Table 20.2

Comparison of Gas-Chromatographic Detectors

Detector	Application	Sensitivity Range	Linearity	Remarks
Thermal conductivity	General, responds to all substances	Fair, 5–100 ng, 10 ppm–100%	Good, except thermistors at higher temperatures	Sensitive to temperature and flow changes; concentration sensitive
Flame ionization	All organic substances; some oxygenated products respond poorly. Good for hydrocarbons	Very good, 10–100 pg, 10 ppb–99%	Excellent, up to 10^6	Requires very stable gas flow; response for water is 10^4–10^6 times weaker than for hydrocarbons; mass-sensitive
Flame photometric	Sulfur compounds (393 nm), phosphorus compounds (526 nm)	Very good, 10 pg S, 1 pg P	Excellent	
Flame thermionic	All nitrogen- and phosphorus-containing substances	Excellent, 0.1–10 pg, 100 ppt–0.1%	Excellent	Needs recoating of sodium salts on screen; mass sensitive
Rubidium silicate bead	Specific for nitrogen- and phosphorus-containing substances	Excellent		Mass sensitive
Argon ionization (β-ray)	All organic substances; with ultrapure He carrier gas, also for inorganic and permanent gases	Very good; 0.1–100 ng, 0.1–100 ppm	Good	Very sensitive to impurities and water; needs very pure carrier gas; concentration sensitive
Electron capture	All substances that have affinity to capture electrons; no resonse for aliphatic and naphthenic hydrocarbons	Excellent for halogen-containing substancs, 0.05–1 pg, 50 ppt–1 ppm	Poor	Very sensitive to impurities and temperature changes; quantitative analysis complicated; concentration sensitive
Mass spectrometry	Nearly all substances. Depends on ionization method	Excellent	Excellent	Can provide structural and molecular weight information

water, and so aqueous solutions can be injected. If oxygen is used as the flame support gas in place of air, then many inorganic compounds can be detected because a hotter flame is produced that can ionize them. Since the flame ionization detector is so sensitive, a portion of the sample can be diverted by an appropriate stream splitter so that it can be collected and analyzed further if necessary.

When sulfur and phosphorus compounds are burned in an FID-type flame, chemiluminescent species are produced that produce light at 393 nm (sulfur) and 526 nm (phosphorous). An optical interference filter passes the appropriate light to a photomultiplier tube, a sensitive photon detector. These detectors are known as flame photometric detectors (FPD).

The **flame thermionic detector** is essentially a two-stage flame ionization detector designed to give an increased specific response for nitrogen- and phosphorus-containing substances. A second flame ionization detector is mounted above the first, with the flame gases from the first passing into the second flame. The two stages are divided by a wire mesh screen coated with an alkali salt or base such as sodium hydroxide.

The column effluent enters the lower flame, which acts as a conventional FID whose response may be recorded. A small current normally flows in the second flame due to evaporation and ionization of sodium from the screen. However, if a substance containing nitrogen or phosphorus is burned in the lower flame, the ions resulting from these greatly increase the volatilization of the alkali metal from the screen. This results in a response that is much greater (at least 100 times) than the response of the lower flame to the nitrogen or phosphorus. By recording the signals from both flames, one can obtain the usual chromatogram of a FID; a second chromatogram is obtained where the peaks corresponding to the nitrogen- and phosphorus-containing compounds are amplified over the others, which will be practically missing. This detector is also known as a nitrogen–phosphorous detector (NPD).

In the **β-ray**, or **argon ionization, detector**, the sample is ionized by bombardment with β rays from a radioactive source (e.g., strontium-90). The carrier gas is argon, and the argon is excited to a metastable state by the β particles. Argon has an excitation energy of 11.5 eV, which is greater than the ionization potential of most organic compounds, and the sample molecules are ionized when they collide with the excited argon atoms. The ions are detected in the same manner as in the flame ionization detector. This detector is very sensitive but less accurate than others, and the β-ray source is a potential hazard, although with proper shielding, no danger exists. The sensitivity is about 300 times greater than that of the conventional thermal conductivity cell.

The ECD is very sensitive for halogen-containing compounds, for example, pesticides.

The **electron capture detector** (ECD) is extremely sensitive for compounds that contain electronegative atoms and is selective for these. It is similar in design to the β-ray detector, except that nitrogen or methane doped with argon is used as the carrier gas. These gases have low excitation energies compared to argon and only compounds that have high electron affinity are ionized, by capturing electrons.

The detector cathode consists of a metal foil impregnated with a β-emitting element, usually tritium or nickel-63. The former isotope gives greater sensitivity than the latter, but it has an upper temperature limit of 220°C because of losses of tritium at high temperatures; nickel-63 can be used routinely at temperatures up to 350°C. Also, nickel is easier to clean than the tritium source; these radioactive sources inevitably acquire a surface film that decreases the β-emission intensity and hence the sensitivity. A 30% KOH solution is usually used to clean the sources.

The cell is normally polarized with an applied potential, and electrons (β rays) emitted from the source at the cathode strike gas molecules, causing electrons to be released. The resulting cascade of thermal electrons is attracted to the anode, and establishes a standing current. When a compound possessing electron

affinity is introduced into the cell, it captures electrons to create a large negative ion. The negative ion has a mobility in an electric field about 100,000 less than electrons, and so a decrease in current results.

Relatively few compounds show significant electronegativities, and so electron capture is quite selective, allowing the determination of trace constituents in the presence of noncapturing substances. High-electron-affinity atoms or groups include halogens, carbonyls, nitro groups, certain condensed ring aromatics, and certain metals. The ECD is widely used for pesticides and polychlorinated biphenyls (PCBs). Electron capture has very low sensitivity for hydrocarbons other than aromatics.

Compounds with low electron affinities may be determined by preparing appropriate derivatives. Most important biological compounds, for example, possesses low electron affinities. Steroids such as cholesterol can be determined by preparing their chloroacetate derivatives. Trace elements have been determined at nanogram and picogram levels by preparing volatile trifluoroacetylacetone chelates. Examples are chromium, aluminum, copper, and beryllium. Methylmercuric chloride, present in contaminated fish, can be determined at the nanogram level.

The gas chromatograph may be interfaced with atomic spectroscopic instruments for specific element detection. This powerful combination is useful for speciation of different forms of toxic elements in the environment. For example, a helium microwave induced plasma atomic emission detector (AED) has been used to detect volatile methyl and ethyl derivatives of mercury in fish, separated by GC. Also, gas chromatographs are interfaced to inductively coupled plasma–mass spectrometers (ICP–MS) in which atomic isotopic species from the plasma are introduced into a mass spectrometer (see Section 20.10 for a description of mass spectrometry), for very sensitive simultaneous detection of species of several elements.

Detectors are either concentration sensitive or mass flow sensitive. The signal from a concentration-sensitive detector is related to the concentration of the solute in the detector and is decreased by dilution with a makeup gas. The sample is usually not destroyed. Thermal conductivity, argon-ionization, and electron capture detectors are concentration sensitive. In mass-flow-sensitive detectors, the signal is related to the rate at which solute molecules enter the detector and is not affected by the makeup gas. These detectors usually destroy the sample, such as flame ionization and flame thermionic detectors. Sometimes two-column GC is used to increase resolution, by taking cuts of eluents from an initial column and directing them to a second column for secondary separation. The first detector must be nondestructive or else the eluent split prior to detection, with a portion going to the second column.

20.4 · Temperature Selection

Chromatographic conditions represent a compromise between speed, resolution, and sensitivity.

The proper temperature selection in gas chromatography is a compromise between several factors. The **injection temperature** should be relatively high, consistent with thermal stability of the sample, to give the fastest rate of vaporization to get the sample into the column in a small volume; decreased spreading and increased resolution result. Too high an injection temperature, though, will tend to degrade the rubber septum and cause dirtying of the injection port. The **column temperature** is a compromise between *speed*, *sensitivity*, and *resolution*. At high column temperatures, the sample components spend most of their time in the gas phase and so they are eluted quickly, but resolution is poor. At low temperatures, they spend more time in the stationary phase and elute slowly; resolution is increased but sensitivity is decreased due to increased spreading of the peaks. The **detector**

Temperature programming from low to higher temperatures speeds up separations. The more difficult to elute solutes are made to elute faster at the higher temperatures. The more easily eluted ones are better resolved at the lower temperatures.

temperature must be high enough to prevent condensation of the sample components. The sensitivity of the thermal conductivity detector decreases as the temperature is increased and so its temperature is kept at the minimum required.

Separations can be facilitated by **temperature programming**, and most gas chromatographs have temperature programming capabilities. The temperature is automatically increased at a preselected rate during the running of the chromatogram; this may be linear, exponential, steplike, and so on. In this way, the compounds eluted with more difficulty can be eluted in a reasonable time without forcing the others from the column too quickly.

Figure 20.6 shows a temperature programmed separation of a complex hydrocarbon mixture with stepwise linear temperature programming. The first 12 gaseous or light compounds are readily eluted and resolved at a low fixed (100°C) temperature for 5.5 min, while the others require higher temperatures. After 5.5 min, the temperature is linearly increased at 5°C/min for 20 min to 200°C, and then the temperature is held at that value until the last two compounds are eluted.

If the constituent to be determined is not volatile at the accessible temperatures, it may be converted to a **volatile derivative**. For example, nonvolatile fatty acids are converted to their volatile methyl esters. Some inorganic halides are sufficiently volatile that at high temperatures they can be determined by gas chromatography. Metals may be made volatile by complexation, for example, with trifluoroacetylacetone.

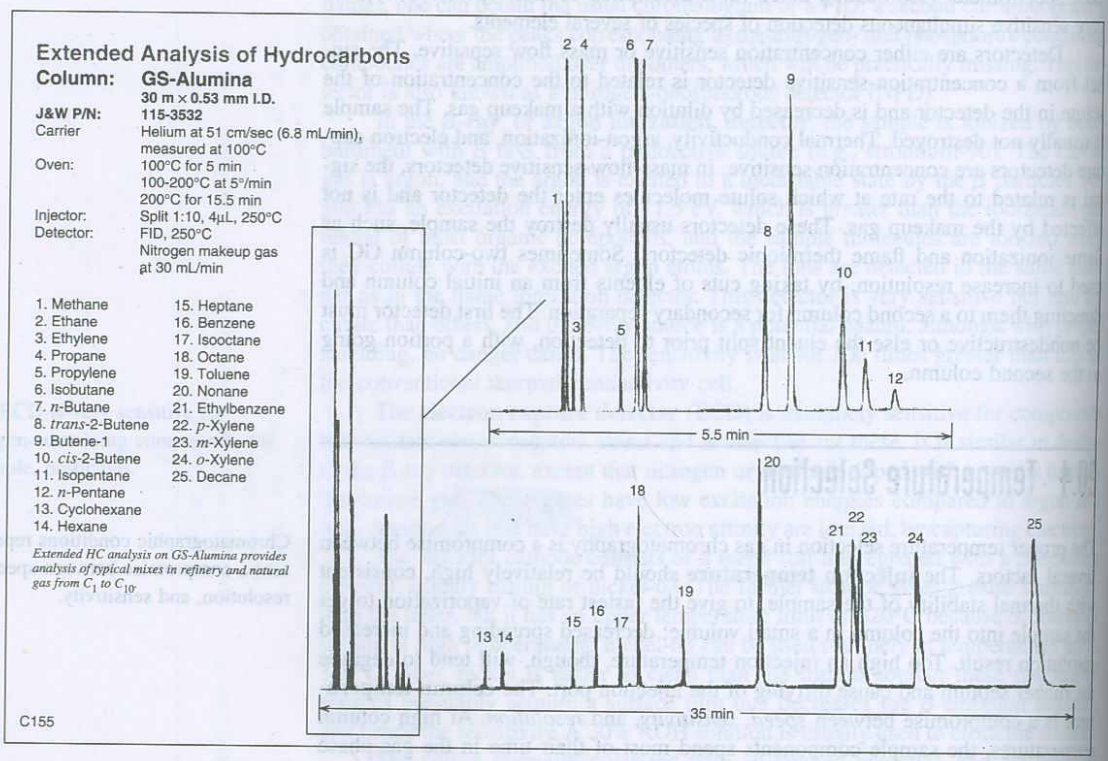


Fig. 20.6. Temperature-programmed analysis. (Courtesy of Agilent Technologies.)

20.5 Quantitative Measurements

The concentrations of eluted solutes are proportional to the areas under the recorded peaks. Electronic integrations in GC instruments print out the areas of peaks, and the retention times of peaks are also generally printed. It is also possible to measure peak height to construct a calibration curve. The linearity of a calibration curve should always be established.

The method of **standard additions** is a useful technique for calibrating, especially for occasional samples. One or more aliquots of the sample are spiked with a known concentration of standard, and the increase in peak area is proportional to the added standard. This method has the advantage of verifying that the retention time of the unknown analyte is the same as that of the standard.

A more important method of quantitative analysis is the use of **internal standards**. Here, the sample and standards are spiked with an equal amount of a solute whose retention time is near that of the analyte. The ratio of the area of the standard or analyte to that of the internal standard is used to prepare the calibration curve and determine the unknown concentration. This method compensates for variations in physical parameters, especially inaccuracies in pipetting and injecting microliter volumes of samples. Also, the *relative* retention should remain constant, even if the flow rate should vary somewhat.

An internal standard is usually added to standard and sample solutions. The ratio of the analyte peak area to internal standard peak area is measured and will remain unaffected by slight variations in injected volume and chromatographic conditions.

SPREADSHEET EXERCISE: INTERNAL STANDARD CALIBRATION

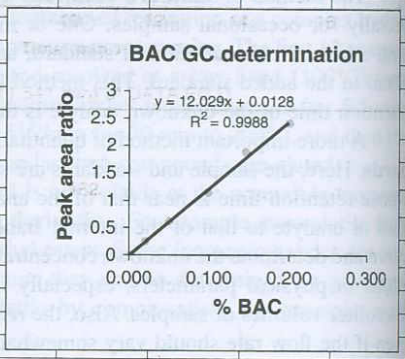
When a driver is arrested for suspicion of driving under the influence of alcohol, the blood alcohol content is determined to see if it exceeds the legal limit. The measurement of breath alcohol is usually done for routine driving arrests because it is noninvasive, and a factor is applied to convert to blood alcohol concentration (BAC). But this is subject to biological variations in individuals. In cases where there is an accident, injury, or death, the blood alcohol is usually determined directly, by analyzing a blood sample by gas chromatography.

A 5.00-mL blood sample from a suspect is spiked with 0.500 mL of aqueous 1% propanol internal standard. A 10-μL portion of the mixture is injected into the GC, and the peak areas are recorded. Standards are treated in the same way. The following results were obtained:

% EtOH (wt/vol)	*Peak Area EtOH*	*Peak Area PrOH*
0.020	114	457
0.050	278	449
0.100	561	471
0.150	845	453
0.200	1070	447
Unknown	782	455

Prepare a spreadsheet to construct a calibration curve of the ratio of the EtOH/PrOH areas vs. EtOH concentration, and to calculate the unknown concentration and its standard deviation. Refer to Equations 16.18 to 16.21 and the spreadsheet that follows in Chapter 16 for a refresher on the statistical calculations.

	A	B	C	D	E	F	G	H
1	GC Determination of blood ethanol using propanol internal standard.							
2	%EtOH	Peak area	Peak area	Ratio peak area				
3		EtOH	PrOH	EtOH/PrOH				
4	0.020	114	457	0.249453				
5	0.050	278	449	0.619154				
6	0.100	561	471	1.191083				
7	0.150	845	453	1.865342				
8	0.200	1070	447	2.393736				
9	Unknown (y_c)	782	455	1.718681				
10								
11	Slope, m:		12.0286923					
12	Intercept, b:		0.01276953					
13	Unknown % BAC, C:		0.14182022					
14								
15	S_r:	0.0345743						
16	N:	5						
17	S_{xx}:	0.02132						
18	y_{ave}:	1.2637535						
19	M:	1						
20	S_c:	0.0033365						
21								
22	Cell D4 = ratio =B4/C4 (copy down)							
23	Cell C11 = m = SLOPE(D4:D8,A4:A8)							
24	Cell C12 = b = INTERCEPT(D4:D8,A4:A8)							
25	Cell C13 = C = $(y_c$-b)/m = (Ratio$_c$ - b)/m = (D9-C12)/C11							
26	Cell B15 = S_r = STEYX(D4:D8,A4:A8)							
27	Cell B16 = N = COUNT(A4:A8)							
28	Cell B17 = S_{xx} = N*VARP(A4:A8) = B16*VARP(A4:A8)							
29	Cell B18 = y_{ave} = AVERAGE(D4:D8)							
30	Cell B19 = sample replicates = M =1							
31	Cell B20 = std. devn. $S_c = S_r/m((1/M + 1/N + (y_c - y_{ave})/m^2*S_{xx}))^{1/2}$							
32	=B15/C11*((1/B19+1/B16+(D9-B18)/C11^2*B17))^0.5							



The blood alcohol concentration is 0.142 ± 0.003%, a high level. The legal limit for driving in most states is 0.08% blood alcohol (wt/vol). The influence on the ability to drive is exponential with alcohol concentration and it roughly doubles for every 0.05% BAC, so someone with 0.20% blood alcohol is 8 times as drunk as one with 0.05%!

