutral, eliminating electroendosmosis. The position of proteins in any electrophoresis separation depends not only on the nature of the protein but also on all other technical variables.

Isoelectric Focusing

Isoelectric focusing is a modification of electrophoresis. An apparatus is used similar to that shown in Figure 5-24. Charged proteins migrate through a support medium that has a continuous pH gradient. Individual proteins move in the electric field until they reach a pH equal to their isoelectric point, at which point they have no charge and cease to move.

Capillary Electrophoresis

In capillary electrophoresis (CE), separation is performed in narrow-bore fused silica capillaries (inner diameter, 2575 mm). Usually, the capillaries are only filled with buffer, although gel media can also be used. A CE instrumentation schematic is shown in Figure 5-24. Initially, the capillary is filled with buffer and then the sample is loaded; applying an electric field performs the separation. Detection can be made near the other end of the capillary directly through the capillary wall.[13]

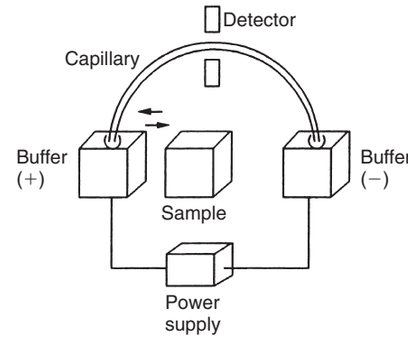


FIGURE 5-24. Schematic of capillary electrophoresis instrumentation. Sample is loaded on the capillary by replacing the anode buffer reservoir with the sample reservoir. (Reprinted with permission from Heiger DN. High-performance capillary electrophoresis. Waldbronn, Germany: Hewlett-Packard, 1992.)

A fundamental CE concept is the electro-osmotic flow (EOF). EOF is the bulk flow of liquid toward the cathode upon application of electric field and it is superimposed on electrophoretic migration. EOF controls the amount of time solutes remain in the capillary. Cations migrate fastest because both EOF and electrophoretic attraction are toward the cathode; neutral molecules are all carried by the EOF but are not separated from each other; and anions move slowest because, although they are carried to the cathode by the EOF, they are attracted to the anode and repelled by the cathode (Fig. 5-25). Widely used for monitoring separated analytes, UV-visible detection is performed directly on the capillary; however, sensitivity is poor because of the small dimensions of the capillary, resulting in a short path length. Fluorescence, laser-induced fluorescence, and chemiluminescence detection can be used for higher sensitivity.

CE has been used for the separation, quantitation, and determination of molecular weights of proteins and peptides; for the analysis of polymerase chain reaction (PCR) products; and for the analysis of inorganic ions, organic acids, pharmaceuticals, optical isomers, and drugs of abuse in serum and urine.[14]

CHROMATOGRAPHY

Chromatography refers to the group of techniques used to separate complex mixtures on the basis of different physical interactions between the individual compounds and the stationary phase of the system. The basic components

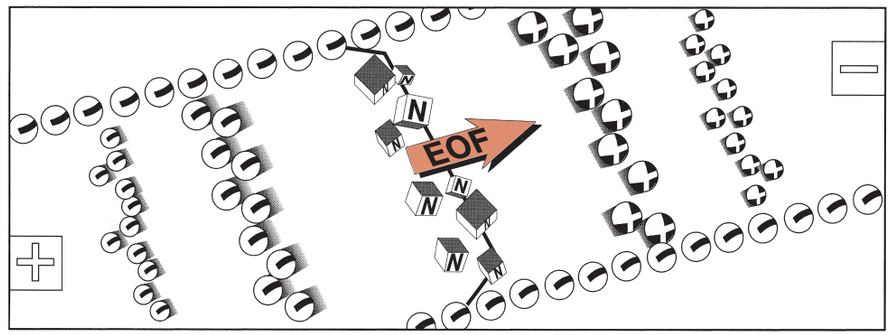


FIGURE 5-25. Differential solute migration superimposed on electro-osmotic flow in capillary zone electrophoresis. (Reprinted with permission from Heiger DN. High-performance capillary electrophoresis. France: Hewlett-Packard, 1992.)

in any chromatographic technique are the mobile phase (gas or liquid), which carries the complex mixture (sample); the stationary phase (solid or liquid), through which the mobile phase flows; the column holding the stationary phase; and the separated components (eluate).

Modes of Separation

Adsorption

Adsorption chromatography, also known as liquid-solid chromatography, is based on the competition between the sample and the mobile phase for adsorptive sites on the solid stationary phase. There is an equilibrium of solute molecules being adsorbed to the solid surface and desorbed and dissolved in the mobile phase. The molecules that are most the soluble in the mobile phase, move fastest; the least soluble, move slowest. Thus, a mixture is typically separated into classes according to polar functional groups. The stationary phase can be either acidic polar (e.g., silica gel), basic polar (e.g., alumina), or nonpolar (e.g., charcoal). The mobile phase can be a single solvent or a mixture of two or more solvents, depending on the analytes to be desorbed. Liquid-solid chromatography is not widely used in clinical laboratories because of technical problems with the preparation of a stationary phase that has homogeneous distribution of absorption sites.

Partition

Partition chromatography is also referred to as liquid-liquid chromatography. Separation of solute is based on relative solubility in an organic (nonpolar) solvent and an aqueous (polar) solvent. In its simplest form, partition (extraction) is performed in a separatory funnel. Molecules containing polar and nonpolar groups in an aqueous solution are added to an immiscible organic solvent. After vigorous shaking, the two phases are allowed to separate. Polar molecules remain in the aqueous solvent; nonpolar molecules are extracted in the organic solvent. This results in the partitioning of the solute molecules into two separate phases.

The ratio of the concentration of the solute in the two liquids is known as the partition coefficient:

$$K = \frac{\text{solute in stationary phase}}{\text{solute in mobile phase}} \qquad \textbf{(Eq. 5-11)}$$

Modern partition chromatography uses pseudo liquid stationary phases that are chemically bonded to the support or high-molecular-weight polymers that are insoluble in the mobile phase.[15] Partition systems are considered normal phase when the mobile solvent is less polar than the stationary solvent and reverse phase when the mobile solvent is more polar.

Partition chromatography is applicable to any substance that may be distributed between two liquid phases. Because ionic compounds are generally soluble only in water, partition chromatography works best with nonionic compounds.

Steric Exclusion

Steric exclusion, a variation of liquid-solid chromatography, is used to separate solute molecules on the basis of size and shape. The chromatographic column is packed with porous material, as shown in Figure 5-26. A sample containing different-sized molecules moves down the column dissolved in the mobile solvent. Small molecules enter the pores in the packing and are momentarily trapped. Large molecules are excluded from the small pores and so move quickly between the particles. Intermediate-sized molecules are partially restricted from entering the pores and, therefore, move through the column at an intermediate rate that is between those of the large and small molecules.

Early methods used hydrophilic beads of cross-linked dextran, polyacrylamide, or agarose, which formed a gel when soaked in water. This method was termed gel filtration. A similar separation process using hydrophobic

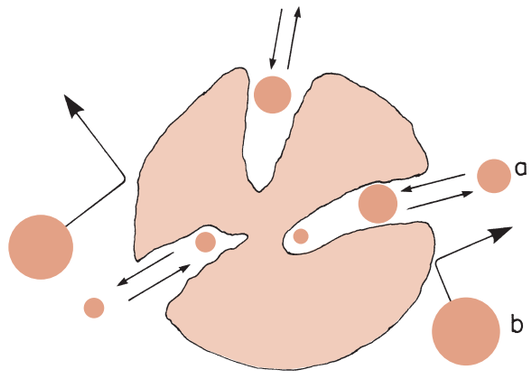


FIGURE 5-26. Pictorial concept of steric exclusion chromatography. Separation of sample components by their ability to permeate pore structure of column-packing material. Smaller molecules **(a)** permeating the interstitial pores; large excluded molecules **(b).** (Reprinted with permission from Parris NA. Instrumental liquid chromatography: a practical manual on high performance liquid chromatographic methods. New York, N.Y.: Elsevier, 1976.)

gel beads of polystyrene with a nonaqueous mobile phase was called gel permeation chromatography. Current porous packing uses rigid inorganic materials such as silica or glass. The term steric exclusion includes all these variations. Pore size is controlled by the manufacturer, and packing materials can be purchased with different pore sizes, depending on the size of the molecules being separated.

Ion-Exchange Chromatography

In ion-exchange chromatography, solute mixtures are separated by virtue of the magnitude and charge of ionic species. The stationary phase is a resin, consisting of large polymers of substituted benzene, silicates, or cellulose derivatives, with charge functional groups. The resin is insoluble in water, and the functional groups are immobilized as side chains on resin beads that are used to fill the chromatographic column. Figure 5-27*A* shows resin with sulfonate functional groups. Hydrogen$^+$ ions are loosely held and free to react. This is an example of a cation-exchange resin. When a cation such as Na^+ comes in contact with these functional groups, an equilibrium is formed, following the law of mass action. Because there are many sulfonate groups, Na^+ is effectively and completely removed from solution. The Na^+ concentrated on the resin column can be eluted from the resin by pouring acid through the column, driving the equilibrium to the left.