20.6 Headspace Analysis

Headspace analysis avoids the need for solvent extraction for volatile analytes.

In Chapter 18, we described solvent extraction and solid-phase extraction sample preparation methods, which are applicable to GC analyses as well as others. A convenient way of sampling volatile samples for GC analysis is the technique of **headspace analysis**. A sample in a sealed vial is equilibrated at a fixed temperature, for example, for 10 min, and the vapor in equilibrium above the sample is sampled and injected into the gas chromatograph. A typical 20-mL glass vial is capped with a silicone rubber septum lined with polytetrafluoroethylene (PTFE). A syringe needle can be inserted to withdraw a 1-mL portion. Or the pressurized vapor is allowed to expand into a 1-mL sample loop at atmospheric pressure, and then an auxiliary carrier gas carries the loop contents to the GC loop injector. Volatile compounds in solid or liquid samples can be determined at parts per million or less. Pharmaceutical tablets can be dissolved in a water–sodium sulfate solution

for headspace analysis. Figure 1 in your CD, Chapter 20, shows a headspace chromatogram of volatile compounds in a blood sample.

20.7 Thermal Desorption

In thermal desorption, the volatile analyte is desorbed from the sample by heating and introduced directly into the GC.

Thermal desorption (TD) is a technique in which solid or semisolid samples are heated under a flow of inert gas. Volatile and semivolatile organic compounds are extracted from the sample matrix into the gas stream and introduced into a gas chromatograph. Samples are typically weighed into a replaceable PTFE tube liner, which is inserted into a stainless steel tube for heating.

The thermal desorption must take place at a temperature below the decomposition point of other materials in the sample matrix. Solid materials should have a high surface area (e.g., powders, granules, fibers). Bulk materials are ground with a coolant such as solid carbon dioxide prior to weighing. This technique simplifies sample preparation and avoids the necessity of dissolving samples or solvent extraction. Thermal desorption is well suited for dry or homogeneous samples such as polymers, waxes, powders, pharmaceutical preparations, solid foods, cosmetics, ointments, and creams. There is essentially no sample preparation required.

See www.markes.com for literature on sorbent selection for thermal desorption.

An example of the use of TD is for the analysis of water-based paints for organic volatiles. The TD tube is used in combination with a second tube containing a sorbent that removes the water, which cannot be introduced into the capillary GC column. A small aliquot of paint (e.g., 5 μL) is placed on glass wool in the TD tube. Solids from the paint, which would harm a GC column, remain behind.

20.8 Purging and Trapping

Purge-and-trap is a form of headspace analysis in which the volatile analyte is trapped on a sorbent and then thermally desorbed.

The **pure-and-trap** technique is a variation of thermal desorption analysis in which volatiles are purged from a liquid sample placed in a vessel by bubbling a gas (e.g., air) through the sample and collecting the volatiles in a sorbent tube containing a suitable sorbent. The trapped volatiles are then analyzed by thermally desorbing them from the sorbent. This is a form of "headspace" analysis in which analytes are concentrated prior to introduction into the GC. A typical sorbent is a hydrophobic one that can collect organics ranging in volatility from hexane up to C16 or greater. Examples are Tenax TA or graphitized carbon. These sorbents allow bulk polar solvents such as water or ethanol to pass through unretained. Whiskey is analyzed for C4 to C6 ethyl esters, which are markers of maturity, and the alcohol does not interfere in the chromatogram.

Purge-and-trap is suitable for nonhomogeneous samples, since fairly large samples can be taken, and high-humidity samples. Examples include foods such as pizza or fruits. The measurement of malodorous organic volatiles in the headspace vapor above a sample of aged food is used to determine whether it still meets the "freshness" requirements. The food sample, placed in a large purge vessel, is heated under a flow of air, and the effluent air is collected on the sorbent.

Sorbents more selective than Tenax-type sorbents may be used, and two or more may be used in series for measurement of different classes of compounds. Other sorbents include many materials used for chromatography such as alumina, silica gel, Florisil (for PCBs), coconut charcoal, Poropak, and Chromosorb. Some may be coated for specific applications, for example, silica gel coated with sulfuric acid or sodium hydroxide for collecting bases or acids.

Another important use of trapping is for the direct analysis of gaseous samples such as air. The sample is passed directly through the sorbent tube, and the trapped volatiles are subsequently desorbed or removed by extraction. This technique is finding widespread use for indoor and outdoor air monitoring.

For more information about the sorbents and thermal desorption, see SKC (www.skcinc.com) and Markes International (www.markes.com). SKC lists sorbents for specific applications (analytes).

20.9 Small and Fast

Thin-film stationary phases, small diameter and short columns, and a light carrier gas gives fast separations.

The advent of automated, computerized data handling has made possible the design of chromatographic columns with thin liquid-phase films that allow very rapid analyses, for example, of a few seconds. Small diameter and short capillary columns are used with hydrogen as the carrier gas (which allows more rapid mass transfer) and fast temperature program rates. Faster flow rates and higher pressures are used. Fast elution of analytes requires fast detector response time and data acquisition rates because peak widths are on the order of only 0.5 s, compared to 0.5 to 2 s or more for conventional capillary GC, improving analysis times 5- to 10-fold. High-speed chromatography with short columns makes column selection (selectivity) more critical than in conventional capillary GC. Figure 20.7 shows a fast GC chromatographic separation of hydrocarbons in less than 10 s using a 0.32-mm-diameter column of 5 m length, and 0.25-μm-thick stationary phase, compared to 10 min using conventional GC.

Shorter columns and faster temperature programming causes some loss in resolution, but this is partially offset by the smaller internal diameter and thinner liquid film.

There are small portable GCs available from a number of manufacturers. There are limited applications, with isothermal ovens, but they perform well for specific applications. [See C. Henry, "Taking the Show on the Road. Portable GC and GC/MS" (Product Review), *Anal. Chem.*, **69**(5) (1997) 195A].

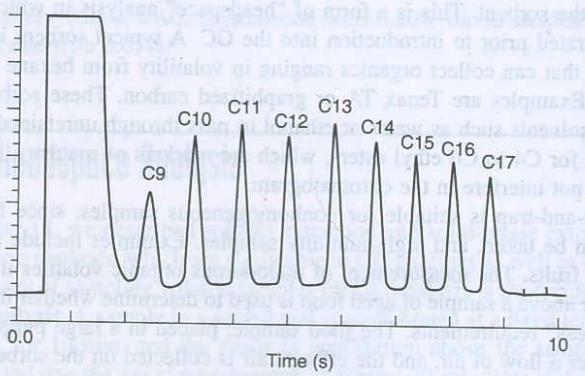


Fig. 20.7. Separation of C9 to C17 hydrocarbons by fast chromatography, with rapid temperature programming. Conditions: 5 m × 0.32 mm, 0.25 μm d_f, 60°C, 19.2°C/s. [From G. L. Reed, K. Clark-Baker, and H. M. McNair. *J. Chromatogr. Sci.*, **37** (1999) 300. Reproduced from the *Journal of Chromatographic Science* by permission of Preston Publications, A Division of Preston Industries, Inc.]

20.10 Gas Chromatography–Mass Spectrometry

The appearance of a chromatographic peak at a particular retention time suggests but does not guarantee the presence of a particular compound. The probability of positive identification will depend on factors such as the type and complexity of the sample and sample preparation procedures employed. A gas chromatogram of an injected blood sample diluted with a solution of an internal standard (to verify retention time and relative peak area) that gives a large peak expected for alcohol strongly suggests the presence of blood alcohol since there are few nontoxic compounds that would likely interfere. Usually, there is indication of alcohol ingestion, and the key legal question is what is the concentration? However, the appearance of a GC peak for cocaine may not be so straightforward in confirming the presence of this drug. Hence, confirmatory evidence is usually sought. Spectral information, such as infrared or ultraviolet spectrometry, may be sought. A very powerful tool is the combination of gas chromatography with mass spectrometry, a technique known as **gas chromatography–mass spectrometry** (GC–MS).

GC–MS is very powerful for positive identification.

GC–MS systems used to fill a room and cost several hundred thousand dollars. Today, relatively inexpensive compact benchtop systems are available and widely used in laboratories. A modern GC–MS instrument is shown in Figure 20.8. We describe first the principles of mass spectrometers and types of instruments, and then discuss how the two techniques of gas chromatography and mass spectrometry are used together.

PRINCIPLES OF MASS SPECTROMETRY

Mass spectrometry is a sophisticated instrumental technique that produces, separates, and detects ions in the gas phase. The basic components of a mass spectrometer are shown in Figure 20.9. A sample with a moderately high vapor pressure is introduced in an inlet system, operated under vacuum (10^{-4} to 10^{-7} torr) and at high temperature (up to 300°C). It vaporizes and is carried to the ionization source. Nonvolatile compounds may be vaporized by means of a spark or other

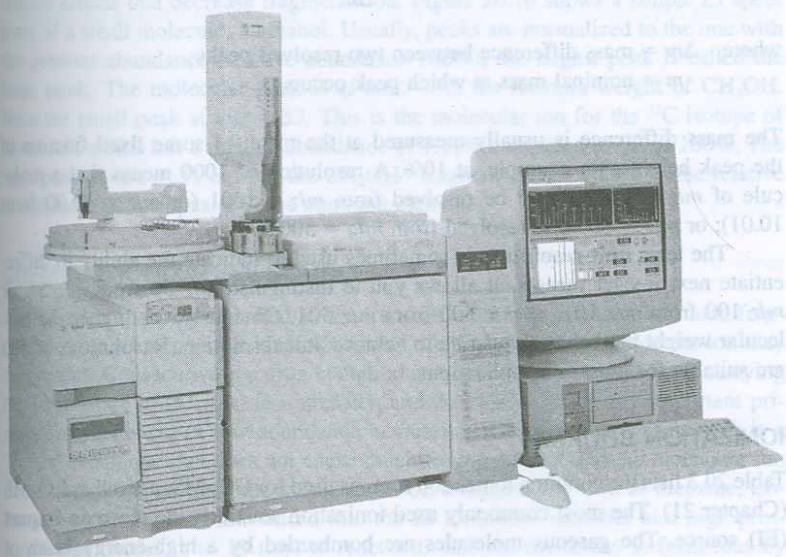


Fig. 20.8. Gas chromatography–mass spectrometry benchtop system. (Courtesy of Agilent Technologies.)

Fig. 20.9. Block diagram of mass spectrometer.

source. Analyte molecules are typically neutral and must be ionized. This is accomplished by various means but typically is done by bombarding the sample with high-energy electrons in an electron-impact source. We describe common ionization sources below.

The ions are separated in the spectrometer by being accelerated through a mass separator described below. Separation actually accomplished based on the mass-to-charge (m/e) ratios of the ions. We will not go into the many useful rules for deducing structural information from mass spectra. But one rule in particular is useful to know for identifying the molecular ion, the **nitrogen rule**. In applying this, M^+ is taken as the highest mass, ignoring isotope contributions. Such a molecular mass will be an even number if it contains an even number (0, 2, 4 . . .) of nitrogen atoms, and will otherwise be an odd number, that is, when it contains an odd number of nitrogens. So if there is no nitrogen, it will be even. There should also be no illogical losses on fragmentation. For example, organic molecules seldom lose more than four H atoms, to give M-4 fragments. Other losses we would expect to see are methyl groups (M-15), NH_2 or O (M-16), OH or NH_3 (M-17), H_2O (M-18), F (M-19), HF (M-20), and C_2H_2 (M-26). So there should be no losses of 4 to 14 or 21 to 25 mass units. If so, the assignment is incorrect or we have a mixture spectrum.

The nitrogen rule tells you whether you have an even or an odd formula weight compound.

RESOLUTION

Resolution tells you how accurately you can differentiate two masses. Unit resolution tells you that you can differentiate one mass difference.

In mass spectrometry, the resolving power, that is, the ability to differentiate two masses, is given by the resolution R, defined as the nominal mass divided by the difference between two masses that can be separated:

$$R = \frac{m}{\Delta m} \tag{20.2}$$

where Δm = mass difference between two resolved peaks
m = nominal mass at which peak occurs

The mass difference is usually measured at the mean of some fixed fraction of the peak heights, for example, at 10%. A resolution of 1000 means that a molecule of $m/z = 1000$ would be resolved from $m/z = 1001$ (or $m/z = 10.00$ from 10.01); or $m/z = 500.0$ is resolved from $m/z = 500.5$.

The term **unit resolution** is sometimes used to indicate the ability to differentiate next integer masses. It allows you to distinguish m/z 50 from m/z 51, or m/z 100 from m/z 101, or m/z 500 from m/z 501. Obviously, the higher the molecular weight the better R must be to achieve unit resolution. Resolutions of 500 are suitable for most GC applications.

IONIZATION SOURCES

Table 20.3 lists the common ionization sources used for GC–MS, as well as LC–MS (Chapter 21). The most commonly used ionization source is the **electron-impact** (EI) source. The gaseous molecules are bombarded by a high-energy beam of

Electron-impact ionization produces many fragments.

Table 20.3
Comparison of Ionization Methods[a]

Ionization Method	Typical Analytes	Sample Introduction	Mass Range	Method Highlights
Electron impact (EI)	Relatively small volatile	GC or liquid–solid probe	To 1000 daltons	Hard method. Versatile, provides structure information
Chemical ionization (CI)	Relatively small, volatile	GC or liquid–solid probe	To 1000 daltons	Soft method. Molecular ion peak $[M + H]^+$
Electrospray (ESI)	Peptides, proteins, nonvolatile	Liquid chromatography or syringe	To 200,000 daltons	Soft method. Ions often multiply charged
Matrix-assisted laser desorption (MALDI)	Peptides, proteins, nucleotides	Sample mixed in solid matrix	To 500,000 daltons	Soft method, very high mass

[a]From Web page of Professor Vicki Wysocki, University of Arizona. Reproduced by permission.

electrons, usually 70 eV, generated from a tungsten filament. An electron that collides with a neutral molecule may impart sufficient energy to remove an electron from the molecule, resulting in a singly charged ion:

$$M + e^- \rightarrow M^+ + 2e^-$$

where M is the analyte molecule and M^+ is the **molecular ion** or **parent ion**. The M^+ ions are produced in different energy states and the internal energy (rotational, vibrational, and electronic) is dissipated by fragmentation reactions, producing fragments of lower mass, which are themselves ionized or converted to ions by further electron bombardment. The fragmentation pattern is fairly consistent for given conditions (electron beam energy). Only a small amount or none of the molecular ion may remain. If it does appear, it will be the highest mass in an EI spectrum, if there are not multiple isotopes. Compounds with aromatic rings, cyclic structures, or double bonds are more likely to give molecular ion peaks because of delocalization effects that decrease fragmentation. Figure 20.10 shows a simple EI spectrum of a small molecule, methanol. Usually, peaks are normalized to the one with the greatest abundance (relative abundance 100%); the largest peak is called the **base peak**. The molecular peak is at $m/z = 32$, the formula weight of CH_3OH. Note the small peak at $m/z = 33$. This is the molecular ion for the ^{13}C isotope of methanol, which has a relative abundance of 1.11% compared to ^{12}C at 100%. The base peak at $m/z = 31$ is from the CH_2OH^+ fragment. Table 20.4 lists the relative abundances of isotopes of some common elements.

CHEMICAL IONIZATION SOURCE

Chemical ionization produces the molecular ion.

The EI source is what is called a "hard source" and may produce too much fragmentation to allow positive identification of the analyte, and no molecular ion may be present. Consecutive ion fragmentation may result in low-mass ions carrying the large share of the total ion intensity, and the more analytically important primary fragments are of low abundance or missing. **Chemical ionization** (CI) is a "softer" technique that does not cause much fragmentation, and the molecular ion is the dominant one in CI mass spectra. In CI, a reagent gas such as methane, isobutane, or ammonia is introduced into the EI ionization chamber at a high pressure (large excess, 1 to 10 torr) to react with analyte molecules to form ions by

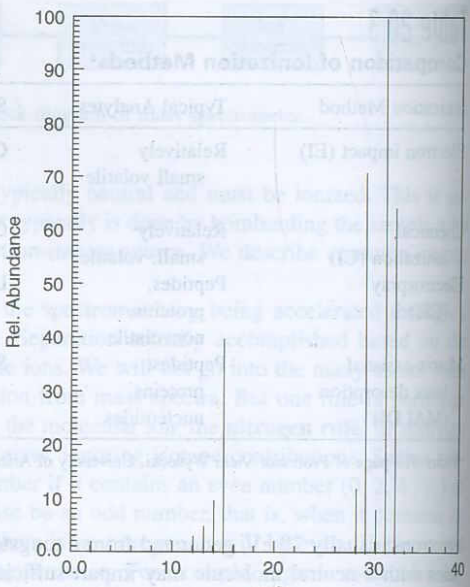


Fig. 20.10. Electron ionization mass spectrum of methanol. From NIST MassSpec Data Center. Reproduced by permission.

either a proton or hydride transfer. The chemical ionization process begins by ionization of the reagent gas. With methane, electron collisions produce CH_4^+ and CH_3^+, which further react with CH_4 to form CH_5^+ and $C_2H_5^+$:

$$CH_4^+ + CH_4 \rightarrow CH_5^+ + CH_3$$
$$CH_3^+ + CH_4 \rightarrow C_2H_5^+ + H_2$$

Table 20.4

Relative Abundances and Exact Masses of Some Common Elements.[a]

Element	Isotope	Mass	Relative Abundance (%)
Hydrogen	^{1}H	1.007825	100.0
	^{2}H	2.014102	0.0115
Carbon	^{12}C	12.000000	100.0
	^{13}C	13.003355	1.07
Nitrogen	^{14}N	14.003074	100.0
	^{15}N	15.000109	0.369
Oxygen	^{16}O	15.994915	100.0
	^{17}O	16.999132	0.038
	^{18}O	17.999160	0.205
Sulfur	^{32}S	31.972071	100.0
	^{33}S	32.971450	0.803
	^{34}S	33.967867	4.522
Chlorine	^{35}Cl	34.968852	100.0
	^{37}Cl	36.965903	31.96
Bromine	^{79}Br	78.918338	100.0
	^{81}Br	80.916291	97.28

[a] The most abundant isotope is assigned an abundance of 100%, and the others are listed relative to it.

These react with the sample by transferring a proton (H^+) or by extracting a hydride (H^-) or electron, which imparts a +1 charge on the sample molecule:

$$CH_5^+ + MH \rightarrow MH_2^+ + CH_4$$
$$C_2H_5^+ + MH \rightarrow M^+ + C_2H_6$$

MH_2^+ and M^+ may fragment to give the mass spectrum. No M^+ ion may be observed, but the molecular weight is readily obtained from the M + H or M − H ions formed. Weaker acid gas-phase ions further simplify spectra. $C_4H_9^+$ from isobutane and NH_4^+ from ammonia also ionize by proton transfer, but with less energy, and fragmentation of MH_2^+ is minimal. Table 20.5 lists the CI characteristics of different reagents. CI is almost universally available on modern GC–MS instruments for determining molecular weights of eluting compounds otherwise difficult to obtain due to extreme fragmentation with "harder" sources.

Other ion sources are described in Chapters 21 and 25.

MASS ANALYZERS

Mass spectrometers based on magnetic sectors are widely used by organic chemists to determine molecular structure. They deflect ions down a curved tube in a magnetic field based on their kinetic energy determined by the mass, charge, and velocity. The magnetic field is scanned to measure different ions. This mass separator is very powerful and capable of very high resolution, but the instruments are quite large and expensive and not very suitable for use with gas chromatographs. Most GC–MS instruments today are benchtop systems that use more compact, inexpensive mass analyzers with lower resolution. The availability of these is the reason GC–MS is so widely used.

The quadrupole analyzer is the most commonly used for GC–MS.

1. Quadrupole Mass Filter. The quadrupole mass analyzer is a "mass filter" that allows only specific ions to pass. Figure 20.11 shows the basic design of the quadrupole analyzer. It consists of four parallel metal rods to which both a dc voltage (U) and an oscillating radiofrequency voltage ($V \cos \omega t$, where ω is the frequency and t the time) is applied. Two opposite poles are positively charged and the other two negatively charged, and their polarities change throughout the experiment. The applied voltages are $U + V \cos \omega t$ and $-(U + V \cos \omega t)$. The applied voltages determine the trajectory of the ions down the flight path between the four poles. As ions from the ionization source enter the RF field along the z axis of the electrodes, they oscillate along the z axis. Only those with a specific mass-to-charge ratio will resonate along the field and have a stable path through to the detector. Others (nonresonating) will be deflected (unstable path) and collide

Table 20.5

Chemical Ionization Characteristics of Different CI Reagents

Reagent	Adducts Produced	Uses/Limitations
Methane	$M–H^+$, $M–CH_3^+$	Most organic compounds. Adducts not always abundant. Extreme fragmentation.
Isobutane	$M–H^+$, $M–C_4H_9^+$	Less universal. Adducts more abundant. Some fragmentation.
Ammonia	$M–H^+$ (basic compounds) $M–NH_4^+$ (polar compounds)	Polar and basic compounds. Others not ionized. Virtually no fragmentation.

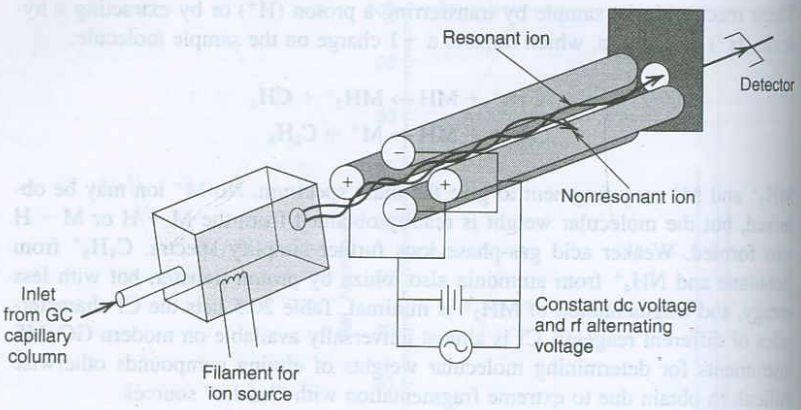


Fig. 20.11. Quadrupole mass spectrometer.

with the electrodes and be lost (they are filtered out). By rapidly varying the voltages, ions of one mass after another will take the stable path and be collected by the detector. Either ω is varied while holding U and V constant or U and V are varied, keeping U/V constant.

The quadrupole analyzer has a number of advantages that make it ideally suited for GC–MS. The path does not depend on the kinetic energy (e.g., velocity) or angular deflection of the incoming ions. So the transmission rate is high. Since only a change in voltage is required, a complete scan can be very fast. As many as eight spectra per second can be recorded over a range of about 800 mass units. Rapid scanning is needed to monitor GC peaks that may be a fraction of a second wide. A resolution of about 1500 can be achieved, and GC systems usually provide unit resolution. Finally, quadrupole instruments are relatively compact and inexpensive.

The TOF analyzer is good for large molecules.

2. Time-of-Flight Analyzer. The time-of-flight (TOF) analyzer is the second most popular mass analyzer for GC–MS. Figure 20.12 shows the basic construction of a TOF mass spectrometer. The ions formed in the ionization chamber are accelerated through the accelerator plates, which have a voltage of 3000 V, pulsating at 3000 to 20,000 times per second, and they enter the drift or flight tube

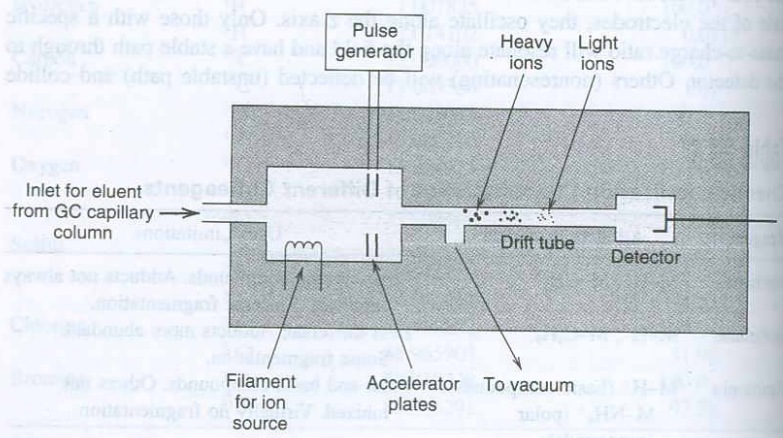


Fig. 20.12. Time-of-flight mass spectrometer.

with constant kinetic energy. Ions of different m/z travel at different velocities. The kinetic energy of ions leaving the source is given by:

$$\frac{mv^2}{2} = Vq \tag{20.3}$$

where m = mass of ion
v = velocity of ion
V = accelerating voltage
q = ion charge

Rearranging,

$$v = \sqrt{\frac{2Vq}{m}} \tag{20.4}$$

So the ion velocity is inversely proportional to the square root of the mass. The time t to reach the detector is $t = L/v$, where L is the length of the flight tube. The difference, Δt, in arrival time that separates two ions is

$$\Delta t = L \frac{(m_1)^{1/2} - (m_2)^{1/2}}{(2Vq)^{1/2}} \tag{20.5}$$

depending, then, on the square root of the masses.

Pulsed-mode acceleration is needed since continuous ionization and acceleration would lead to a continuous stream of all ions with overlapping masses. The sequence of events for pulsed operation is to turn on the electron source for 10^{-9} s to form a packet of ions, and then the accelerating voltage for 10^{-4} s to draw the ions into the drift tube. Then the power is turned off for the remainder of the pulse interval as the ion packets drift down the tube to the detector.

TOF analyzers, like quadrupoles, scan the mass spectrum rapidly. Resolutions of 500 can be obtained. These analyzers are popular for high mass ion detection since they have no real upper mass limit.

MASS SPECTROMETRY WITH GAS CHROMATOGRAPHY

One of the biggest problems in the early development of GC–MS was interfacing the column outlet to the mass spectrometer. Packed columns were used, and the high volumes of both sample and carrier gas overwhelmed the MS system, which operates under low pressure, and special interfaces had to be built. The advent of fused silica capillary columns meant that the GC–MS interface could be dispensed with, and the column eluent is introduced directly into the ion source. It is essential that column bleeding be minimized since the mass spectrometer will detect the stationary-phase materials. Bleeding is prevented by chemically bonding alkylsiloxanes to the column wall. Other low bleeding stationary phases are mentioned above.

Of course, monitoring GC peaks (several times per second) generates enormous amounts of data. The evolution of fast and high-capacity computers is the other technology advancement that made GC–MS routine.

The separated ions are detected by means of an electron multiplier, which is similar in design to photomultiplier tubes described in Chapter 16. Detection sensitivities at the nanogram level are common.

Nanogram quantities are detected by MS.

The mass spectrometer may be operated in various modes. In the **total ion current** (TIC) monitoring mode, it sums the currents from all fragment ions as

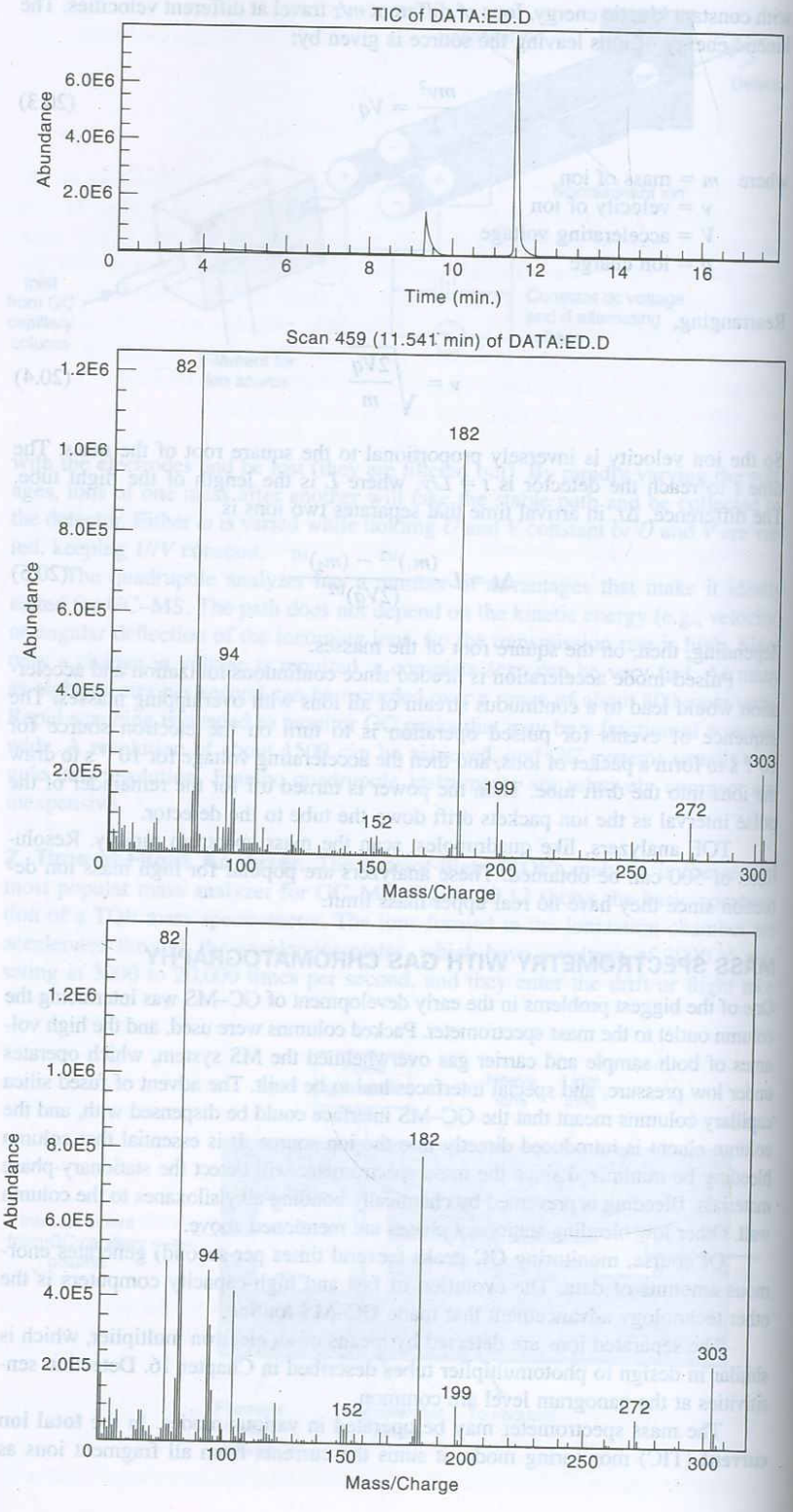


Fig. 20.13. Confirmation of cocaine by GC–MS. *Top:* Total ion current gas chromatogram of cocaine in urine sample. *Middle:* Mass spectrum taken from peak at 11.5 min. *Bottom:* Mass spectrum taken from GC peak of cocaine standard at same elution time.

a molecule (or molecules) in a GC peak passes through the detector, to provide a conventional looking gas chromatogram of several GC peaks. In the **selective ion** mode, a specific *m/z* ratio is monitored, and so only molecules that give a molecular or fragment ion at that ratio will be sensed. The **mass spectrum** of each molecule detected is stored in the system's computer, and so the mass spectrum corresponding to a given GC peak can be read out. The mass spectrum is generally characteristic for a given compound (if only one compound is present under the GC peak), giving a certain "fingerprint" of peaks at various *m/z* ratios. Certain peaks will dominate in intensity.

Figure 20.13 illustrates the application of GC–MS for positive identification of cocaine in a suspected powder sample, dissolved in methanol. Shown at the top is the gas chromatogram of the sample obtained from the TIC. The peak at 11.5 minutes corresponds to the retention time expected for cocaine. The middle figure is the mass spectrum corresponding to the compound at that peak, and the bottom one is the mass spectrum of a cocaine standard. The mass spectrum of the sample compound is essentially the same as for the cocaine standard. Furthermore, the parent ion peak is present at the *m/z* corresponding to M^+ for cocaine (formula weight 303.35). (There is a small peak at *m/z* 304 corresponding to MH^+, which is often formed in the ion chamber.)

The highest *m/z* peak often corresponds to MH^+. It may be very small.

The fragmentation pattern often exhibits peaks corresponding to loss of specific groups in the molecule, for example, —CO_2 or —NH, which lends further credence to the presence of a given molecule or which can be used to gain structural information about a molecule. Manufacturers of mass spectrometers provide computer libraries of mass spectra of thousands of compounds, and spectral computer searches can be made to match an unknown spectrum.

The NIST/EPA/NIH Mass Spectral Library 1998 database (www.nist.gov/srd/analy.htm) is the product of a multiyear, comprehensive evaluation and expansion of the world's most widely used mass spectral reference library, and is sold in ASCII or Windows versions. It contains 108,000 compounds with electron ionization spectra, chemical structures, and molecular weights. It is available with the NIST MS Search Program for GC/MS deconvolution, MS interpretation, and chemical substructure analysis. The NIST chemistry WebBook (http://webbook.nist.gov) is a *free* online system that contains the mass spectra of over 12,000 compounds (this Standard Reference Data Program also has IR and UV–Vis spectra).

The Wiley/NIST Registry of Mass Spectral Data, 7th edition, 1999, contains over 390,000 reference spectra. The registry has 32-bit search software to identify unknown mass spectra. You can view reference spectra by mass, molecular weight, or peaks.

The marriage of capillary gas chromatography with mass spectrometry provides an extremely powerful analytical tool. Capillary GC, with thousands of theoretical plates, can resolve hundreds of molecules into separate peaks, and mass spectrometry can provide identification. Even if a peak contains two or more compounds, identifying peaks can still provide positive identification, especially when combined with retention data.

Learning Objectives

WHAT ARE SOME OF THE KEY THINGS WE LEARNED FROM THIS CHAPTER?