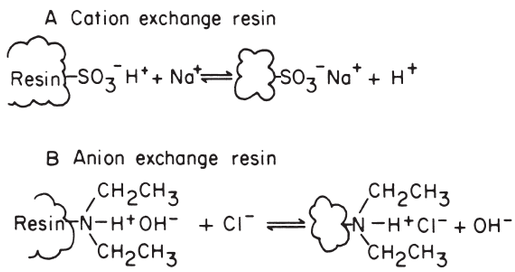


FIGURE 5-27. Chemical equilibrium of ion-exchange resins. **(A)** Cation exchange resin. **(B)** Anion exchange resin.

Anion-exchange resins are made with exchangeable hydroxyl ions such as the diethylamine functional group illustrated in Figure 5-27*B*. They are used like cation-exchange resins, except that hydroxyl ions are exchanged for anions. The example shows Cl^- in sample solution exchanged for OH^- from the resin functional group. Anion and cation resins mixed together (mixed-bed resin) are used to deionize water. The displaced protons and hydroxyl ions combine to form water. Ionic functional groups other than the illustrated examples are used for specific analytic applications. Ion-exchange chromatography is used to remove interfering substances from a solution, to concentrate dilute ion solutions, and to separate mixtures of charged molecules, such as amino acids. Changing pH and ionic concentration of the mobile phase allows separation of mixtures of organic and inorganic ions.

Chromatographic Procedures

Thin-Layer Chromatography

Thin-layer chromatography (TLC) is a variant of column chromatography. A thin layer of sorbent, such as alumina, silica gel, cellulose, or cross-linked dextran, is uniformly coated on a glass or plastic plate. Each sample to be analyzed is applied as a spot near one edge of the plate, as shown in Figure 5-28. The mobile phase (solvent) is usually placed in a closed container until the atmosphere is saturated with solvent vapor. One edge of the plate is placed in the solvent, as shown. The solvent migrates up the thin layer by capillary action, dissolving and carrying sample molecules. Separation can be achieved by any of the four processes previously described, depending on the sorbent (thin layer) and solvent chosen. After the solvent reaches a predetermined height, the plate is removed and dried. Sample components are identified by comparison with standards on the same plate. The distance a component migrates, compared with the distance the solvent front moves, is called the retention factor, R_f:

$$R_f = \frac{\text{distance leading edge of component moves}}{\text{total distance solvent front moves}} \quad \textbf{(Eq. 5-12)}$$

Each sample-component R_f is compared with the R_f of standards. Using Figure 5-28 as an example, standard A has an R_f value of 0.4, standard B has an R_f value of 0.6, and standard C has a value of 0.8. The first unknown

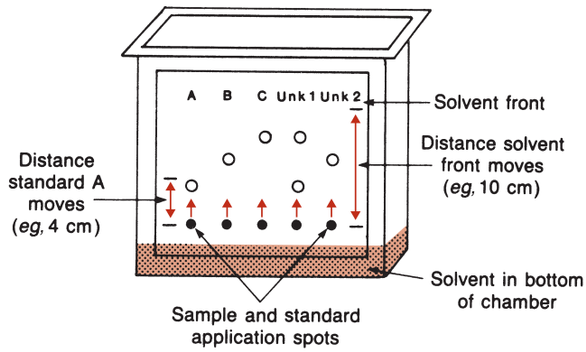


FIGURE 5-28. TLC plate in chromatographic chamber.

contains A and C, because the R_f values are the same. This ratio is valid only for separations run under identical conditions. Because R_f values may overlap for some components, further identifying information is obtained by spraying different stains on the dried plate and comparing colors of the standards.

TLC is most commonly used as a semiquantitative screening test. Technique refinement has resulted in the development of semiautomated equipment and the ability to quantitate separated compounds. For example, sample applicators apply precise amounts of sample extracts in concise areas. Plates prepared with uniform sorbent thickness, finer particles, and new solvent systems have resulted in the technique of high-performance thin-layer chromatography (HPTLC).[16] Absorbance of each developed spot is measured using a densitometer, and the concentration is calculated by comparison with a reference standard chromatographed under identical conditions.

High-Performance Liquid Chromatography

Modern liquid chromatography uses pressure for fast separations, controlled temperature, in-line detectors, and gradient elution techniques.[17,18] Figure 5-29 illustrates the basic components.

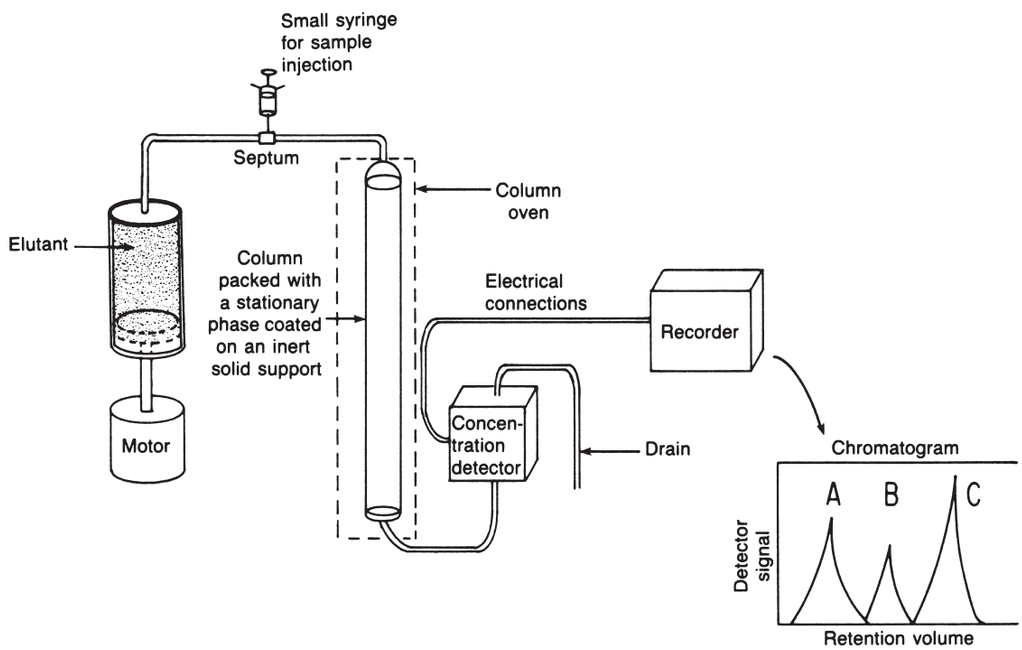


FIGURE 5-29. HPLC basic components. (Reprinted with permission from Bender GT. Chemical instrumentation: a laboratory manual based on clinical chemistry. Philadelphia, Pa.: WB Saunders, 1972.)

Pumps

A pump forces the mobile phase through the column at a much greater velocity than that accomplished by gravity-flow columns and includes pneumatic, syringe, reciprocating, or hydraulic amplifier pumps. The most widely used pump today is the mechanical reciprocating pump, which is now used as a multihead pump with two or more reciprocating pistons. During pumping, the pistons operate out of phase (180 degrees for two heads, 120 degrees for three heads) to provide constant flow. Pneumatic pumps are used for preoperative purposes; hydraulic amplifier pumps are no longer commonly used.

Columns

The stationary phase is packed into long stainless steel columns. HPLC is usually run at ambient temperatures, although columns can be put in an oven and heated to enhance the rate of partition. Fine, uniform column packing results in much less band broadening but requires pressure to force the mobile phase through. The packing also can be either pellicular (an inert core with a porous layer), inert and small particles, or macroporous particles. The most common material used for column packing is silica gel. It is very stable and can be used in different ways. It can be used as solid packing in liquid-solid chromatography or coated with a solvent, which serves as the stationary phase (liquid-liquid). As a result of the short lifetime of coated particles, molecules of the mobile-phase liquid are now bonded to the surface of silica particles.

Reversed-phase HPLC is now popular; the stationary phase is nonpolar molecules (e.g., octadecyl C-18 hydrocarbon) bonded to silica gel particles. For this type of column packing, the mobile phase commonly used is acetonitrile, methanol, water, or any combination of solvents. A reversed-phase column can be used to separate ionic, nonionic, and ionizable samples. A buffer is used to produce the desired ionic characteristics and pH for separation of the analyte. Column packings vary in size (3 mm to 20 mm), using smaller particles mostly for analytic separations and larger ones for preparative separations.

Sample Injectors

A small syringe can be used to introduce the sample into the path of the mobile phase that carries it into the column (Fig. 5-29). The best and most widely used method, however, is the loop injector. The sample is introduced into a fixed-volume loop. When the loop is switched, the sample is placed in the path of the flowing mobile phase and flushed onto the column.

Loop injectors have high reproducibility and are used at high pressures. Many HPLC instruments have loop injectors that can be programmed for automatic injection of samples. When the sample size is less than the volume of the loop, the syringe containing the sample is often filled with the mobile phase to the volume of the loop before filling the loop. This prevents the possibility of air being forced through the column because such a practice may reduce the lifetime of the column packing material.

Detectors

Modern HPLC detectors monitor the eluate as it leaves the column and, ideally, produce an electronic signal proportional to the concentration of each separated component. Spectrophotometers that detect absorbances of visible or UV light are most commonly used. PDA and other rapid scanning detectors are also used for spectral comparisons and compound identification and purity. These detectors have been used for drug analyses in urine. Obtaining a UV scan of a compound as it elutes from a column can provide important information as to its identity. Unknowns can be compared against library spectra in a similar manner to MS. Unlike **gas chromatography** (GC)/MS, which requires volatilization of targeted compounds, liquid chromatography/photodiode array (LC/PDA) enables direct injection of aqueous urine samples.

Because many biologic substances fluoresce strongly, fluorescence detectors are also used, involving the same principles discussed in the section on spectrophotometric measurements. Another common HPLC detector is the amperometric or electrochemical detector, which measures current produced when the analyte of interest is either oxidized or reduced at some fixed potential set between a pair of electrodes.

A mass spectrometer (MS) can also be used as a detector, as described later.

Recorders

The recorder is used to record detector signal versus the time the mobile phase passed through the instrument, starting from the time of sample injection. The graph is called a chromatogram (Fig. 5-30). The retention time is used to identify compounds when compared with standard retention times run under identical conditions. Peak area is proportional to concentration of the compounds that produced the peaks.

When the elution strength of the mobile phase is constant throughout the separation, it is called isocratic elution. For samples containing compounds of widely differing relative compositions, the choice of solvent is a compromise. Early eluting compounds may have retention times close to zero, producing a poor separation (resolution), as shown in Figure 5-30*A*. Basic compounds often have low retention times because C-18 columns cannot tolerate high pH mobile phases. The addition of cation-pairing reagents to the mobile phase

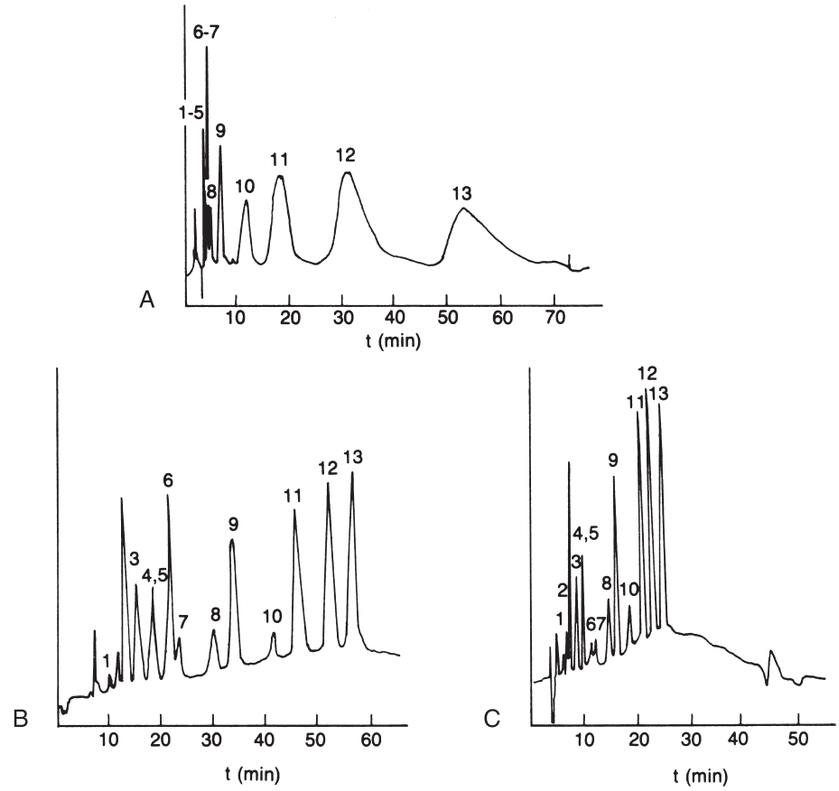


FIGURE 5-30. Chromatograms. **(A)** Isocratic ion-exchange separation mobile phase contains 0.055 mol/L $NaNO_3$. **(B)** Gradient elution mobile phase gradient from 0.01 to 0.1 mol/L $NaNO_3$ at 2% per minute. **(C)** Gradient elution—5% per minute. (Reprinted with permission from Horváth C. High performance liquid chromatography, advances and perspectives. New York, N.Y.: Academic Press, 1980.)

(e.g., octane sulfonic acid) can result in better retention of negatively charged compounds onto the column.

The late-eluting compounds may have long retention times, producing broad bands resulting in decreased sensitivity. In some cases, certain components of a sample may have such a great affinity for the stationary phase that they do not elute at all. Gradient elution is an HPLC technique that can be used to overcome this problem. The composition of the mobile phase is varied to provide a continual increase in the solvent strength of the mobile phase entering the column (Fig. 5-30*B*). The same gradient elution can be performed with a faster change in concentration of the mobile phase (Fig. 5-30*C*).

Gas Chromatography

Gas chromatography is used to separate mixtures of compounds that are volatile or can be made volatile.[19] Gas chromatography may be gas-solid chromatography (GSC), with a solid stationary phase, or gas-liquid chromatography (GLC), with a nonvolatile liquid stationary phase. GLC is commonly used in clinical laboratories. Figure 5-31 illustrates the basic components of a GC system. The setup is similar to HPLC, except that the mobile phase is a gas and samples are partitioned between a gaseous mobile phase and a liquid stationary phase. The carrier gas can be nitrogen, helium, or argon. The selection of a carrier gas is determined by the detector used in the instrument. The instrument can be operated at a constant temperature or programmed to run at different temperatures if a sample has components with different volatilities. This is analogous to gradient elution described for HPLC.

The sample, which is injected through a septum, must be injected as a gas or the temperature of the injection port must be above the boiling point of the components so that they vaporize upon injection. Sample vapor is swept through the column partially as a gas and partially dissolved in the liquid phase. Volatile compounds that are present mainly in the gas phase will have a low partition coefficient and will move

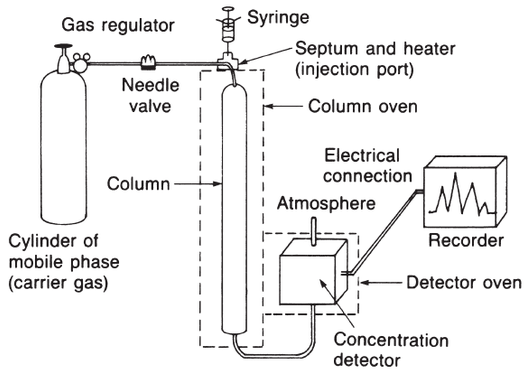


FIGURE 5-31. GLC basic components. (Reprinted with permission from Bender GT. Chemical instrumentation: a laboratory manual based on clinical chemistry. Philadelphia, Pa.: WB Saunders, 1972.)

quickly through the column. Compounds with higher boiling points will move slowly through the column. The effluent passes through a detector that produces an electric signal proportional to the concentration of the volatile components. As in HPLC, the chromatogram is used both to identify the compounds by the retention time and to determine their concentration by the area under the peak.

Columns

GLC columns are generally made of glass or stainless steel and are available in a variety of coil configurations and sizes. Packed columns are filled with inert particles such as diatomaceous earth or porous polymer or glass beads coated with a nonvolatile liquid (stationary) phase. These columns are usually ⅛ to ¼ inch wide and 3 to 12 feet long. Capillary-wall coated open tubular columns have inside diameters in the range of 0.25 mm to 0.50 mm and are up to 60 m long. The liquid layer is coated on the walls of the column. A solid support coated with a liquid stationary phase may in turn be coated on column walls.

The liquid stationary phase must be nonvolatile at the temperatures used, must be thermally stable, and must not react chemically with the solutes to be separated. The stationary phase is termed nonselective when separation is primarily based on relative volatility of the compounds. Selective liquid phases are used to separate polar compounds based on relative polarity (as in liquid-liquid chromatography).