- Gas chromatograph, p. 575
- GC columns—packed, capillary, p. 578
- Stationary phases—polar to nonpolar, p. 581

- GC detectors (see Table 20.2), p. 584
- Temperature programming, p. 588
- Quantitative measurements—internal standards, spreadsheets for calculation, p. 589
- Headspace analysis, thermal desorption, purging, and trapping, pp. 590, 591
- Small columns for fast separations, p. 592
- GC–MS, mass analyzers, pp. 593, 597, 599

Questions

1. Describe the principles of gas chromatography.
2. What compounds can be determined by gas chromatography?
3. What are the main types of gas chromatography?
4. Compare packed and capillary columns in number of plates.
5. Compare WCOT, SCOT, and PLOT columns.
6. Describe the principles of the following gas chromatography detectors: (a) thermal conductivity, (b) flame ionization, (c) electron capture.
7. Compare the detectors in Question 6 with respect to sensitivity and types of compounds that can be detected.
8. How does temperature programming improve separations?
9. What is required for fast GC analysis?
10. Describe the principles of gas chromatography–mass spectrometry. What are its benefits?
11. What is the molecular ion?
12. What is the nitrogen rule?
13. What ion sources are commonly used for GC–MS?
14. What mass analyzers are commonly used for GC–MS?

Problems

15. What mass spectrometry resolution is required for unit resolution of molecular mass 600 and 601?
16. A mass spectrometer has a resolution of 5000. How closely are two peaks at nominal mass 600 resolved?
17. Gas reduction valves used on gas tanks in gas chromatography usually give the pressure in psig (pounds per square inch above atmospheric pressure). Given that atmospheric pressure (760 torr) is 14.7 psi, calculate the inlet pressure to the gas chromatograph in torr, for 40.0 psig, if the ambient pressure is 745 torr.

SPREADSHEET PROBLEM

18. A water sample is analyzed for traces of benzene using headspace analysis. Samples and standards are spiked with a fixed amount of toluene as internal standard. The following data are obtained:

ppb Benzene	Peak Area Benzene	Peak Area Toluene
10.0	252	376
15.0	373	370
25.0	636	371
Sample	533	368

What is the concentration of benzene in the sample? Prepare a spreadsheet similar to the one described in the chapter, and print the calibration curve.

LITERATURE SEARCH

19. Using Chemical Abstracts or SciFinder Scholar (the online access to Chemical Abstracts—see Appendix A) if your library subscribes to it, find at least one article on the gas chromatography determination of ethanol in blood. Read the journal article and write a summary of the principle of the method and prepare a synopsis of the procedure employed, including any sample preparation. Is there information on the accuracy and precision of the method?

Recommended References

GENERAL

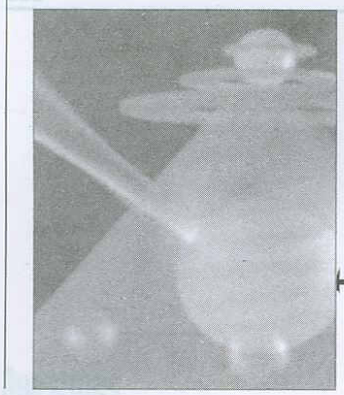
1. H. M. McNair and J. M. Miller, *Basic Gas Chromatography*. New York: Wiley-Interscience, 1997.
2. W. Jennings, E. Mittlefehldt, and P. Stremple, eds., *Analytical Gas Chromatography*, 2nd ed. San Diego: Academic, 1997.
3. D. Rood, *A Practical Guide to Care, Maintenance, and Troubleshooting of Capillary Gas Chromatography*. New York: Wiley, 1999.
4. A. Van Es, *High-Speed Narrow-Bore Capillary Gas Chromatography*, Heidelberg: Huthig, 1992.
5. B. Kolb and L. S. Ettre, *Static Headspace-Gas Chromatography*. New York: Wiley, 1997.
6. W. O. McReynolds, "Characterization of Some Liquid Phases," *J. Chromatogr. Sci.*, **8** (1970) 685.
7. D. M. Ottenstein, "Column Support Materials for Use in Gas Chromatography," *J. Gas Chromatog.*, **1**(4) (1963) 11.
8. W. R. Supina and L. P. Rose, "The Use of Rohrschneider Constants for Classification of GLC Columns," *J. Chromatogr. Sci.*, **8** (1970) 214.

GAS CHROMATOGRAPHY/MASS SPECTROMETRY

9. M. C. McMaster and C. McMaster, *GC/MS: A Practical User's Guide*. New York: Wiley, 1998.
10. H.-J. Hubschmann, *Handbook of GC/MS*. New York: Wiley, 2001.
11. M. Oehme, *Practical Introduction to GC-MS Analysis with Quadrupoles*. New York: Wiley, 1999.

WEB PAGES

12. Scientific Instrument Services, www.sisweb.com. Has exact mass calculator.
13. JEOL USA, Inc., www.jeol.com. Has tutorials on basic mass spectrometry and on mass analyzers, and links to other MS websites.



Chapter Twenty-One

LIQUID CHROMATOGRAPHY

HPLC is the liquid chromatography analog of GC. The secret to its success is small uniform particles to give small eddy diffusion and rapid mass transfer.

Gas chromatography (GC), because of its speed and sensitivity and quite broad applicability, has been more widely used since its development than the various modes of liquid chromatography. But the latter techniques have potentially broader use because approximately 85% of known compounds are not sufficiently volatile or stable to be separated by gas chromatography. The wealth of chromatographic theory accumulated, primarily from gas chromatography, has led to the development of techniques of **high-performance liquid chromatography (HPLC)** that rival gas chromatography in performance and allows separations and measurements to be made in a matter of minutes. The driving force for the rapid acceptance of gas chromatography in the 1950s was its immediate application to the petrochemical industry. Conversely, the applicability of HPLC to the pharmaceutical industry made it the mainstay of pharmaceutical laboratories in the 1970s, and today the HPLC market for new instruments is larger than the more mature GC market.

In this chapter, we describe the principles of HPLC and developments that have led to its success. The techniques of normal and reverse-phase liquid chromatography (separations based on polarity), size exclusion chromatography (separations based on molecular size), and ion exchange chromatography (separations based on charge) are described. Thin-layer chromatography, a planar form, is discussed. In addition, we describe electrophoresis, in which separations are accomplished in an electric field gradient, based on the sign and magnitude of charge on the analyte.

21.1 High-Performance Liquid Chromatography

In 1964, J. Calvin Giddings from the University of Utah published a paper entitled, "Comparison of the Theoretical Limit of Separation Ability in Gas and Liquid Chromatography" [*Anal. Chem.*, **36** (1964) 1890]. Conventional liquid chromatography up to that time was primarily accomplished in large columns with large particles under gravity feed, with manual collection of fractions of eluents for measurement in a spectrophotometer. Giddings predicted improved liquid chromatography performance if one could use small particles under increased flow pressure and that theoretically very high plate numbers could be achieved. A couple of years later, Csaba Horvath and colleague S. R. Lipsky at Yale University built the first HPLC, and called it "high pressure liquid chromatography." But it was not until the early

1970s that the technology of producing small silanized silica particles allowed the use of small-volume longer columns necessary to give the high-resolution performance. Today, HPLC has become known as "high performance liquid chromatography."

PRINCIPLES

Classical liquid chromatography has largely been supplanted by the much more powerful and analytically useful form of high-performance liquid chromatography. Figure 21.1 shows the basic components of an HPLC system, and Figure 21.2 illustrates a modern HPLC instrument. These instruments tend to be assembled in modular form, unlike most GC instruments, allowing the user to change different components.

The rate of distribution of solutes between the stationary and the mobile phase in traditional liquid chromatography is largely diffusion-controlled. Diffusion in liquids is extremely slow compared to that in gases. To minimize diffusion and the time required for the movement of sample components to and from the interaction sites in the column, two criteria should be met. First, the packing should be finely divided and have high spherical regularity to allow for optimum homogeneity and packing density; and second, the stationary phase should be in the form of a thin uniform film with no stagnant pools. The former results in a smaller A value in the van Deemter equation (smaller eddy diffusion) and the latter results in a small C value (more rapid mass transport between the phases—necessary for high flow rates). Because molecular diffusion in liquids is small, the B term in Equation 19.8 is small. Hence, the detrimental increase in H at slow flow rates does not occur as in Figure 19.4. This is illustrated in Figure 21.3 and is expressed by the Huber equation (Equation 19.21) and the Knox equation (Equation 19.23) in Chapter 19.

Molecular or longitudinal diffusion in liquids is slow and can be neglected.

Mass transfer is the primary determinant of H in HPLC.

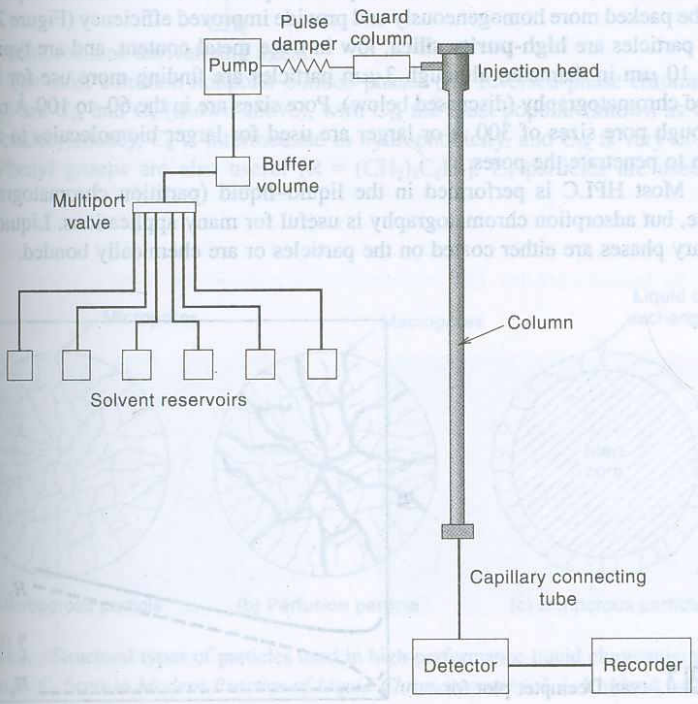


Fig. 21.1. Basic components of high-performance liquid chromatograph. (Adapted from Analabs, Inc. *Research Notes*. Copyright © 1971. Reproduced by permission.)

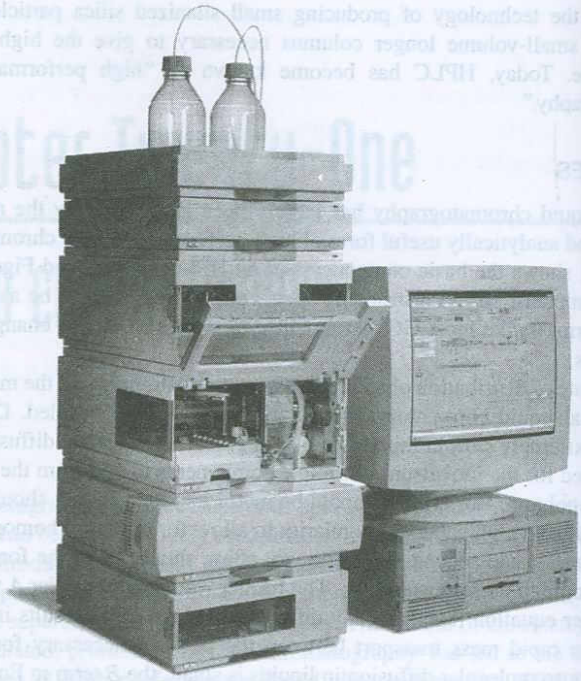


Fig. 21.2. High-performance liquid chromatograph. (Courtesy of Agilent Technologies.)

STATIONARY PHASES

Original HPLC microparticles were irregularly shaped porous silica gel or alumina particles of 10 μm or less. Since then, spherical particles have been developed that can be packed more homogeneously and provide improved efficiency (Figure 21.4). The particles are **high-purity silica**, low in trace metal content, and are typically 5 to 10 μm in diameter, although 3-μm particles are finding more use for high-speed chromatography (discussed below). Pore sizes are in the 60- to 100-Å range, although pore sizes of 300 Å or larger are used for larger biomolecules to allow them to penetrate the pores.

Most HPLC is performed in the liquid–liquid (partition chromatography) mode, but adsorption chromatography is useful for many applications. Liquid stationary phases are either coated on the particles or are chemically bonded.

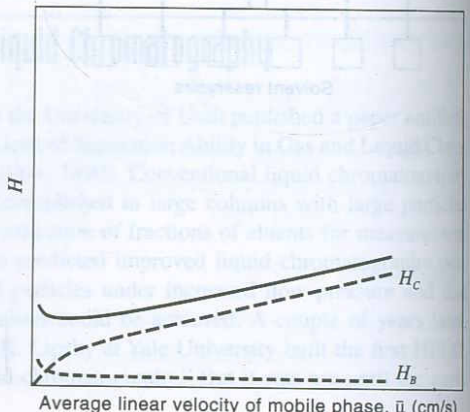
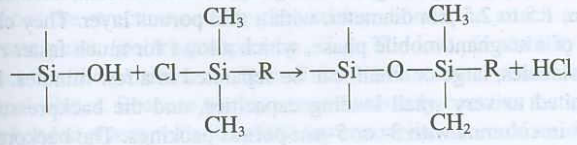


Fig. 21.3. van Deempter plot for HPLC.

The most commonly used particles are **microporous** or **diffusive particles**, permeable to solvent (Figure 21.5*a*). The majority of the surface area is within the pores. Mobile phase moves around the particles and solute diffuses into the stagnant mobile phase within the pores to interact with the stationary phase, and then diffuses out into the moving mobile phase. The use of small particles minimizes the pathlength of the diffusion pathlength, and hence band broadening. Those in Figure 21.4 are microporous. Figure 21.6 shows an aggregated spherical particle with 50% porosity, with 100-Å pore size. Also shown is a widely used one with a spongelike structure with about 70% porosity and the same nominal pore size with greater surface area, but with greater distribution in pore size and less resistance to solubility in alkaline solution.

Silica tends to dissolve above pH 8, and crosslinked polymeric particles, for example, polystyrene or polymethacrylates, are used for separation of bases. These can withstand strongly basic mobile phases but exhibit somewhat lower efficiency.

Silica particles have surface silanol groups, —SiOH. These are used for chemical bonding of stationary phases by silination reactions with chlorosilanes:



About half the silanol groups are chemically bonded, and the remainder are "endcapped" with trimethyl silyl groups to render them inert. Zorbax also makes particles in which the siloxane bonds are protected by steric hindrance provided by larger isopropyl groups, $-\overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{\overset{|}{\underset{|}{C}}}}-CH_3$, in place of the two methyl groups on the organochlorosilane derivatizing agent.

The most common nonpolar bonded phases (for reversed-phase chromatography) are C_{18} and C_8 (shown above), with C_{18} the most popular (known as ODS for octadecylsilane); C_8 is intermediate in hydrophobicity, and C_{18} is very nonpolar. Phenyl groups are also useful [$R = (CH_2)_3C_6H_5$]. C_5 particles are used for

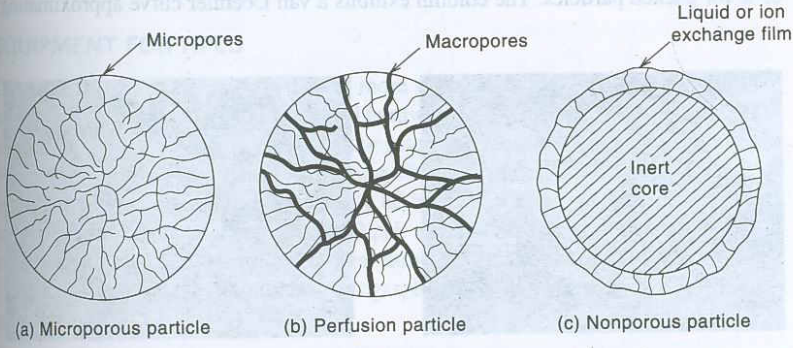


Fig. 21.5. Structural types of particles used in high-performance liquid chromatography. (From D. C. Scott in *Modern Practice of Liquid Chromatography*, J. J. Kirkland, ed. New York: Wiley-Interscience, 1971; with permission.)

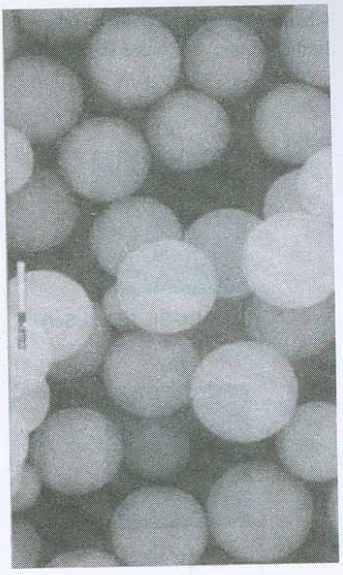
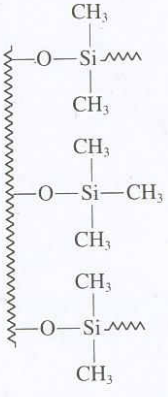


Fig. 21.4. Spherical porous silica particles, 10 μm, 800× magnification. Particles are fully porous with 100-Å pores. They are available as base silica for adsorption chromatography, or with bonded phases. (Astrosil® from Stellar Phases, Inc. Courtesy of Stellar Phases, Inc.)



Endcapping. The middle free SiOH group has been endcapped.

Chemically bonded phases (functional groups) are more stable.