Detectors

Although there are many types of detectors, only thermal conductivity (TC) and flame ionization detectors are discussed because they are the most stable (Fig. 5-32). TC detectors contain wires (filaments) that change electrical

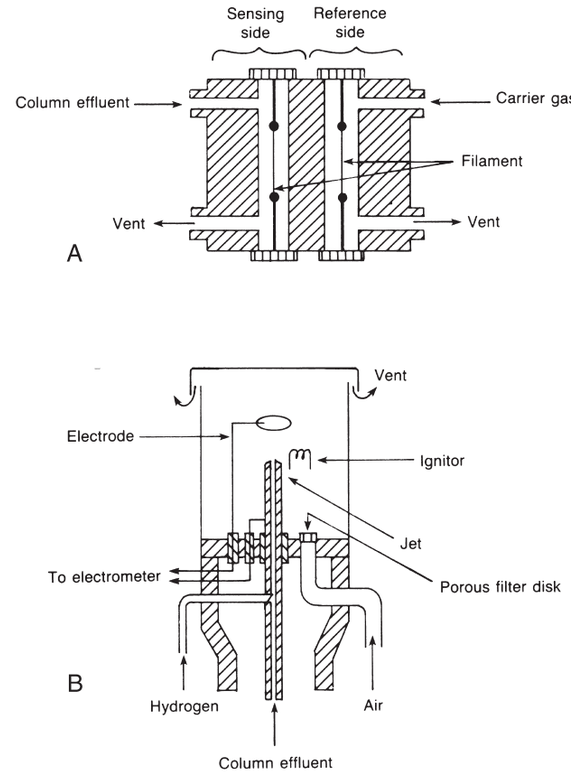


FIGURE 5-32. (A) Schematic diagram of a thermal conductivity detector. **(B)** Schematic diagram of a flame ionization detector. (Reprinted with permission from Tietz NW, ed. Fundamentals of clinical chemistry. Philadelphia, Pa.: WB Saunders, 1987.)

resistance with change in temperature. The filaments form opposite arms of a Wheatstone bridge and are heated electrically to raise their temperature. Helium, which has a high thermal conductivity, is usually the carrier gas. Carrier gas from the reference column flows steadily across one filament, cooling it slightly. Carrier gas and separated compounds from the sample column flow across the other filament. The sample components usually have a lower thermal conductivity, increasing the temperature and resistance of the sample filament. The change in resistance results in an unbalanced bridge circuit. The electrical change is amplified and fed to the recorder. The electrical change is proportional to the concentration of the analyte.

Flame ionization detectors are widely used in the clinical laboratory. They are more sensitive than TC detectors. The column effluent is fed into a small hydrogen flame burning in excess air or atmospheric oxygen. The flame jet and a collector electrode around the flame have opposite potentials. As the sample burns, ions form and move to the charged collector. Thus, a current

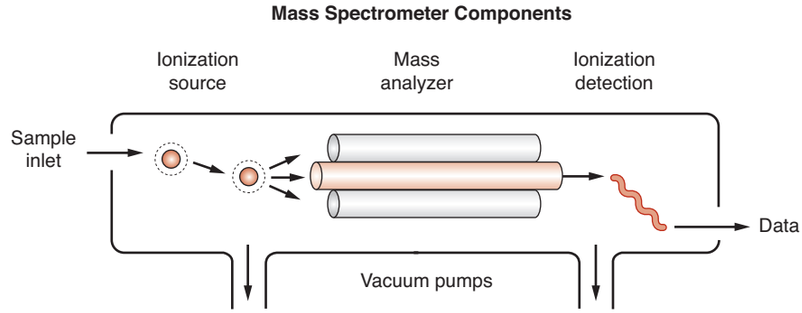


FIGURE 5-33. The components of a mass spectrometer. In this case, the ionization source pictured is electrospray ionization and the mass analyzer is a quadrupole.

proportional to the concentration of the ions is formed and fed to the recorder.

MASS SPECTROMETRY

Definitive identification of samples eluting from GC or HPLC columns is possible when an MS is used as a detector.[20] The coupled techniques, GC/MS and LC/MS, have powerful analytic capabilities with widespread clinical applications. The sample in an MS is first volatilized and then ionized to form charged molecular ions and fragments that are separated according to their mass-to-charge (*m/z*) ratio; the sample is then measured by a detector, which gives the intensity of the ion current for each species. These steps take place in the four basic components that are standard in all MSs: the sample inlet, ionization source, mass analyzer, and ion detector (Fig. 5-33). Ultimately, molecule identification is based on the formation of characteristic fragments. Figure 5-34 illustrates the mass spectrum of Δ9-carboxytetrahydrocannabinol, a metabolite of marijuana.

Sample Introduction and Ionization

Direct infusion is commonly used to interface a GC or LC with an MS; however, the challenge of introducing a liquid sample from an LC column into an MS was a significant barrier until recent technological advances in ionization techniques.

Electron Ionization

The most common form of ionization used in GC/MS is electron ionization (EI). This method requires a source of electrons in the form of a filament to which an electric potential is applied, typically at 70 electron volts.[21] The molecules in the source are bombarded with high-energy electrons, resulting in the formation of charged molecular ions and fragments. Molecules break down into characteristic fragments according to their molecular structure (Fig. 5-35). The ions formed and their relative proportions are reproducible and can be used for qualitative identification of the compound. Since most instruments use the same 70 eV potential, the fragmentation of molecules on different days and different

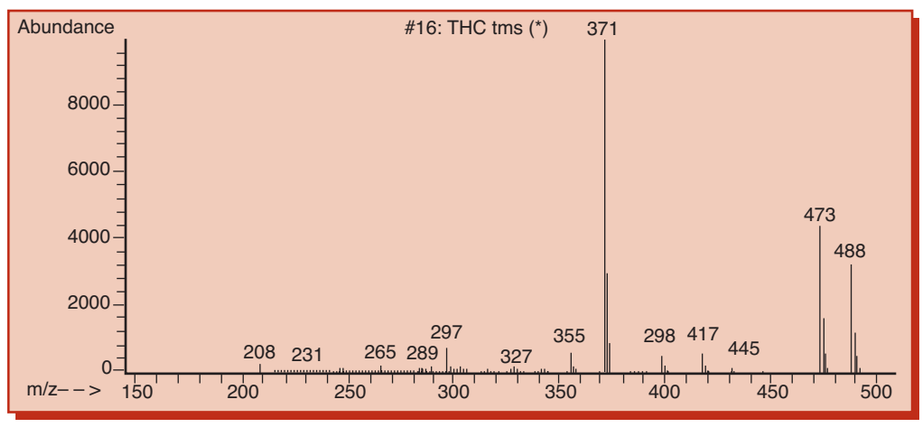


FIGURE 5-34. Mass spectrum of the trimethylsilane derivative of Δ9-carboxytetrahydrocannabinol (marijuana metabolite).

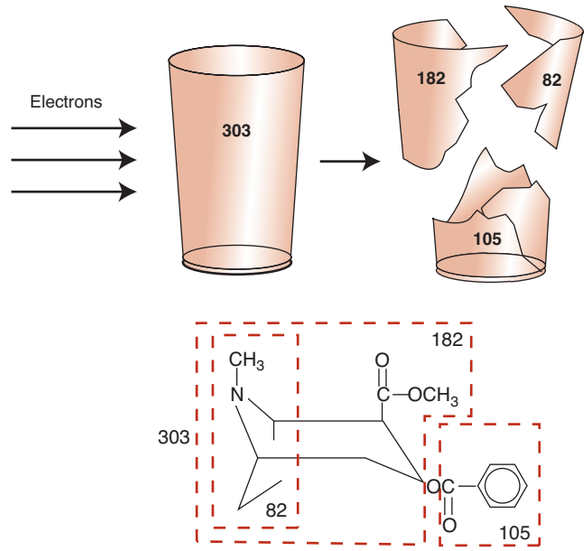


FIGURE 5-35. Electron bombardment breaks cocaine into fragments, with number and size quantified. Unlike the illustrative glass tumbler, the result of mass fragmentation of cocaine or other chemical compounds is both predictable and reproducible, especially with electron ionization.

instruments is remarkably similar, allowing the comparison of unknown spectra to spectra in a published reference library.[21]

Atmospheric Pressure Ionization

Unlike EI in GC/MS, most LC/MS ionization techniques are conducted at atmospheric pressure. As such, the ion source of this type of instrument is not included in the high vacuum region of the instrument. Two types of ionization for LC/MS will be discussed here: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), while matrix-assisted laser desorption (MALDI) and surface-enhanced laser desorption (SELDI) ionization will be discussed later in the section on proteomics. ESI and APCI also differ from EI in that they are "soft" ionization techniques that leave the molecular ion largely intact in the source. Many LC/MS techniques employ technologies after the source, in the mass analyzer, to fragment molecules and generate the "fingerprint" spectra used in identification. However, ionization techniques used in LC/MS produce fragments and therefore mass spectra that are somewhat less reproducible between instruments than EI used in GC/MS. This may prove to limit the utility of reference library spectra produced on other instruments.

Electrospray Ionization

Thanks to its wide mass range and high sensitivity, ESI can be applied to a wide range of biological macromolecules in addition to small molecules and has become the most common ionization source for LC/MS.

ESI involves passing the LC effluent through a capillary to which a voltage has been applied. The energy is transferred to the solvent droplets, which become charged.[21] Evaporation of the solvent through heat and gas causes the droplets to decrease in size, which increases the charge density on the surface. Eventually, the Coulombic repulsion of like charges lead to the ejection of ions from the droplet[22] (Fig. 5-36). The individually charged molecules are drawn into the MS for mass analysis.

ESI is adept at forming singly charged small molecules, but larger molecules can also be ionized using this method. Larger molecules such as proteins become multiply charged in ESI, and since MSs measure the *m/z,* even these large molecules can be observed in an instrument with a relatively small mass range[22] (Fig. 5-37).

Atmospheric Pressure Chemical Ionization

Another important ionization source is APCI, which is similar to ESI in that the liquid from LC is introduced directly into the ionization source. However, the droplets are not charged and the source contains a heated vaporizer to allow rapid desolvation of the drops.[22] A high voltage is applied to a corona discharge needle, which emits a cloud of electrons to ionize compounds after they are converted to the gas phase.

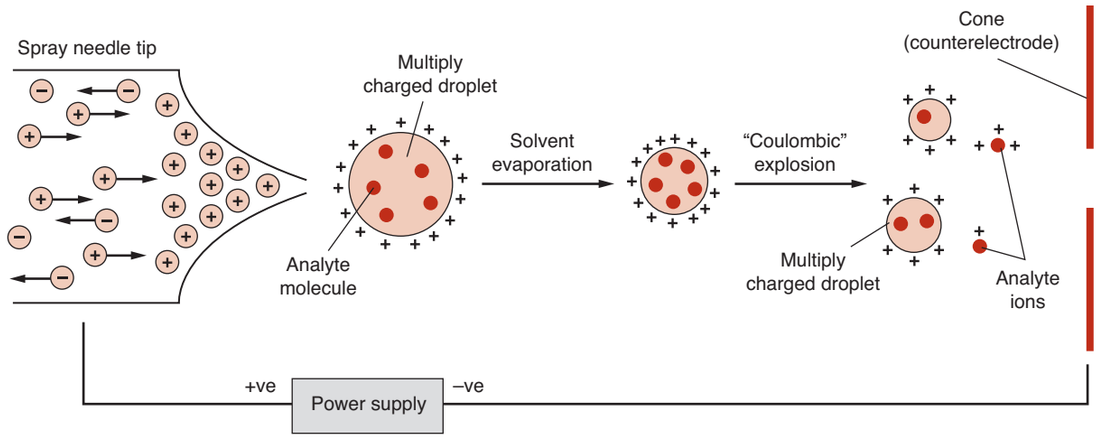


FIGURE 5-36. Diagram of electrospray ionization, the most common ionization source for LC/MS.

Mass Analyzer

The actual measuring of the *m/z* occurs when the gas phase ions pass into the mass analyzer. Two types of mass analyzers will be discussed here: the quadrupole and ion trap, while time-of-flight (TOF) will be discussed later in the proteomics section. Both quadrupole and ion trap mass analyzers contain rods or plates that are charged with varying AC and DC voltages to form electric fields. These electric fields manipulate the charged molecules to sort them according to their *m/z*.

Quadrupole

A diagram of a quadrupole MS is shown in Figure 5-38. The quadrupole is the most common mass analyzer in use today. The electric field on the two sets of diagonally opposed rods allows only ions of a single selected *m/z* value to pass through the analyzer to the detector. All other ions are deflected into the rods. The rods can be scanned from low to high mass to allow ions of increasing mass to form stable sinusoidal orbits and traverse the filtering sector. This technique will generate a full scan mass spectrum. Alternatively, specific masses can be selected to monitor a few target analytes. This technique is called selected ion monitoring (SIM) and it allows for a longer dwell time (time spent monitoring a single ion) and therefore higher sensitivity.[21] A full scan provides more information than SIM since ions not specifically selected in SIM are not detected. Therefore, a full scan would be preferable for general unknown screening while SIM analysis is more suitable for target compound analysis.

Ion Trap

The ion trap can be thought of as a modified quadrupole. A linear ion trap employs a stopping potential on the end

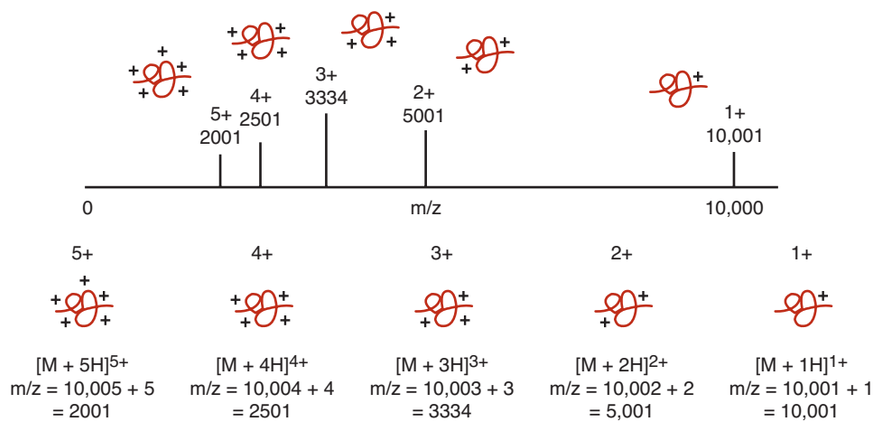


FIGURE 5-37. A theoretical protein with a molecular weight of 10,000 can be multiply charged, which will generate numerous peaks. A mass spectrometer with a relatively small mass range can still detect the multiply charged ions since the *m/z* is reduced.

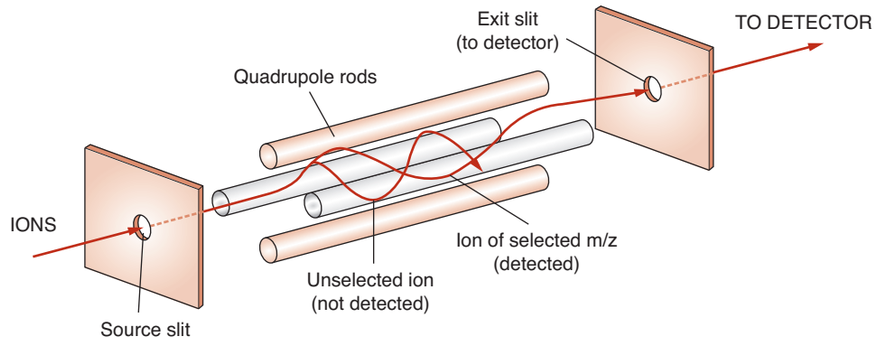


FIGURE 5-38. Single quadrupole mass spectrometer.

electrodes to confine ions along the two-dimensional axis of the quadrupoles. In a three-dimensional ion trap, the four rods, instead of being arranged parallel to each other, form a three-dimensional sphere in which ions are "trapped." In all ion traps, after a period of accumulation, the electric field adjusts to selectively destabilize the trapped ions, which are mass-selectively ejected from the cavity to the detector based on their *m/z*.[21] The unique feature of ion trap MSs is that they trap and store ions generated over time, effectively concentrating the ions of interest and yielding a greater sensitivity.

MS/MS

Tandem MSs (GC/MS/MS and LC/MS/MS) can be used for greater selectivity and lower detection limits. A common form of MS/MS is to link three quadrupoles in series; such an instrument is referred to as a triple quad (Fig. 5-39). Generally, each quadrupole has a separate function.[22] Following an appropriate ionization method, the first quadrupole (Q1) is used to scan across a preset *m/z* range and select an ion of interest. The second quadrupole (Q2) functions as a collision cell. In a process called collision-induced dissociation (CID), the ions are accelerated to high kinetic energy and allowed to collide with neutral gas molecules (usually nitrogen, helium, or argon) to fragment the ions. The single ion passed through the first analyzer is called the precursor (or parent) ion while the ions formed during fragmentation of the precursor ions are called product (or daughter) ions. The third quadrupole (Q3) serves to analyze the product ions generated in Q2. This last quadrupole can be set to scan all of the product ions to produce a full product ion scan, or to selectively allow one or more of these product ions through to the

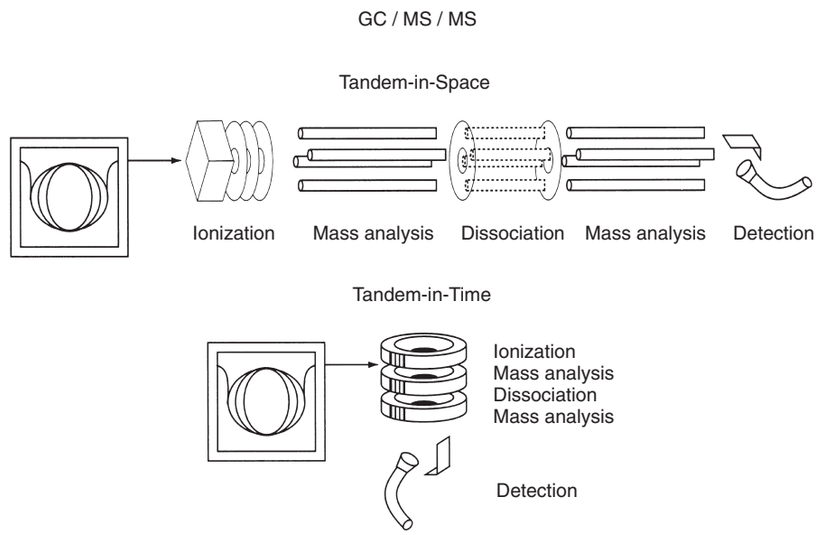


FIGURE 5-39. Triple quadrupole mass spectrometer.