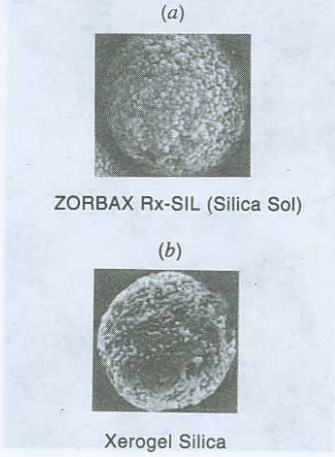


Fig. 21.6. (*a*) Zorbax porous silica microsphere particle, 50% porosity, 100-Å pores. (*b*) Xerogel silica particle, 70% porosity, 100-Å pores. (Courtesy of Agilent Technologies.)

HPLC–MS because of their low bleed properties. Polar phases for normal-phase chromatography in increasing order of polarity include cyano [R = $(CH_2)_3CN$], diol [R = $(CH_2)_2OCH_2CH(OH)CH_2OH$], amino [R = $(CH_2)_3NH_2$], and dimethylamino [R = $(CH_2)_3N(CH_3)_2$].

When particles are coated with liquid phases, rather than bonded, they are first treated with trimethylcholosilane to deactivate the silanol groups.

Perfusion packings, developed by Fred Regnier and co-workers at Purdue University, are made of a mixture of large and small pores (larger through pores and small diffusive pores, Figure 21.5*b*). The diffusive pores, as in microporous particles, provide the sorption capacity. The through pores allow the mobile phase to pass directly through the packing, hence increasing the rate of mass transfer in the mobile phase. Since the solute spends less time undergoing mass transfer, peaks are narrower; the process is actually a combination of diffusion plus convection. These packings are larger than the microporous ones, being about 12 μm in diameter. They can be used at higher flow rates and give better efficiency for large molecules such as proteins. They are also useful for preparative chromatography.

Nonporous packings (Figure 21.5*c*), either silica or resin, have much smaller particle size, 1.5 to 2.5 μm diameter, with a thin porous layer. They eliminate the occurrence of a stagnant mobile phase, which allows for much faster rate of mass transfer. Molecules, large or small, can be separated in a few minutes. But the thin layer is limited to very small loading capacities, and the backpressure is much greater than in columns with 3- or 5-μm porous packings. The backpressure is inversely proportional to the square of the particle diameter, so cutting the size in half causes the pressure to increase fourfold. For these reasons, small porous particles are preferred for most applications, and a 3.5-μm porous packing can give comparable separation times as a column containing 1.5-μm nonporous silica bonded phase, with less pressure, when operated at high flow rates. Nonporous columns are useful for separating complex peptide mixtures in seconds to minutes, and are used in ion chromatography (discussed below).

Monolithic columns are another approach to provide lower pressure drops and higher rates of mass transfer. These are continuous solid columns of porous silica stationary phase instead of packed particles. Like perfusion packings, they have a bimodal pore structure (Figure 21.7). Macropores, which act as flowthrough pores, are about 2 μm in diameter. The silica skeleton contains mesopores with diameters of about 13 nm (130 Å). It can be surface modified with stationary phases like C_{18}. The rod is shrink-wrapped in a polyetheretherketone (PEEK) plastic holder to prevent "wall effects" of solution flowing along the walls. The surface area of the mesopores is about 300 m^2/g, and the total porosity is 80%, compared with 65% for packed particles. The column exhibits a van Deemter curve approximating

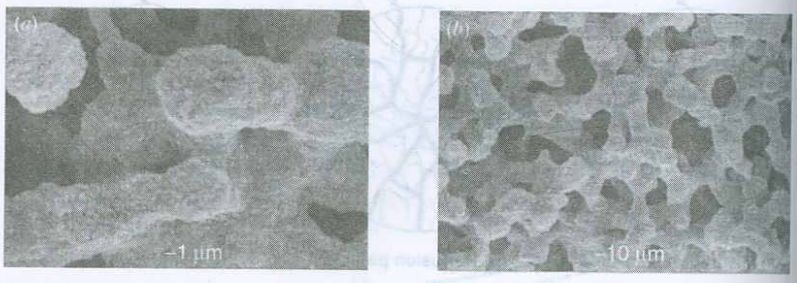


Fig. 21.7. Electron micrograph of (*a*) mesoporous and (*b*) macroporous structures in monolithic rod. [From D. Lubda, K. Cabrera, W. Krass, C. Schaefer, and D. Cunningham, *LC·GC* **19** (12) (2001) 1186. Reproduced by permission.]

that for 3.5-μm packed particles, giving significantly better efficiency than conventional 5-μm particles. Because of low pressure drops, flow rates as high as 9 mL/min can be used, resulting in fast separations. Mass transport is facilitated by convection in addition to diffusion, making these columns well suited for efficient separations of proteins, peptides, and polynucleotides, as well as small molecules. Polymeric monolithic columns are rather short, and the plate counts are too low to perform isocratic separation of complex mixtures, although several columns can be linked in series. These columns are manufactured by Merck KGaA, Darmstadt, Germany (www.merck.de), and are called Chromolith.

Various approaches have been taken to develop HPLC particles, besides polymer-based ones, that can be used at high pH, as well as in acid solution. High pH is required for the separation of basic compounds. Silica particles are generally limited to about pH 8 because the silane bonds hydrolyze about this pH, and they have a lower limit of about pH 2. Waters Corporation (www.waters.com) developed a **hybrid silica/polymer** particle they call Xterra. The particles are derived from a mixture of two high-purity monomers, one forming SiO_2 units and the other $RSiO_{1.5}$ units, where R is a methyl group. The organasilane groups are incorporated throughout the structure and are then surface-bonded to attach a variety of different reverse-phase (polar) groups (e.g., C_{18}). The low pH stability is in part due to the fact that the particles are formed from trifunctional silanes rather than monofunctional silanes. Because the base particle already contains methylsiloxane units, it gives bonded phases with 30 to 50% lower concentrations of residual silanol groups, which gives reduced peak tailing. The particles exhibit good stability at both high (greater than pH 11) and low pH.

Peter Carr at the University of Minnesota has developed **porous zirconia** (ZrO_2) particles that are chemically stable over the pH range 1 to 14 and are also thermally stable up to 200°C. These are manufactured by ZirChrom Separations, Inc. (www.zirchrom.com). They are produced through the controlled polymerization-induced aggregation of 1000-Å colloidal zirconia, resulting in monodisperse 3-μm porous zirconia spheres. These are then sintered at temperatures up to 900°C to produce a monoclinic crystallographic form of zirconia. The particles are bonded with a variety of reverse-phase stationary phases. Thin layers of polybutadiene or polystyrene are coated on the zirconia. Particles are also produced with a very thin layer of elemental carbon that can be used as a stationary phase. A proprietary covalent bonding technology is used to graft C_{18} groups to the carbon surface. Visit the ZirChrom website for a list of representative uses for each type of particle. (Its site also has a free automated buffer preparation calculator.) See also *Anal. Chem.*, **73** (2001) 598A for an excellent review of the properties of these phases.

EQUIPMENT FOR HPLC

The price that must be paid for faster, more efficient separations using finer column packings is pressure and the special hardware to handle this. Pressures of 1000 to 3000 psi are required to provide flow rates of 1 to 2 mL/min in columns of 3- to 5-mm diameter and 10 to 30 cm long, although in certain instances pressures up to 6000 psi may be required. Probably 80 to 90% of HPLC separations are performed with pressures of less than 1200 psi, and even some polyurethane column materials require very low pressures near atmospheric pressures.

High-performance liquid chromatography apparatus consists of four principal parts:

1. Mobile-Phase Supply System. This system contains a pump to provide the high pressures required and usually contains some means of providing gradient elution (i.e., changing concentrations of the eluent, such as solvent, salt, or H^+).

The solvent reservoirs can be filled with a range of solvents of different polarities, provided they are miscible, or they can be filled with solutions of different pH and are mixed in the buffer volume. The solvents must be pure and be degassed to avoid formation of gas bubbles when they prevent proper check valve function or enter the piston chamber. And they generate spurious peaks when they pass through the detector. The problem is most serious when mixing solvents (usually acetonitrile or methanol with water) because the solubility of air in mixtures is less than in the same proportion of pure solvents; and, when solvents saturated with air are mixed, bubbles are released. Degassing systems only need to remove enough air to reduce it to below saturation levels. The most common ways of degassing are sparging with helium, which removes about 80% of the air from solution, or vacuum degassing, which removes around 60%. When sparging with helium, some of the more volatile solvent is evaporated, and commercial systems incorporate pressure reservoirs to minimize this. The air is replaced with another gas, helium, but its solubility is such that outgassing is not a problem. Many manufacturers incorporate an online vacuum degassing system in which the solvent passes through a thin-walled porous polymer tube, for example, PTFE or Teflon AF, an amorphous fluoropolymer with two- to threefold higher gas diffusion rates, in a vacuum chamber. Some workers prefer to first briefly sparge with helium, and then rely on the vacuum degasser to maintain low gas content.

Typical flow rates are 1 to 2 mL/min for conventional 4.6-mm i.d. columns. Solvents used for HPLC should be of "HPLC" grade, that is, solvents that have been filtered through 0.2-μm filters. This extends the pump life by preventing scoring and reduces contamination or plugging of the column.

The most commonly used pump for HPLC is the reciprocating pump. This has a small cylindrical piston chamber that is alternately filled with mobile phase and emptied via back-and-forth movement of the piston. This produces a pulsed flow that must be damped. Reciprocating pumps have a number of advantages. They have a small internal volume, are capable of high output pressures, and they can readily be used for gradient elution. They provide constant flow rates, independent of solvent viscosity or column backpressure. Other pumps used are motor-driven syringe pumps and pneumatic (constant-pressure) pumps.

2. Sample Injection System. A typical injection system is shown in Figure 21.8. This consists of a stainless steel ring with six different ports, one to the column. A movable Teflon cone within the ring has three open segments, each of which connects a pair of external ports. Two of the ports are connected by an external sample loop of known or fixed volume. In one configuration, the cone permits direct flow of effluent into the column, and the loop can be filled with the sample. The cone can then be rotated 30° to make the sample loop part of the moving stream, which sweeps the sample into the column. Samples of a few microliters can be injected at pressures up to 6000 psi.

Samples can be introduced manually into the valve with a syringe to fill the sample loop. Automated sampling valves are routinely used today in which samples are taken from an autosampler for unattended operation. The major limitation of valve injectors is that the sample size is fixed, and the loop must be changed in order to vary the injected sample size. There are automated motor-driven adjustable syringes that provide enough pressure to inject the sample past a check valve that prevents backflow.

3. Column. Straight lengths of stainless steel tubing make excellent columns (Figure 21.9). These come in various diameters and lengths, depending on the particular application. Usually the internal diameter is 3.9 or 4.6 mm. A well-packed

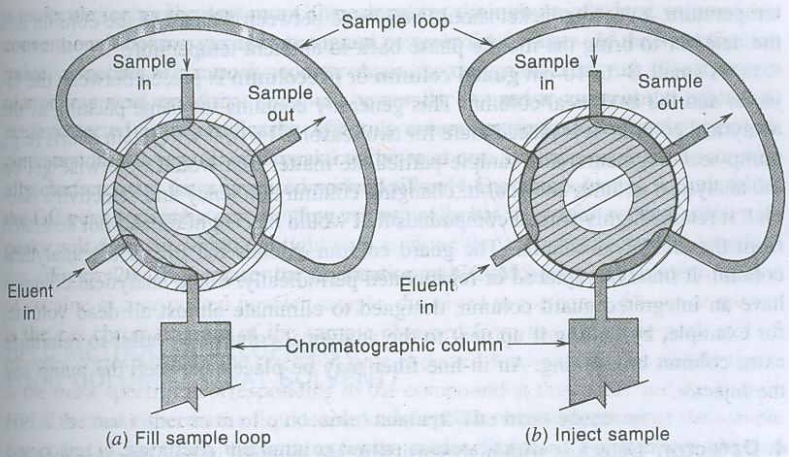


Fig. 21.8. Sample loop injector. (From D. C. Scott in *Modern Practice of Liquid Chromatography*, J. J. Kirkland, ed. New York: Wiley-Interscience, 1971. With permission.)

4.6-mm column of 5-μm-diameter (d_p) particles should give a plate count on the order of 60,000 to 90,000 plates/meter at flow rates of 1 mL/min. A typical 15-cm-long column with 4.6-mm i.d. will give 15,000 plates with 3-μm particles, 9000 plates with 5-μm particles, and 5000 plates with 10-μm particles. The smaller particles are limited to 25 cm length or shorter columns because of high-pressure drops. At 25 cm, 50,000 plates are possible at a pressure of 5000 psi with water–methanol mixtures. They can resolve 50 to 100 peaks, about the upper limit for conventional HPLC. We will see below that fast chromatography can be performed with wide-bore, short columns, but narrow columns give better resolution.

Some rules for selecting column stationary and mobile phases are given below. Temperature control of the column is usually not necessary for liquid–solid chromatography, unless it has to be operated at elevated temperatures, but is generally required for other forms of liquid chromatography (liquid–liquid, size exclusion, ion exchange). Some detectors, especially refractometers, are very sensitive to temperature changes; and so, if the column is operated at greater than ambient

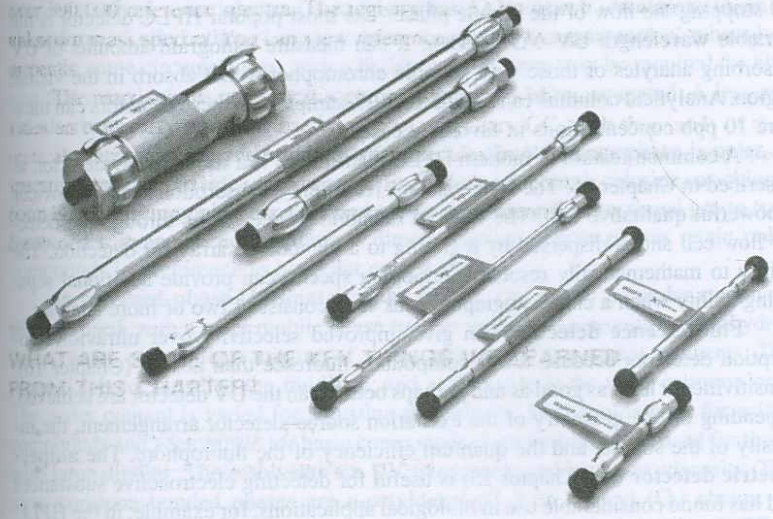


Fig. 21.9. Typical HPLC columns. (Courtesy of Waters Corporation.)

temperature, a cooling jacket should be placed between the end of the column and the detector to bring the mobile phase back to ambient temperature.

A guard column extends the life of the analytical column and improves separations by retaining strongly sorbed compounds and debris.

A small, 3- to 10-cm **guard column** or **precolumn** is placed between the injector and the analytical column. This generally contains the same packing as the analytical column. It is placed there for two reasons. First, it will retain debris (e.g., pump-seal fragments) and sample particulate matter that would otherwise get on the analytical column and foul it, changing column efficiency and selectivity. Second, it retains highly sorbed compounds that would be caught on and not be eluted from the analytical column. The guard column extends the life of the analytical column. It must be replaced or regenerated periodically. Some analytical columns have an integrated guard column, designed to eliminate almost all dead volume, for example, by butting it up next to the analytical column, in order to minimize extra column broadening. An in-line filter may be placed between the pump and the injector.

4. Detector. Detectors with high sensitivity are required in high-performance liquid chromatography, usually with sensitivities in the microgram to nanogram range. Widely used detectors are refractometer detectors and ultraviolet (UV) detectors. The **differential refractometer detector** is often called a "universal detector." This measures changes in refractive index of the eluent that result from the presence of solutes as they emerge from the column. It cannot be used effectively with gradient elution due to a change in the baseline (a change in the solvent index of refraction as the gradient is changed) nor when the solvent has an index of refraction close to that of the solutes. As mentioned, it is very sensitive to temperature changes. This detector is rugged and will detect concentrations of about 10^{-5} to 10^{-6} g/mL (10 to 1 ppm). The **ultraviolet detector** has much better sensitivity, about 10^{-8} g/mL (0.01 ppm). It is not temperature sensitive, is relatively inexpensive, and can be used with gradient elution. It is sensitive to a large number of organic compounds. Because of its advantages, the UV detector represents about 80% of the measurements made. Of course, it cannot be used with solvents that have significant absorption in the UV or with sample components that do not absorb in the UV.

Many UV detectors are simple interference filter devices that can measure the absorbance at only a few selected wavelengths. The more expensive detectors have a monochromator that allows selection of a particular wavelength. Scanning of the spectrum can even be achieved for qualitative identification by momentarily stopping the flow of the mobile phase. The most popular HPLC detector is the variable wavelength UV–Vis detector. It can measure nanogram amounts of UV absorbing analytes or those with suitable chromophores that absorb in the visible region. Analytical columns can handle 100-μL samples, so these detectors can measure 10 ppb concentrations in favorable cases.

A diode array detector provides additional resolving power and fingerprinting.