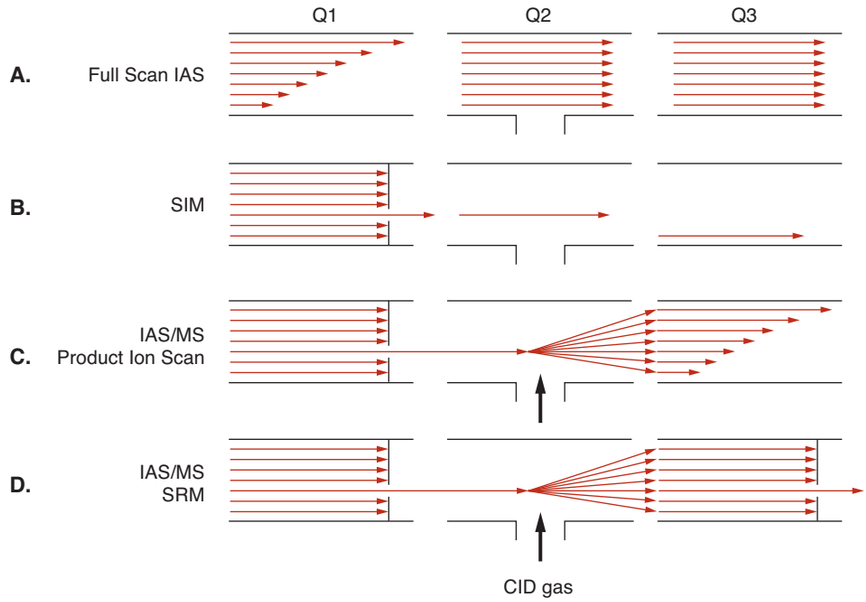


FIGURE 5-40. Scanning modes used in a triple quadrupole mass spectrometer. **(A)** Full scan MS detects all ions. **(B)** Selected ion monitoring (SIM) detects ions of one selected *m/z*. **(C)** Product ion scans select ions of one *m/z* in Q1 to pass on to Q2, the collision cell, where the ion is fragmented. All ion fragments are allowed to pass through to the detector. **(D)** Selected reaction monitoring (SRM) is similar to the product ion scan, but only fragments of one selected *m/z* are allowed to pass on to the detector. Both **C** and **D** are examples of tandem mass spectrometry (MS/MS).

detector in a process called selected reaction monitoring (SRM). Various scanning modes commonly used in a triple quad are shown in Figure 5-40. In some triple quad instruments, the third quadrupole can also function as a linear ion trap to add further sensitivity to MS/MS.

Detector

The most common means of detecting ions employs an electron multiplier (Fig. 5-41). In this detector, a series of dynodes with increasing potentials are linked. When ions strike the first dynode surface, electrons are emitted. These electrons are attracted to the next dynode where more secondary electrons are emitted due to the higher potential of subsequent dynodes. A cascade of electrons is formed by the end of the chain of dynodes, resulting in overall signal amplification on the order of 1 million or greater.[22]

Applications of Mass Spectrometry in the Clinical Laboratory

Mass spectrometers coupled to GC or LC can be used not only for the identification and quantitation of compounds but also for structural information and molecular weight

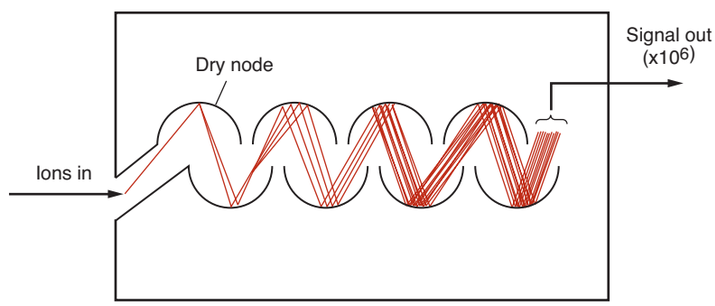


FIGURE 5-41. Diagram of an electron multiplier detector in a mass spectrometer.

determination.[23] GC/MS systems are widely used for measuring drugs of abuse in urine toxicology confirmations. Drugs and metabolites must be extracted from body fluids and typically reacted with derivatizing reagents to form compounds that are more volatile for the GC process. Computerized libraries and matching algorithms are available within the instrument to compare mass spectral results of an unknown substance obtained from a sample to the reference library.

Increasingly, LC/MS (including LC/MS/MS) technology is taking its place alongside GC/MS in clinical laboratories. LC offers a number of advantages over GC. Typically, LC requires less extensive extraction procedures and derivatization is rarely used, saving time and expense. In addition, polar and heat-labile compounds fare better in LC.[21] However, the chromatography itself in LC can be somewhat less robust than in GC, resulting in wider peaks, more variable retention times and potentially requiring more frequent maintenance. Another disadvantage of LC/MS is the less reproducible mass spectra, as mentioned earlier.

Besides its use in toxicology, LC/MS also has great potential for measuring low-level and mixed-polarity analytes such as vitamin D, testosterone, and immunosuppressant drugs due to its superior sensitivity and specificity over immunoassays. In addition, LC/MS has the advantage of being able to detect multiple analytes (such as a panel of drugs or a series of metabolites) in one run. LC/MS is free from the antibody interferences seen in immunoassays, although LC/MS has its own type of interference in the form of ion suppression. This effect is seen when a co-eluting chemical in the sample prevents a compound of interest from being ionized, thereby reducing or eliminating its signal. LC/MS also requires highly skilled operators and is not nearly as automated as immunoassay instruments.

INSTRUMENTATION FOR PROTEOMICS

The next generation of biomarkers for human diseases will be discovered using techniques found within the research fields of genomics and proteomics. Genomics use the known sequences of the entire human genome for determining the role of genetics in certain human diseases. Proteomics is the investigation of the protein products encoded by these genes. Protein expression is equal to and, in many cases, more important for disease detection than genomics because these products determine what is currently occurring within a cell, rather than the genes, which indicate what a cell might be capable of performing. Moreover, many (posttranslational) changes can occur to the protein, as influenced by other proteins and enzymes that cannot be easily predicted by knowledge at the genomic level.

A "shotgun" approach is often used in the discovery of new biochemical markers. The proteins from samples (e.g., serum, urine, tissue extract) from normal individuals are compared with those derived from patients with the disease being studied. Techniques such as two-dimensional electrophoresis can be used to separate proteins into individual spots or bands. Proteins that only appear in either the normal or diseased specimens are further studied. Computer programs are available that digitally compare gels to determine spots or areas that are different. When candidate proteins have been found, the spots can be isolated and subjected to sophisticated MS analysis to identify the protein and possibly any posttranslational modifications that may have occurred. Using this approach, the researcher does not have any preconceptions or biases as to what directions or particular proteins to look for.

Two-Dimensional Electrophoresis

This electrophoresis assay combines two different electrophoresis dimensions to separate proteins from complex matrices such as serum or tissue. In the first dimension, proteins are resolved according to their isoelectric points (pIs), using immobilized pH gradients. Commercial gradients are available in a variety of pH ranges. In the second dimension, proteins are separated according to their relative size (molecular weight), using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A schematic of this procedure is shown in Figure 5-42. Gels can be run under denaturing or nondenaturing conditions (e.g., for the maintenance of enzyme activity) and visualized by a variety of techniques, including the use of colorimetric dyes (e.g., Coomassie blue or silver stain), radiographic, fluorometric, or chemiluminescence of appropriately labeled polypeptides. These latter techniques are considerably more sensitive than the colorimetric dyes.

MALDI-TOF and SELDI-TOF Mass Spectrometry

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) is used for the analysis of biomolecules, such peptides and proteins. Protein samples, such as those isolated from a two-dimensional electrophoretogram, are mixed with an appropriate matrix solvent and spotted onto a stainless steel plate. The solvent is dried and the plate is introduced into the vacuum system of the MALDI-TOF analyzer. As shown in Figure 5-43, a laser pulse irradiates the sample causing desorption and ionization of both the matrix and sample. Because the monitored mass spectral range is high (>500 daltons), the ionization

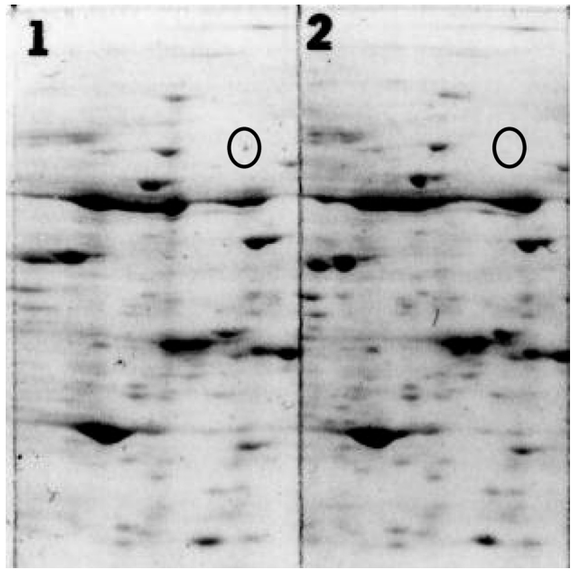


FIGURE 5-42. Hypothetical example of a two-dimensional electrophoretogram from a patient with a disease (*panel 1*) compared with a normal subject (*panel 2*). The patient exhibits a protein (*oval*) that is not expressed in the normal subject. This protein might be a potential marker for this disease. (Gels courtesy of Kendrick Laboratories, Madison, Wis.)

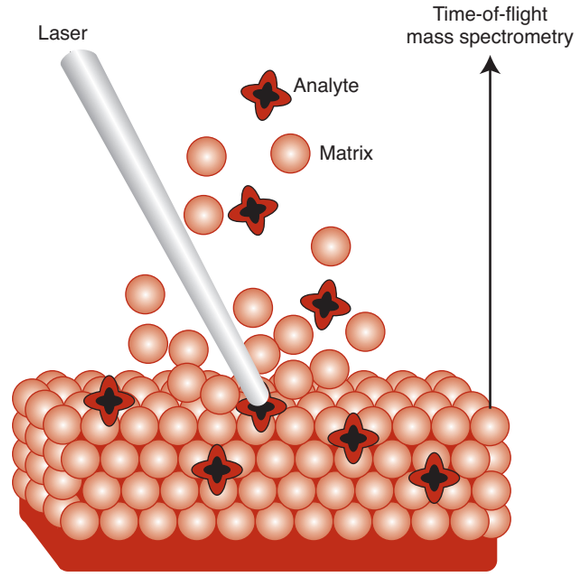


FIGURE 5-43. Sample desorption process prior to MALDI-TOF analysis. (Diagram courtesy of Stanford Research Systems, Sunnyvale, Ca.)

of the low-molecular-weight matrix can be readily distinguished from high-molecular-weight peptides and proteins and do not interfere with the assay of the protein. Ions from the sample are focused into the mass spectrum. The time required for a mass to reach the detector is a nonlinear function of the mass, with larger ions requiring more time than smaller ions. The molecular weight of the proteins acquired by mass spectrum is used to determine the identity of the sample and is helpful in determining posttranslational modifications that may have occurred. For very large proteins, samples can be pretreated with trypsin, which cleaves peptide bonds between lysine and arginine, to produce lower-molecular-weight fragments that can then be measured. The detection limit of this assay is about 10^{-15} to 10^{-18} moles. A modification of MALDI-TOF MS is surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) MS, in which proteins are directly captured on a chromatographic biochip without the need of sample preparation. Figure 5-44 illustrates the SELDI-TOF process.

OSMOMETRY

An osmometer is used to measure the concentration of solute particles in a solution. The mathematic definition is

$$\text{Osmolality} = \varphi \times n \times C \qquad \textbf{(Eq. 5-13)}$$

where φ is osmotic coefficient, n is number of dissociable particles (ions) per molecule in the solution, and C is = concentration in moles per kilogram of solvent.

The osmotic coefficient is an experimentally derived factor to correct for the fact that some of the molecules, even in a highly disociated compound, exist as molecules rather than ions.

The four physical properties of a solution that change with variations in the number of dissolved particles in the solvent are osmotic pressure, vapor pressure, boiling point, and freezing point. Osmometers measure osmolality indirectly by measuring one of these colligative properties, which change proportionally with osmotic pressure. Osmometers in clinical use measure either freezing-point depression or vapor-pressure depression; results are expressed in milliosmolal per kilogram (mOsm/kg) units.

Freezing-Point Osmometer

Figure 5-45 illustrates the basic components of a freezing-point osmometer. The sample in a small tube is lowered into a chamber with cold refrigerant circulating from a cooling unit. A thermistor is immersed in the sample. To measure temperature, a wire is used to gently stir the sample until it is cooled to several degrees below its freezing point. It is possible to cool water to as low as −40°C and still have liquid water, provided no crystals or particulate matter are present. This is

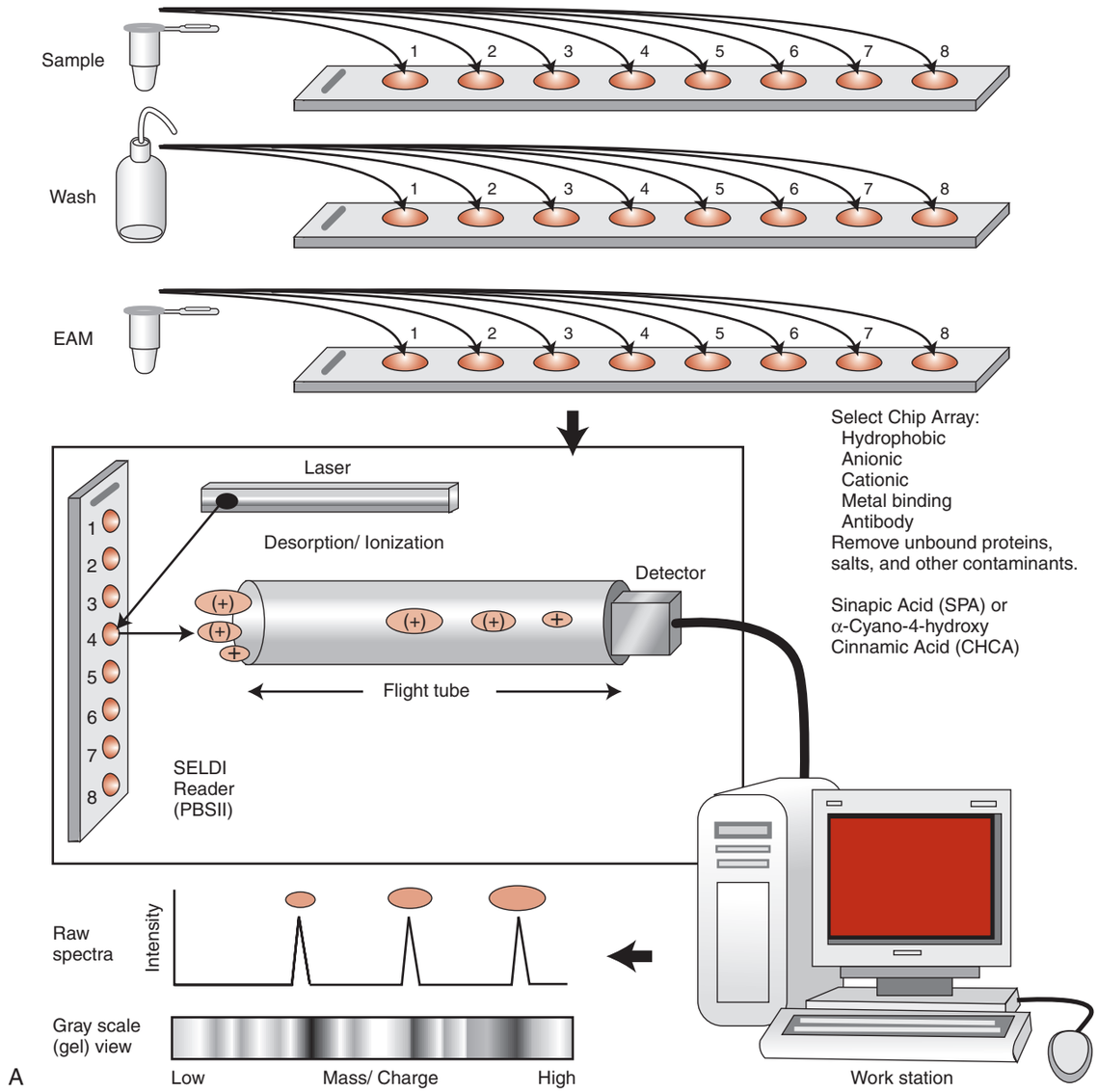
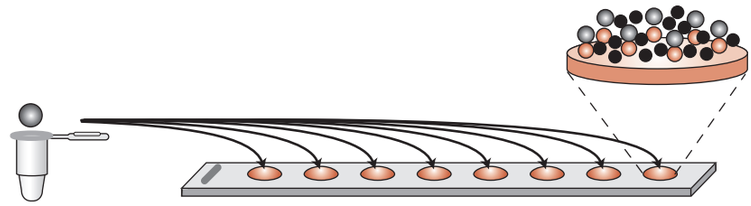


FIGURE 5-44. (A, B) Overview of the SELDI-TOF process. (Diagram courtesy of Ciphergen Biosystems, Fremont, Ca.) *(continues)*

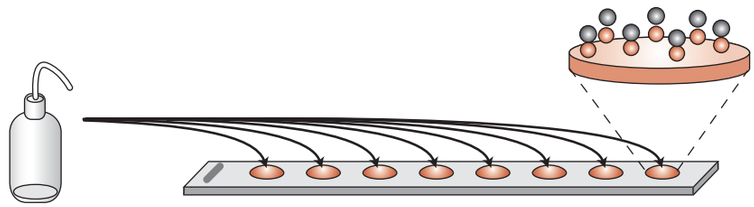
1. Apply supernatant from CD8$^+$ cells

Supernatant from stimulated and unstimulated CD8$^+$ cell cultures from Normal, LTPN, and Progressors is added to a Protein Chip Array. Proteins bind to chemical or biological "docking" sites on the array surface through an affinity interaction.