A common feature of modern HPLC instruments is a **diode array** detector, as described in Chapter 16. The instantaneous recording of absorption spectra provides a powerful qualitative tool. The focused radiation source passes through the detector flow cell and is dispersed by a grating to a photodiode array for detection. The ability to mathematically resolve overlapping spectra can provide additional separating ability when a chromatographic peak may consist of two or more analytes.

Fluorescence detectors can give improved selectivity over ultraviolet absorption detectors because fewer compounds fluoresce than absorb (Chapter 16). Sensitivities at least as good as and perhaps better than the UV detector are achieved, depending on the geometry of the excitation source–detector arrangement, the intensity of the source, and the quantum efficiency of the fluorophore. The **amperometric detector** (see Chapter 15) is useful for detecting electroactive substances and has found considerable use in biological applications, for example, in the HPLC separation and detection of trace quantities of catecholamines from the brain.

In arranging the apparatus, there must be a minimum of "dead volume" between the injection port and column and between the column and detector, to minimize spreading of the peaks and to obtain maximum efficiency. A 20-cm length of stainless steel capillary tubing can generally be used to connect the column to the detector without significantly affecting column performance. The detector volume must also be small, and typical volumes are on the order of 1 μL or so, with high-performance detectors used with capillary LC systems having submicroliter volumes. A spectrophotometric flow cell typically has a "Z-cell" configuration with quartz windows, in which the light path is along the axis of a horizontal length of the effluent flow to increase the absorbance pathlength and the sensitivity.

HPLC METHOD DEVELOPMENT: WHAT COLUMN? WHAT SOLVENT?

HPLC is used either in the liquid–solid **adsorption chromatography** mode or the liquid–liquid **partition chromatography** mode. The most common is partition chromatography, either normal or reversed-phase. Both partition and adsorption chromatography operate on differences in solute polarity since polarity is important in determining both adsorption and solubility. Liquid–liquid partition processes are quite sensitive to small molecular weight differences and so are preferred for the separation of members of a homologous series. Adsorption processes, on the other hand, are sensitive to steric effects and are preferred for the separation of isomers having different steric configurations. The most common adsorption phases are alumina or silica particle.

As a general rule, highly polar materials are best separated using partition chromatography, while very nonpolar materials are separated using adsorption chromatography. Between extremes, either process might be applicable. Compound polarity follows the approximate order of: hydrocarbons and derivatives < oxygenated hydrocarbons < proton donors < ionic compounds; that is, RH < RX < RNO_2 < ROR (ethers) = RCO_2R (esters) = RCOR (ketones) = RCHO (aldehydes) = RCONHR (amides) < RNH_2, R_2NH, R_3N (amines) < ROH (alcohols) < H_2O < ArOH (phenols) < RCO_2H (acids) < nucleotides < $^+NH_3RCO_2^-$ (amino acids). In adsorption chromatography the adsorbent is usually kept constant, and the eluting solvent polarity is increased until elution is achieved. Some commonly used solvents in order of increasing polarity are: light petroleum solvents (hexane, heptane, petroleum ether) < cyclohexane < trichloroethane < toluene < dichloromethane < chloroform < ethyl ether < ethyl acetate < acetone < *n*-propanol < ethanol < water. In some circumstances, such a highly polar solvent may be required for elution that many solutes are eluted together rather than being separated. A less polar adsorbent should then be used.

In **normal-phase chromatography** (NPC), the stationary phase is polar. A nonpolar mobile phase is used, such as *n*-hexane, methylene chloride, or chloroform. The stationary phase is a bonded siloxane with a polar functional group (polarity order: cyano < diol < amino < dimethylamino). These phases retain polar compounds in preference to nonpolar compounds.

In **reversed-phase chromatography** (RPC), a relatively nonpolar stationary phase is used, with a polar mobile phase such as methanol, acetonitrile, tetrahydrofuran, water, or usually a mixture of water with one of the organic solvents. The organic solvent is called the **modifier**, and acetonitrile is the most common one. The water content is varied for adjusting the polarity. Methanol is used for acidic compounds and acetonitrile for basic compounds. Tetrahydrofuran is used for those with large dipoles. These solvents are UV transparent and have low viscosity. The most common bonded phases are *n*-octyldecyl (C_{18}) or *n*-decyl (C_8) chains, or phenyl groups. Polar reversed-phase columns such as polyethylene glycol (PEG)

Reversed-phase HPLC is used to separate organic compounds.

contain ether groups that interact with polar analytes. They are useful for phenolic compounds and multiaromatic ring, hydroxyl-containing compounds. While many of these may by separated on C_{18} columns, there may be overlaps. Different orders of elution can be expected.

A wide range of organic compounds can dissolve in the mixed water–organic solvent phases and be separated, so RPC is by far the most popular form of HPLC. For those that can be separated either by NPC or RPC, the elution order is generally reversed, although not always exactly. Of course, if a sample is very nonpolar and insoluble in water mixtures, normal–phase chromatography is used. Otherwise, reversed-phase chromatography is used.

1. Solvent Selection. In contrast to gas–liquid chromatography, where the mobile gas phase is fixed and separation conditions are changed by varying the polar stationary phase, in liquid–liquid partition chromatography we can also vary the mobile phase, that is, the "solvent strength." This is easier to do than changing columns, and adjusting the mobile phase is part of developing an LC separation method.

In most applications, a pure solvent will not provide efficient separation of a range of compounds, and a blend of two or more solvents is used. The weak solvent is designated the A solvent and the strong solvent the B solvent. Trial-and-error can be used to obtain an optimum % B solvent. We are interested in two factors to achieve efficient separations with baseline resolution: the retention factor, k, a *polarity* term, and the separation factor, α, a *selectivity* term (see Equation 19.28). Generally, we try to adjust the solvent strength to position all solute bands within a k range of about 0.5 to 20. Adjusting for acceptable retention will provide adequate resolution for many compounds. If we have incomplete separation, a change in % B from say 35 to 40 or 45% will often result in significant change in band spacing and give better resolution. But while some bands may now be better separated, others may now overlap that did not before. A mixture of two strong solvents, for example, acetonitrile and methanol, with water can provide intermediate band spreading and acceptable resolution, as illustrated in Figure 21.10. The two experiments with 80 and 40% acetonitrile (ACN) gives an idea of k-value range

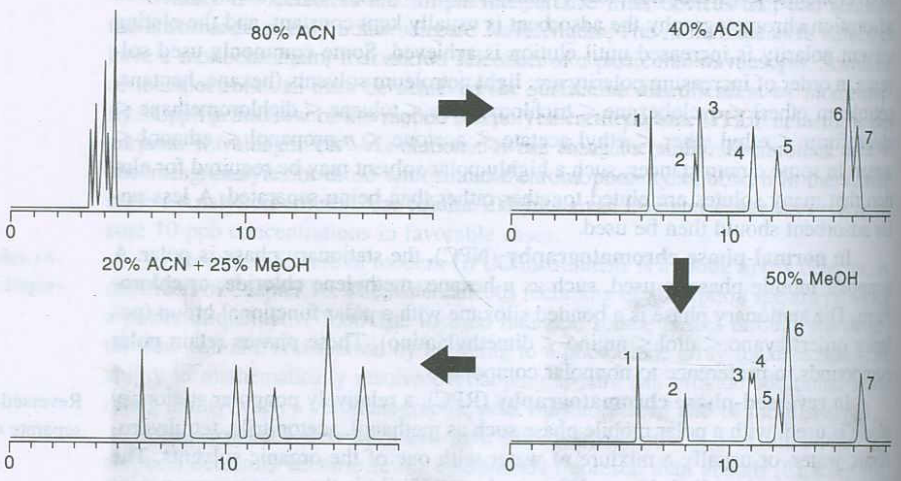


Fig. 21.10. Hypothetical series of method development experiments, beginning with a strong mobile phase of 80% acetonitrile–water. (From L. R. Snyder, J. J. Kirkland, and J. L. Glajch, *Practical HPLC Method Development*, 2nd ed. New York: Wiley-Interscience, 1998. With permission.)

with solvent strength; 40% ACN gives increased but still incomplete resolution. Changing from ACN to 50% methanol (MeOH) changes the spacing but results in new overlaps. Finally, mixing the two solvents (equal volumes of 40% ACN and 50% MeOH used before) gives a separation intermediate between the two, with acceptable resolution. This multi-solvent selection method works for many cases.

2. Column Differences. There are over 100 different brands of reversed-phase columns. Column manufacturers provide information such as plate count, unique selectivity, reproducibility, pressure drop, and so forth. While these are important to know, they are of little help to you in selecting the most appropriate column for most of your applications.

While indexes similar to those available for gas chromatography columns have not been developed, there have been attempts to try to classify columns. One way is to use a single compound such as aspirin to characterize a series of columns for a fixed polarity analyte, to determine the relative time it elutes. Others use a chemometrics (statistical) approach to group columns according to efficiency, peak symmetry, tailing, and free silane. These methods at present are of limited value in discerning relatively small differences. The bottom line is that empirical evaluation, starting with manufacturers' recommended applications, is the best way to settle on the appropriate column.

See www.mac-mod.com/comparison_guide.html for a very detailed comparison of 60 commonly used C_{18} phases. They are categorized by relative hydrophobicity and polarity, and column efficiencies for neutral and basic compounds are given. See also http://ois.nist.gov/srmcatalog/certificates/870.pdf complete NIST report.

3. Gradient Elution. In gas chromatography, we described how separations can be improved and speeded up by using temperature programming, but in HPLC this is a limited option (but see the discussion below on fast chromatography for exceptions). A more viable approach, not available in gas chromatography, is to employ mobile-phase gradient elution. Gradient elution LC is one of the most effective ways of separating several analytes with differing relative retention times. In isocratic elution, there is generally poor resolution of peaks early in the chromatogram and broadening of peaks at the longer retention times. In RPC, the gradient is accomplished by increasing the fraction of organic modifier solvent (e.g., methanol). This allows weakly retained compounds to be eluted later and be better resolved than they would in isocratic elution, and longer retained ones to be eluted more quickly, giving a chromatogram with compact, well-spaced peaks, with improved peak shape and lower detection limits since band broadening is lessened.

As in GC temperature programming, the gradient can be stepwise or continually ramped. The pumps are programmed to keep the total flow rate constant. The starting solvent should have a polarity that rapidly elutes and resolves the first components, and the solvent strength (polarity) is increased to a value that resolves the last peaks in a reasonable time. The multi-solvent method described above can help determine the starting and finishing polarities.

One of the problems encountered with gradient elution in reversed-phase chromatography is that the column has to be reequilibrated with the beginning solvent between runs. This requires flushing with 15 to 20 column volumes of the initial mobile phase. John Dorsey at Florida State University developed a procedure to dramatically reduce the reequilibration time by controlling the solvation of the bonded alkyl chains throughout the chromatographic run [L. A. Cole and J. G. Dorsey, *Anal. Chem.*, **62** (1990) 16]. A 3% solution of 1-propanol provides nearly monolayer coverage of a C_{18} surface, and adding 3% 1-propanol to each mobile-phase component reduces the reequilibration time about 75%. Also, the wetting effect provided by the 1-propanol may give increased column efficiencies at the beginning of the chromatogram with highly aqueous initial mobile phase. See also D. L. Warner and J. G. Dorsey, *LC·GC*, **15**(3) (1997) 254, for additional details on practical applications.

Gradient elution works well with bonded-phase partition chromatography and adsorption chromatography but is difficult to use with liquid–liquid chromatography.

FAST LIQUID CHROMATOGRAPHY: SMALL IS BETTER

Faster separations result from using smaller particles and short columns.

By making columns shorter and packing them with smaller particles, separations can be performed in one-tenth the time of conventional HPLC (typically on the order of 20 min), and some separations require only a few seconds. Fast liquid chromatography (LC) columns have the same diameter as conventional columns (4.6 mm) but are only 1 to 3 cm in length, instead of the typical 25 cm, and are packed with 3-μm particles instead of 5 μm, operating in the 1- to 3-mL/min range. The H vs. $\bar{u}$ plot is fairly flat at high flow rates (see Figure 19.5) because the smaller particles are less resistant to mass transfer (C term in the van Deemter equation). The columns are short because of the increased backpressure with the smaller particles, but a typical column can generate 4000 plates, enough for many separations. As many as 20 peaks have been separated in 30 s. While not high resolution, such separations solve many of the analytical problems in the pharmaceutical industry, which accounts for about half the HPLC market. In pharmaceutical quality control, analysts generally need to look for a few active ingredients in a tablet or formulation.

Why use fast LC? Besides the obvious advantage of faster analyses, there is 50 to 80% less solvent use (and less waste generated). Sensitivity is increased 3 to 5 times because there is less dilution of the analyte peaks with the smaller column volumes as a result of tighter packing. These densely packed columns are more prone to clogging. A scavenger column or filter is placed before the injector, in addition to the guard column placed before the column.

If resolution needs to be increased when using these short columns, the mobile phase can be adjusted so that all analytes are eluted in a k range of 1 to 10, with slower elution, for example, 10 min instead of 30 s. A modifier of 50% acetonitrile may be used in high-speed LC, with only 20% for high-resolution LC.

As in high-speed gas chromatography, high-speed LC requires fast-responding detectors and electronics to measure the narrow (0.5 s or 3 μL), fast eluting peaks. The detector should have a time constant capable of measuring rates of change of signal on the order of 50 to 100 ms or less. The sampling rate of the data system should be >10 points/second, and diode array spectrometers should read >5 scans/second. These measurements are more subject to extra column broadening effects, and dead volumes must be minimized, meaning a small detector flow cell (<3 μL), a low dispersion injector (these are commercially available), and shorter small-diameter connection tubing. Smaller flow cells will have shorter pathlength, and so there is some compromise in sensitivity.

NARROW-BORE COLUMNS—HIGHER SENSITIVITY, LOWER SOLVENT USE

Narrow columns improve sensitivity.

If we make columns narrower, lower detection results from narrower and taller peaks. If a 4-μL sample is injected into a 2.1-mm-diameter column, it will be diluted about fivefold less, and the peak will be five times higher than when injected into a 4.6-mm column of the same length. The flow rate is proportional to the square of the column diameter, and the optimum flow rate for the same resolution is about five times less for the smaller column, resulting in less volumetric dilution of the sample. Of course, the same sensitivity can be achieved with conventional columns by injecting a fivefold larger sample, 20 μL. So only if we are sample

limited do we really gain any sensitivity advantage. But solvent use is cut fivefold. Injected sample volumes for the 2.1-mm columns should be no more than 5 μL to minimize the injection contribution to the peak volumes. And the flow cell volume should be 3 to 5 μL. The main use of these columns is when interfacing to a mass spectrometer (LC–MS—see below) because of the decreased peak volumes and low volumetric flow rates required for introduction into the mass spectrometer.

Example 21.1

Calculate the volume, in microliters, of a sample zone corresponding to 1 mm in (a) a 2.1-mm i.d. column and (b) a 4.6-mm i.d. column, assuming that the mobile phase occupies 65% of the column volume.

Solution

(a) Calculate the volumes in cm^3, and so use centimeter dimensions:

$$V = l\pi r^2 = 0.1 \text{ cm} \times 3.14\ (0.105 \text{ cm})^2 = 0.0035 \text{ cm}^3 = 3.5\ \mu\text{L}$$

Since the mobile phase occupies 65% of the volume, multiply by 0.65 to obtain the actual volume:

$$3.5\ \mu\text{L} \times 0.65 = 2.3\ \mu\text{L}$$

(b) $$0.1 \text{ cm} \times 3.14\ (0.23 \text{ cm})^2 \times 0.65 = 0.0108 \text{ cm}^3 = 10.8\ \mu\text{L}$$

This means that the peak height will be the same with a 2.1-mm i.d. column for a 2.3-μL sample as for a 10.8-μL sample with a 4.6-mm i.d. column, about one-fifth the sample size. It also means a small column is not needed for high sensitivity if you are not sample limited.

Example 21.2

If the optimum volumetric flow rate for a 4.6-mm i.d. column is 1.5 mL/min with 5-μm particles, what would it be for a 2.1-mm i.d. column with the same particles? How much solvent would be consumed for a 10-min separation for each column?

Solution

The flow rate is proportional to the square root of the column i.d.:

$$\text{Flow rate (2.1 mm i.d.)} = 1.5 \text{ mL/min} \times \frac{(2.1 \text{ mm})^2}{(4.6 \text{ mm})^2}$$

$$= 0.31 \text{ mL/min}$$

For the 4.6-mm i.d. column, we would use 15 mL solvent, while for the 2.1-mm i.d., column, we would use 3.1 mL, one-fifth as much.

Figure 21.11 shows the difference in throughput for a narrow-bore (2.1-mm × 20-cm long) column packed with 2.5 μm C_{18} beads, compared with a 4.6-mm × 5-cm, 5-μm d_p column. A threefold increase in achieved. The narrow-bore column is heated to reduce the solvent viscosity for flow through the more densely packed column.

Fused silica capillaries of 0.25 or 0.32 mm i.d. packed with 3- or 5-μm particles are widely used for LC–MS. These columns require small samples, on the order of a microliter or less. The volmetric flow rate is several microliters per minute. Resolution is comparable to that of standard analytical columns.

WHAT ABOUT TEMPERATURE?

The zirconia particles described above are stable at high temperatures. Polystyrene-coated porous zirconia particles have been used at 200°C. Peter Carr at the University of Minnesota, in studies on high-temperature fast LC, demonstrated that column efficiency at high velocity improves at higher temperatures, especially for solutes that are strongly retained. Also, the van Deemter plot flattens out significantly as the temperature is increased. Carr performed separations at 150°C at flow rates of 15 mL/min in a 4.6-mm × 5-cm column with 3-μm particles.

LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

Mass spectrometry detection in liquid chromatography, like with gas chromatography, has become a powerful analysis tool for sensitive and selective mass detection in characterizing complex samples. Review the principles of mass spectrometry and the types of instruments used for chromatography detection in Chapter 20.

It is more difficult to interface a liquid chromatograph to a mass spectrometer because of the necessity to remove the solvent. Also, the analytes are nonvolatile and may be thermally labile but must be presented in gaseous form. Hence, the combination of LC and MS was termed an "unlikely marriage," and it took several years to develop interfaces to the stage of reliable and easy use. Today, there are several types of interfaces that make LC–MS a routine technique. Compact and benchtop systems are commercially available (Figure 21.12).

Electrospray ionization is the most common HPLC–MC interface.

The commonly used interfaces are the electrospray ionization (ESI) source, thermospray ionization (TSI), atmospheric pressure chemical ionization (APCI), and particle beam ionization. The choice depends largely on the polarity and thermal stability of the analyte. Electrospray ionization is preferred for polar and ionic and very large molecules such as proteins and peptides. Atmospheric pressure

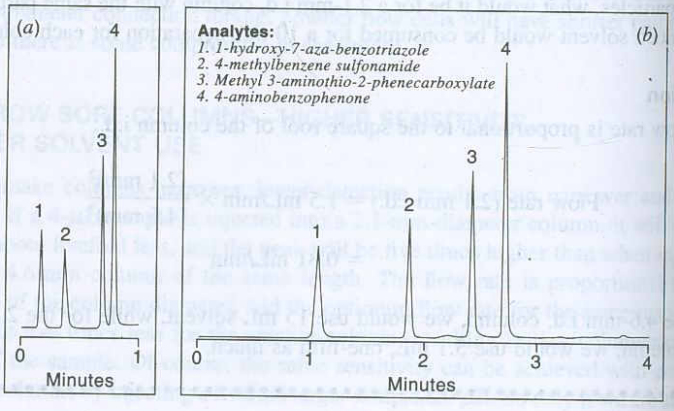


Fig. 21.11. Increased throughput with narrow-bore column. (*a*) Column: 2.5 μm C_{18} particles, 2.1 mm i.d., 2.0 cm long, 60°C. Injection volume 1 μL. (*b*) 5.0 μm C_{18} particles, 4.6 mm i.d., 5.0 cm long, 30°C. Injection volumes 3 μL. (Courtesy of Waters Corporation.)

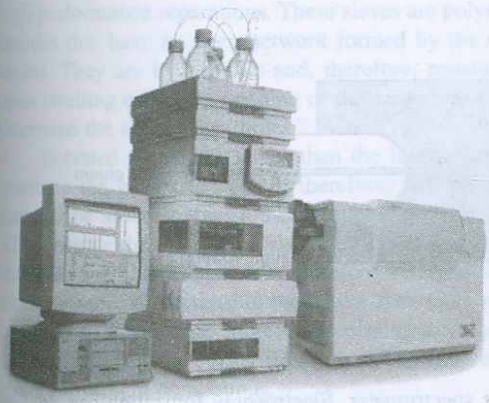


Fig. 21.12. Liquid chromatography–mass spectrometry benchtop system. (Courtesy of Agilent Technology.)

chemical ionization is also suited for large molecules and for nonpolar compounds. Thermospray, the first really successful technique, used for polar as well as nonpolar compounds, has largely been replaced by the atmospheric techniques. Particle beam ionization is useful for relatively volatile, small polar and nonpolar molecules (<1000 daltons).

A schematic of a **particle beam** interface is shown in Figure 21.13. The eluent from the HPLC column is nebulized using helium gas to form an aerosol in a reduced pressure chamber heated at 70°C. A cone with a small orifice is at the end of the chamber, which leads into a lower pressure area. The difference in pressure causes a supersonic expansion of the aerosol. The helium and the solvent molecules are lighter than the analyte molecules and tend to diffuse out of the stream and are pumped away. The remaining stream passes through a second cone into a yet lower pressure area, and then the analyte vapor passes into the ion source. The particle beam interface produces electron ionization (EI) spectra similar to those of GC–MS, so the vast knowledge of EI spectra can be used for analyte identification.

In the **thermospray** interface, the sample from the column passes through a heated tube and rapidly expands as a jet spray into a heated vacuum chamber. The solvent mist is electrostatically charged. The solvent is rapidly pumped away from the droplets, and a static charge is imparted to the particle, which enters through a skimmer into the mass spectrometer. Thermospray produces "soft ionization" with little or no fragmentation, allowing gentle ionization of nonvolatile, thermally labile organic compounds. The spectrum generally displays a protonated molecular ion.

The **electrospray ionization** interface is the most popular. It is also a soft ionization technique. The sample solution is sprayed across a high potential difference of a few thousand volts from a needle into an orifice in the interface (Figure 21.14). Heat and gas flow desolvate the charged droplets, giving charged analyte

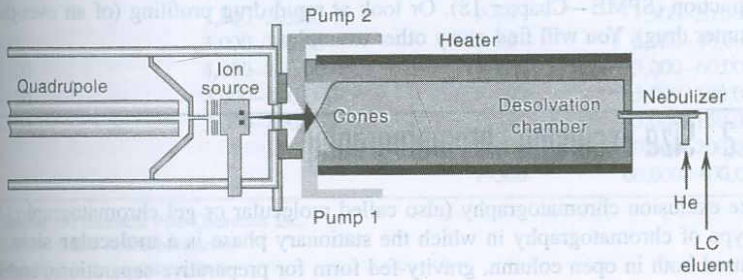


Fig. 21.13. Particle beam interface. [From A. N. Eaton, *Today's Chemist at Work*, October (1994) 34. Copyright 1994 by the American Chemical Society. Reproduced by permission of Micromass Ltd.]

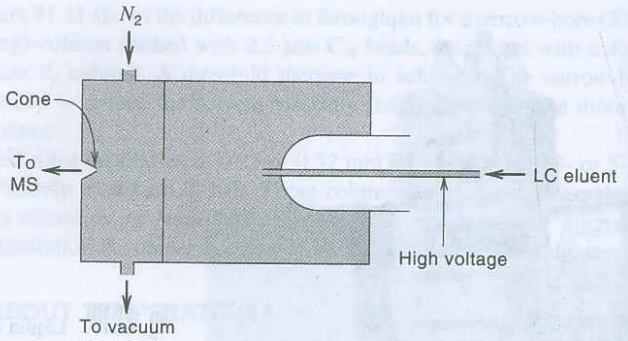


Fig. 21.14. Electrospray ionization interface for LC–MS.

molecules that enter the mass spectrometer. Electrospray ionization can produce multiply charged ions, with the number of charges increasing with molecular weight. For example, multiple charge sites are produced on proteins and peptides. This allows determination of relatively large molecular weights, well beyond the normal 2000- to 3000-dalton m/z ratio limit of the quadrupole mass spectrometer since the m/z ratio decreases by a factor of, for example, 5. Proteins of 50,000 daltons or larger can be measured. Of course, the number of charges needs to be evaluated. Techniques include: comparing two charge states that differ by a single charge and solving simultaneous equations; identifying species with the same charge but different adduct masses; and looking at m/z ratios for resolved isotopic clusters. Electrospray is good for charged, polar, or basic compounds.

Atmospheric pressure chemical ionization uses an atmospheric pressure ionization interface. The interface is similar to that used for ESI, but a corona discharge is used to ionize the analyte in an atmospheric pressure region. (Note: This is different than the chemical ionization source described in Chapter 20 for GC–MS.) The gas-phase ionization of less polar analytes is more efficient than with ESI, but the mass range is limited to about 2000 daltons. ESI and APCI are complementary. APCI has largely replaced thermal ionization as an interface in commercial instruments.

The quadrupole mass filter is the most popular mass analyzer for LC–MS because of its low cost, compactness, and ruggedness. In recent years, time-of-flight analyzers have been developed for interfacing with electrospray ionizers.

CHECK OUT SOME HPLC APPLICATIONS

High-performance liquid chromatography is widely used for many clinical, forensic, environmental, and industrial applications. It has replaced many gas chromatography procedures for forensic drug-screening analysis. You can find examples of specific types of analyses by perusing the chromatogram databases listed in the websites at the end of Chapter 19. For example, go to the Supelco database and look for determination of explosives extracted from water using solid-phase microextraction (SPME—Chapter 18). Or look at rapid drug profiling (of an over-the-counter drug). You will find many other examples.

21.2 Size Exclusion Chromatography

Molecules that can penetrate the gel particles are separated based on size and shape. Others pass straight through the column.

Size exclusion chromatography (also called molecular or gel chromatography) is a type of chromatography in which the stationary phase is a **molecular sieve**. It is used both in open column, gravity-fed form for preparative separations, and in

high-performance separations. These sieves are polymeric carbohydrates and acrylamides that have an open network formed by the crosslinking of the polymeric chains. They are hydrophilic and, therefore, capable of absorbing water, whereupon swelling causes an opening of this structure. The degree of crosslinking will determine the size of the "holes."

Solvated molecules larger than the largest pores of the swollen gel cannot penetrate the gel particles and, therefore, will pass straight through the column through the spaces between the individual particles. Smaller molecules, however, will penetrate the open network in the particles to a varying degree, depending on their size and shape. They are, therefore, retarded to varying degrees and will be eluted in order of decreasing molecular size. Gels with a high degree of swelling are used to fractionate large molecules (generally high-molecular-weight substances), whereas the denser (lower swelling) gels are used for separation of low-molecular-weight compounds.

Names such as **gel filtration chromatography** (mobile phase is water), used by biochemists, and **gel permeation chromatography** (mobile phase is an organic solvent), used by polymer chemists, describe this technique. **Size exclusion chromatography**, however, is the recommended term. Molecular weight distribution of polymers can be obtained by this technique, and proteins, enzymes, peptides, nucleic acids, hormones, polysaccharides, and so on can be separated.

The **exclusion limit** is the molecular weight of that molecule that will just permeate the gel and be retarded. This can range from a molecular weight of 1000 to several million, depending on the gel. It should be emphasized that separations are based on a molecule's size and configuration rather than just its molecular weight, but there is generally a correlation. Generally, *molecules smaller than the exclusion limit can be fractionated down to a certain limiting size* (see Table 21.1).

The gels must be equilibrated for a few hours to a day or more with the solvent to be used, depending on the solvent uptake. Those with loose crosslinking designed for high-molecular-weight substances require the longer periods of soaking.

Sephadex is a popular molecular-sieve material for the separation of proteins. It is a polymeric carbohydrate material that, because of hydroxyl groups along the polymer chain, is fairly polar and so will adsorb water. The amount of crosslinking

Proteins can be separated by molecular exclusion chromatography.

Table 21.1
Sephadex Gels[a] and Bio-Gels[b]

Sephadex Type	Fractionation Range[c] for Peptides and Globular Proteins (MW)	Bio-Gel Type	Fractionation Range (MW)
G-10	Up to 700	P-2	100–1,800
G-15	Up to 1,500	P-4	800–4,000
G-25	1,000–5,000	P-6	1,000–6,000
G-50	1,500–30,000	P-10	1,500–20,000
G-75	3,000–70,000	P-30	2,500–40,000
G-100	4,000–150,000	P-60	3,000–60,000
G-150	5,000–400,000	P-100	5,000–100,000
G-200	5,000–800,000	P-150	15,000–150,000
		P-200	30,000–200,000
		P-300	60,000–400,000

[a] Courtesy of Pharmacia Fine Chemicals Inc.
[b] Courtesy of Bio-Rad Laboratories.
[c] Upper limit is the exclusion limit.

in the preparation can be carefully controlled to give different pore sizes and exclusion limits. Gels are characterized with respect to their swelling ability by their "water regain." This represents the amount of water imbibed by the gels on swelling. The type numbers of the Sephadex gels refer to the water-regaining values of the gels. Sephadex G-10, thus, has a water-regaining value of about 1 mL/g dry gel, and Sephadex G-200 has a value of about 20 mL/g. Several types of Sephadex gels and the fractionation range of molecules are listed in Table 21.1. These gels are insoluble in water and are stable to mild redox agents as well as to bases and weak acids.

Bio-Gel is a more chemically inert series of molecular-sieve gels, consisting of polyacrylamides. These are insoluble in water and common organic solvents and can be used in the pH range of 2 to 11. The inert gel decreases the possibility of adsorption of polar substances; adsorption can be a variable with Sephadex, causing changes in the chromatographic behavior of these substances. Table 21.1 lists the different Bio-Gel preparations and their separation properties.

Styragel is a polystyrene gel that is useful for purely nonaqueous separations in methylene chloride, toluene, trichlorobenzene, tetrahydrofuran, cresol, dimethylsulfoxide, and so on. It cannot be used with water, acetone, or alcohols. Gels of this can be prepared with exclusion limits for molecular weights of from 1600 to 40 million.

Molecular sieves are useful for the desalting of proteins that have been partially fractionated by salting out with a high concentration of some salt. A gel with a low exclusion limit, such as Sephadex 25, will allow the proteins to pass right through the column while the salts are retained. The protein dilution is limited to the elution volume of the column (the volume external of the swelled gel to fill the column).

For high-performance analytical applications, small polystyrene or microporous silica particles of 5- to 10-μm diameter are used, with pore sizes of a few nanometers to several hundred nanometers. The controlled pore silica particles are coated with a hydrophilic phase to reduce solute adsorption. The polymeric particles can be used over a wider pH range (2 to 12) since silica is limited to pH 2 to 7.

21.3 Ion Exchange Chromatography

Cations or anions are separated by ion exchange chromatography.

While most other types of chromatography are used principally for separations of complex organic substances, ion exchange chromatography is particularly well suited for the separation of inorganic ions, both cations and anions, because the separation is based on exchange of ions in the stationary phase. It has also proved to be extremely useful for the separation of amino acids.

The stationary phase in ion exchange chromatography consists of beads made of a polystyrene polymer crosslinked with divinylbenzene. The crosslinked polymer (resin) has free phenyl groups attached to the chain, which can easily be treated to add ionic functional groups. There are basically four types of ion exchange reins used in analytical chemistry, and these are summarized in Table 21.2. Like size exclusion chromatography, ion exchange is used either in packed open tube, gravity-fed form, or in a high-performance mode known as *ion chromatography* (discussed below).

CATION EXCHANGE RESINS

These resins contain acidic functional groups added to the aromatic ring of the resin. The strong-acid cation exchangers have sulfonic acid groups, $—SO_3H$, which

Table 21.2
Types of Ion Exchange Resins

Type of Exchanger	Functional Exchanger Group	Trade Name
Cation		
Strong acid	Sulfonic acid	Dowex[a] 50; Amberlite[b] IR120; Ionac[c] CGC-240; Rexyn[d] 101; Permutit[e] Q
Weak acid	Carboxylic acid	Amberlite IRC 50; Ionac CGC-270; Rexyn 102; Permutit H-70
Anion		
Strong base	Quaternary ammonium ion	Dowex 1; Amberlite IRA 400; Ionac AGA-542; Rexyn 201; Permutit S-1
Weak base	Amine group	Dowex 3; Amberlite IR 45; Ionac AGA-316; Rexyn 203; Permutit W

[a] Dow Chemical Company.
[b] Mallinckrodt Chemical Works.
[c] J. T. Baker Chemical Company.
[d] Fisher Scientific Company.
[e] Matheson Coleman & Bell.

are strong acids much like sulfuric acid. The weak-acid cation exchangers have carboxylic acid groups, $—CO_2H$, which are only partially ionized. The protons on these groups can exchange with other cations:

$$n\text{RzSO}_3^-\text{H}^+ + \text{M}^{n+} \rightleftharpoons (\text{RzSO}_3)_n\text{M} + n\text{H}^+ \qquad (21.1)$$

and

$$n\text{RzCO}_2^-\text{H}^+ + \text{M}^{n+} \rightleftharpoons (\text{RzCO}_2)_n\text{M} + n\text{H}^+ \qquad (21.2)$$

where Rz represents the resin. The equilibrium can be shifted to the left or right by increasing $[H^+]$ or $[M^{n+}]$ or decreasing one with respect to the amount of resin present.

Cation exchange resins are usually supplied in the hydrogen ion form, but they can easily be converted to the sodium ion form by treating with a sodium salt. The sodium ions then undergo exchange with other cations. The **exchange capacity** of a resin is the total number of equivalents of replaceable hydrogen per unit volume or per unit weight of resin, and it is determined by the number and strength of fixed ionic groups on the resin. The ion exchange capacity affects solute retention, and exchangers of high capacity are most often used for separating complex mixtures, where increased retention improves resolution.