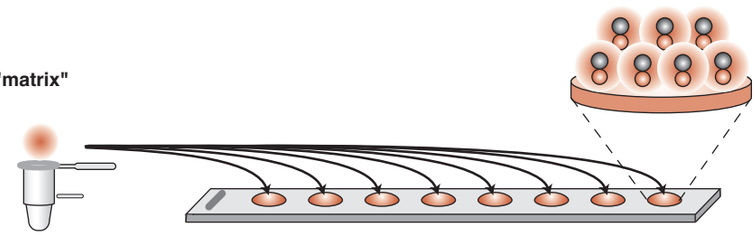
2. Wash Protein Chip Array

Proteins that bind non-specifically and buffer contaminants are washed away, eliminating sample noise.



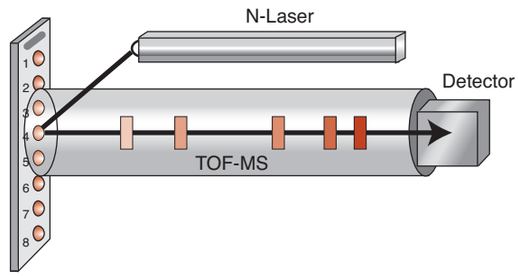
3. Add energy absorbing molecules or "matrix"

After sample processing, the array is dried and EAM is applied to each spot to facilitate desorption and ionization.



4. Analyze in a Protein Chip Reader

The proteins that are retained on the array are detected on the Protein Chip Reader.



B

FIGURE 5-44. *(Continued)*

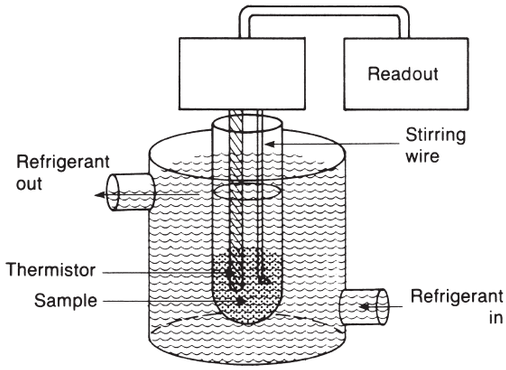


FIGURE 5-45. Freezing-point osmometer. (Reprinted with permission from Coiner D. Basic concepts in laboratory instrumentation. Bethesda, Md.: ASMT Education and Research Fund, 1975–1979.)

referred to as a supercooled solution. Vigorous agitation when the sample is supercooled results in rapid freezing. Freezing also can be started by "seeding" a supercooled solution with crystals. When the supercooled solution starts to freeze as a result of the rapid stirring, a slush is formed and the solution actually warms to its freezing-point temperature. The slush, an equilibrium of liquid and ice crystals, will remain at the freezing-point temperature until the sample freezes solid and drops below its freezing point.

Impurities in a solvent will lower the temperature at which freezing or melting occurs by reducing the bonding forces between solvent molecules so that the molecules break away from each other and exist as a fluid at a lower temperature. The decrease in the freezing-point temperature is proportional to the number of dissolved particles present.

The thermistor is a material that has less resistance when the temperature increases. The readout uses a Wheatstone bridge circuit that detects temperature change as proportional to change in thermistor resistance. Freezing-point depression is proportional to the number of solute particles. Standards of known concentration are used to calibrate the instruments in mOsm/kg.

ANALYTIC TECHNIQUES FOR POINT-OF-CARE TESTING

Point-of-care testing (POCT) devices are widely used for a variety of clinical applications, including physician offices, emergency departments, intensive care units, and even for self-testing. Because analyses can be done at patient-side by primary caregivers, the major attraction of POCT is the reduced turnaround time needed to deliver results. In some cases, total costs can be reduced if the devices eliminate the need for laboratory-based instrumentation or if increased turnaround times lead to shorter hospital stays. POCT relies on the same analytic techniques as laboratory-based instrumentation: spectrometry, electroanalytic techniques, and chromatography. As such, the same steps needed to perform an analysis from the central laboratory are needed for POCT, including instrument validation, periodic assay calibration, quality control testing, operator training, and proficiency testing. Chapter 9 provides an in-depth discussion of this technology. The analytic techniques used in these devices are given in this section.

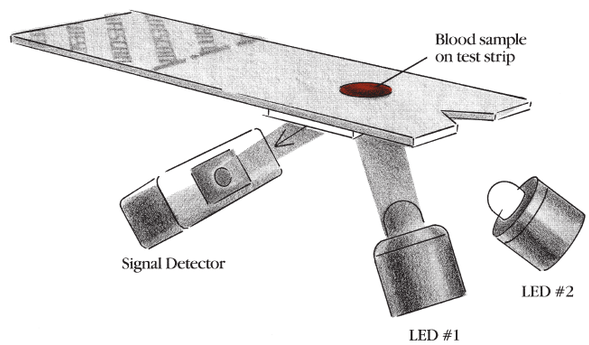


FIGURE 5-46. Dual-wavelength reflectance photometry used in point-of-care glucose monitors. A microporous hydrophilic membrane is used as a reservoir of the sample to filter out solid cellular material from the reservoir and provide a smooth, optical surface for reflectance measurements. (Figure courtesy of Lifescan Inc. One touch system technology. Challenges in diabetes management: clinical protocols for professional practice. New York, N.Y.: Health Education Technologies, 1988.)

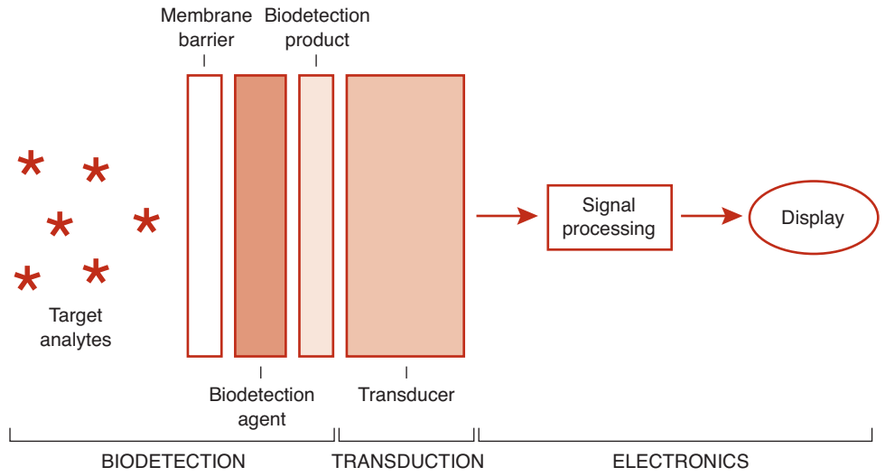


FIGURE 5-47. Schematic diagram of a biosensor. (Reprinted with permission from Rosen A. Biosensors: where do we go from here? MLO Med Lab Obs 1995;27:24.)

The most commonly used POCT devices used at bedside, in physician offices, and at home are the fingerstick blood glucose monitors. The first-generation devices use a photometric approach, whereby glucose produces hydrogen peroxide (H_2O_2) with glucose oxidase immobilized onto test strips. The H_2O_2 is coupled to peroxidase to produce a color whose intensity is measured as a function of concentration and measured using reflectance photometry. A schematic of this technique is shown in Figure 5-46. These strips are measured for glucose concentration without the need to wipe the blood off the strips.

The strip technology in a POCT platform can also be used to measure proteins and enzymes, such as cardiac markers. The separation of analytes from the matrix is accomplished by paper chromatography, in which specific antibodies immobilized onto the chromatographic surface capture the target analyte as it passes through. For qualitative analysis, detection is made by visual means. Reflectance meters similar to those used for glucose are also available for quantitative measurements.

The next generation of POCT devices use biosensors.[22] A biosensor couples a specific biodetector, such as an enzyme, antibody, or nucleic acid probe, to a transducer for the direct measurement of a target analyte without the need to separate it from the matrix[24] (Fig. 5-47). The field has exploded in recent years with the development of microsilicon chip fabrication because biosensors can be miniaturized and made available at low costs. An array of biosensors can be produced onto a single silicon wafer to produce a multipanel of results, such as an electrolyte profile. Commercial POCT devices use electrochemical (e.g., microion-selective electrodes) and optical biosensors for the measurement of glucose, electrolytes, and arterial blood gases. With the immobilization of antibodies and specific DNA sequences, biosensor probes will soon be available for detection of hormones, drugs and drugs of abuse, and hard-to-culture bacteria and viruses such as *Chlamydia,* tuberculosis, or human immunodeficiency virus.[22]

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