Weak-acid cation exchange resins are more restricted in the pH range in which they can be used, from 5 to 14, while the strong-acid resins can be used from pH 1 to 14. At low pH values, the weak-acid exchangers will "hold on" to the protons too strongly for exchange to occur. Also, the weak-acid cation exchangers will not completely remove the cations of very weak bases, while strong-acid resins will. This is analogous to the incompleteness of a weak acid–weak base reaction. Weak-acid resins are generally used for separating strongly basic or multifunctional ionic substances such as proteins or peptides that are often firmly retained on strong-acid exchangers, while strong-acid resins are more generally preferred, especially for complex mixtures.

Strong-acid resins are used for most separations. Weak-acid resins are preferred for proteins and peptides that are retained too strongly by the strong acids.

ANION EXCHANGE RESINS

Basic groups on the resin in which the hydroxyl anion can be exchanged with other anions make up the anion exchange resins. There are strong-base groups (quaternary ammonium groups) and weak-base groups (amine groups). The exchange reactions can be represented by

$$n\mathrm{RzNR_3^+OH^-} + \mathrm{A}^{n-} \rightleftharpoons (\mathrm{RzNR_3})_n\mathrm{A} + n\mathrm{OH^-} \tag{21.3}$$

and

$$n\mathrm{RzNH_3^+OH^-} + \mathrm{A}^{n-} \rightleftharpoons (\mathrm{RzNH_3})_n\mathrm{A} + n\mathrm{OH^-} \tag{21.4}$$

where R represents organic groups, usually methyl.

Strong-base resins are generally applicable. Weak-base resins are used for separating strong acids.

The strong-base exchangers can be used over the pH range 0 to 12, but the weak-base exchangers only over the range of 0 to 9. The latter exchangers will not remove very weak acids, but they are preferred for strong acids that may be retained by strong-base resins, such as sulfonates.

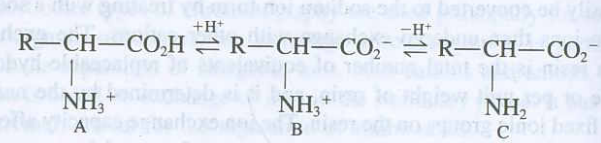
CROSSLINKAGE

The greater the crosslinkage of the resin, the greater the difference in selectivities. Increasing the crosslinkage is expressed by manufacturers as percent of divinylbenzene. Generally, crosslinkage also increases the rigidity of the resin, reduces swelling, reduces porosity, and reduces the solubility of the resin. In general, medium-porosity materials are used for low-molecular-weight ionic species and high-porosity materials are used for high-molecular-weight ionic species. The degrees of crosslinkage is expressed by the manufacturers as percent of divinylbenzene. Generally, crosslinkage of 8 to 10% is used.

EFFECT OF pH—SEPARATION OF AMINO ACIDS

Amino acids may be positively or negatively charged or neutral (isoelectric point). These three forms may be separated by a combination of cation and anion exchange resins.

The ionic forms of many substances will be affected by the pH of the effluent solution. Hydrolysis of metal ions and of salt of weak acids and bases is controlled by adjusting the pH. Weak acids will not dissociate in high acid concentrations and will not exchange, and the same is true for weak bases in high alkaline concentrations. Control of pH is especially important in the separation of amino acids, which are **amphoteric** (can act as acids or bases). There are three possible forms:



Form B, called a **zwitterion**, is the dominant form at the pH corresponding to the **isoelectric point** of the amino acid. The isoelectric point is the pH at which the net charge on the molecule is zero. In more acid solutions than this, the $—CO_2^-$ group is protonated to form a cation (form A), while in more alkaline solutions, the $—NH_3^+$ group loses a proton to form an anion (form C). The isoelectric point will vary from one amino acid to another, depending on the relative acidity and basicity of the carboxylic acid and amino groups. Thus, group separations based on the isoelectric points are possible by pH control.

At a given pH, the amino acids can be separated into three groups by being passed successively through an anion and a cation exchange column. The uncharged

zwitterions (isoelectric point) will pass through both columns, while the positively and negatively charged amino acids will each be retained by one of the columns. The groups can be further subdivided by changing the pH.

Moore and Stein [*J. Biol. Chem.*, **192** (1951) 663] successfully separated up to 50 amino acids and related compounds on a single Dowex-50 cation exchange column by a combination of pH and temperature control. (The temperature affects the equilibria involved.)

Automatic amino acid analyzers based on ion exchange separation are commercially available. The elution of each amino acid is automatically recorded by measuring the color formed between the amino acid and ninhydrin as it is eluted. By operating at pressures of several hundred psi, these perform separations in about 200 min. They have proved valuable to the biochemist as an aid in the elucidation of protein structure. The protein fragments are degraded to amino acids, which must be determined.

EFFECT OF COMPLEXING AGENTS—SEPARATION OF METAL IONS ON ANION EXCHANGE COLUMNS

Negatively charged chloro complexes of metal ions in HCl solutions are retained by anion exchange resins. They are dissociated and eluted by dilution of the acid.

Many metals can be separated on anion exchange columns by being converted to anions by complexation. The complexing agent is an anion such as chloride, bromide, or fluoride. Uncharged complexing agents also affect the equilibrium if they form a complex that must dissociate before the metal is exchanged or if they change its size. Many complexing agents are either weak acids or bases or are salts of these, and so a complicated interdependence of pH and complexation often results.

Some of the most successful separations of metals have been on anion exchange columns. A complexing acid is added in high concentration to form anionic complexes of the metals. Concentrated hydrochloric acid forms anionic chlorocomplexes with all the common metals, with the exception of the alkali and alkaline earth metals and Al(III), Ni(II), and Cr(III), and so all of these can be adsorbed on a quaternary ammonium anion exchange column.

21.4 Ion Chromatography

Ion chromatography is the high-performance form of ion exchange chromatography.

The application of HPLC techniques to ion exchange chromatography has become known as **ion chromatography**. This technique combines the separating power of ion exchange with the universality of the conductivity detector. In ordinary ion exchange chromatography, a conductivity detector is limited in use because of the high background conductance (millimhos) of the eluting agent. In 1970, William Bauman at Dow Chemical Company suggested a way to remove the background eluent using a second ion exchange column, and thus permit detection of analyte ions with a highly sensitive conductivity detector (micromhos). This second column is called the **suppressor column**. For anion analysis, this is a cation exchange column in the acid form; and for cation analysis, it is an anion exchange column in the base form. For ion chromatography, weak exchange resins are usually used, although strong ones are also used. The principles are illustrated in Figure 21.15.

The suppressor column removes the eluent ions and exchanges the analyte ion for H^+ (cations) or OH^- (anions), so that a high-sensitivity conductivity detector can be used.

Suppose the salt MA of an anion A^- is separated on an anion exchange resin and eluted with NaOH. Eluting from the column will be a mixture of MA and NaOH. Upon passing through the cation exchange suppressor column, the following reactions take place:

$$RzSO_3^-H^+ + M^+ + A^- \rightarrow RzSO_3^-M^+ + H^+ + A^-$$

$$RzSO_3^-H^+ + Na^+ + OH^- \rightarrow RzSO_3^-Na^+ + H_2O$$

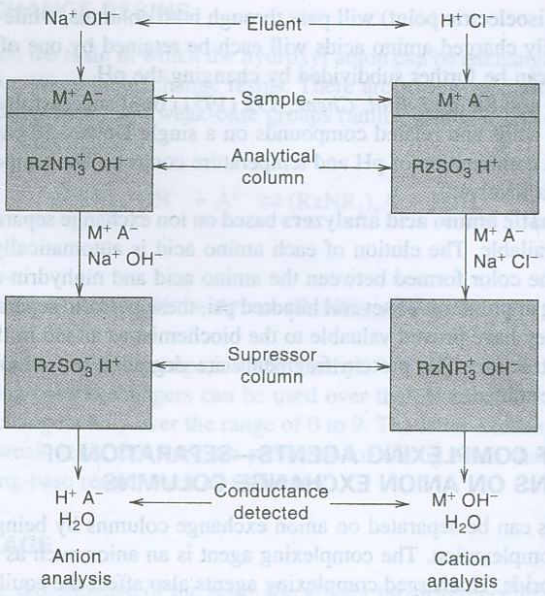


Fig. 21.15. Principles of ion chromatography.

So the NaOH is converted to H_2O, and the analyte ion is converted to the corresponding acid HA. The acid HA is detected by the conductivity detector. For cation analytes, a mixture of MA and HCl emerges from the first (analytical) column. These react in the suppressor anion exchange column as follows:

$$RzNR_3^+OH^- + M^+ + A^- \rightarrow RzNR_3^+A^- + M^+ + OH^-$$
$$RzNR_3^+OH^- + H^+ + Cl^- \rightarrow RzNR_3^+Cl^- + H_2O$$

The suppressor column must be regenerated periodically.

The HCl is converted to H_2O and the analyte cation is converted to the corresponding base MOH, which is detected by the conductivity detector.

The suppressor column obviously will eventually become depleted and will have to be regenerated (with HCl for the cation exchanger and with NaOH for the anion exchanger). The suppressor column is usually a small-volume bed of a high-capacity resin to minimize band spreading in the column. Since microgram or smaller quantities of analytes are usually separated, a low-capacity analytical column is used coupled with relatively low eluent concentration (1 to 10 m*M*).

Ion chromatography is particularly useful for the determination of anions. A typical eluting agent consists of a mixture of $NaHCO_3$ and Na_2CO_3, and these are converted to low-conductivity carbonic acid. Anions such as F^-, Cl^-, Br^-, I^-, NO_2^-, NO_3^-, SO_4^{2-}, PO_4^{3-}, SCN^-, IO_3^-, and ClO_4^-, as well as organic acids or their salts, can be readily determined in a matter of minutes, down to parts per million or lower levels.

If solutions are too dilute for direct analysis, analytes can be concentrated first on an ion exchange concentrator column. Low parts-per-billion concentrations can be measured in this way.

Electronic suppression of background conductance avoids the use of a suppressor column.

The advent of high-performance conductivity detectors with a wide dynamic range and electronic suppression of background conductance has allowed the development of ion chromatography without a suppressor column. A low-capacity analytical column is combined with low-concentration eluent, typically phthalate buffers, for anion measurements. The advantages of avoiding a suppressor column

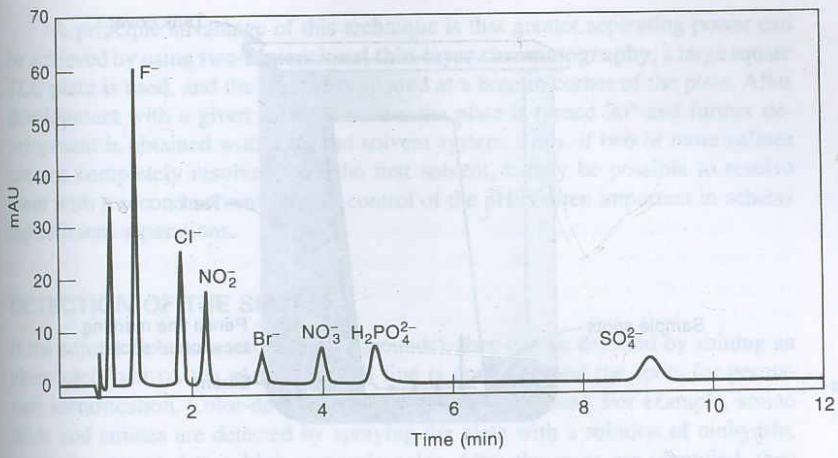


Fig. 21.16. Ion chromatography separation of anions. (Courtesy of Hewlett-Packard Company.)

are that band broadening from the column is eliminated, and anions of weak acids, such as cyanide and borate, are more readily detected because they are only weakly ionized in neutral or weakly acidic solution.

Ion exchange particles for ion chromatography are either resin particles with functionalized surfaces or nonporous silica particles coated with an ion exchange film or small ion exchange particles (Figure 21.4). Resins include functionalized polystyrene–divinylbenzene copolymers and polymethacrylate.

Figure 21.16 shows a typical anion analysis by ion chromatography. Such analyses would be difficult to perform by other methods. Ion chromatography forms the basis of automatic amino acid analysis.

Samples are often pretreated by passing through a small ion exchange precolumn. This concentrates the ions of interest and allows the sample matrix material to pass through unretained. Then the ions are eluted onto the analytical column. The same eluent used for the separation can be used for stripping the concentrator column, provided the capacity of the concentrator column is less than 40% of that of the analytical column. By concentrating ions from a 20-mL sample, low parts-per-billion can be measured. See C. A. Lucy, *LC·GC*, **14**(5) (1996) 406, for a good discussion of the practical aspects of ion chromatographic separation.

21.5 Thin-Layer Chromatography

Thin-layer chromatography (TLC) is a planar form of chromatography useful for wide-scale qualitative analysis screening and can also be used for quantitative analysis. The stationary phase is a thin layer of finely divided adsorbent supported on a glass or aluminum plate, or plastic strip. Any of the solids used in column liquid chromatography can be used, provided a suitable binder can be found for good adherence to the plate.

A sample is spotted onto the plate with a micropipet, and the chromatogram is "developed" by placing the bottom of the plate or strip (but not the sample spot) in a suitable solvent (see Figure 21.17). The solvent is drawn up the plate by capillary action, and the sample components move up the plate at different rates,

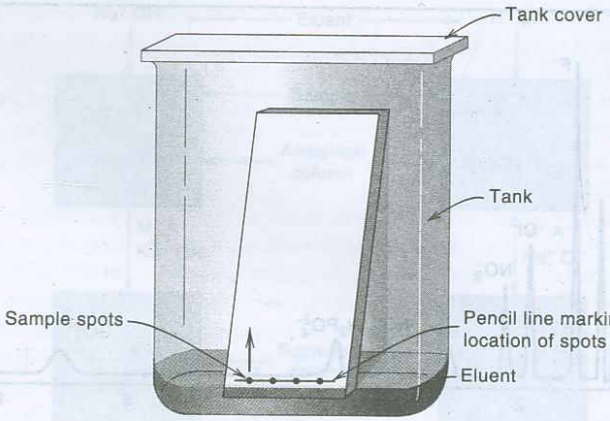


Fig. 21.17. Thin-layer chromatography setup.

depending on their solubility and their degree of retention by the stationary phase. Following development, the individual solute spots are noted or are made visible by treatment with a reagent that forms a colored derivative. The spots will generally move at a certain fraction of the rate at which the solvent moves, and they are characterized by the **R_f value**:

$$R_f = \frac{\text{distance solute moves}}{\text{distance solvent front moves}} \tag{21.5}$$

where the distances are measured from the center of where the sample was spotted at the bottom of the plate. The solvent front will be a line across the plate. The distance the solute moves is measured at the center of the solute spot or at its maximum density, if tailing occurs. The R_f value, then, is characteristic for a given stationary phase and solvent combination. Because of slight variation in plates, it is always a good idea to determine the R_f value on each set of plates.

DEVELOPING THE CHROMATOGRAM

A typical setup for thin-layer chromatography is shown in Figure 21.17. A thin pencil line is drawn across the plate a few centimeters from the bottom, and the sample is spotted on this for future reference in R_f measurements. The spot must be made as small as possible for maximum separation and minimum tailing. It is best done dropwise with a warm air blower (e.g., a hair dryer) to evaporate the solvent after each drop. The plate is placed in a chamber with its end dipping in the developing solvent. A closed (presaturated) chamber must be used to saturate the atmosphere with the solvent and prevent it from evaporating from the surface of the plate as it moves up. The developing may take 10 min to 1 h, but it requires no operator time. The amount of development will depend on the complexity of the mixture of solutes being separated. If a wide plate is used, several samples and standards can be spotted along the bottom and developed simultaneously.

Development times of 5 min can be accomplished by using small microscope slides for TLC plates, and preliminary separations with these can be conveniently used to determine the optimum developing conditions. Typically, sample sizes range from 10 to 100 μg per spot (e.g., 1 to 10 μL of a 1% solution). Sample spots should be 2 to 5 mm in diameter.

A principle advantage of this technique is that greater separating power can be achieved by using **two-dimensional thin-layer chromatography**; a large square TLC plate is used, and the sample is spotted at a bottom corner of the plate. After development with a given solvent system, the plate is turned 90° and further development is obtained with a second solvent system. Thus, if two or more solutes are not completely resolved with the first solvent, it may be possible to resolve them with a second solvent. Proper control of the pH is often important in achieving efficient separations.

DETECTION OF THE SPOTS

If the solutes fluoresce (aromatic compounds), they can be detected by shining an ultraviolet light on the plate. A pencil line is drawn around the spots for permanent identification. Color-developing reagents are often used. For example, amino acids and amines are detected by spraying the plate with a solution of ninhydrin, which is converted to a blue or purple color. After the spots are identified, they may be scraped off and the solutes washed off (eluted) and determined quantitatively by a micromethod.

Frequently, colorless or nonfluorescent spots can be visualized by exposing the developed plate to iodine vapor. The iodine vapor interacts with the sample components, either chemically or by solubility, to produce a color. Thin-layer plates and sheets are commercially available that incorporate a fluorescent dye in the powdered adsorbent. When held under ultraviolet light, dark spots appear where sample spots occur due to quenching of the plate fluorescence.

A common technique for organic compounds is spraying the plate with a sulfuric acid solution and then heating it to char the compounds and develop black spots. This precludes quantitative analysis by scraping the spots off the plate and eluting for measurement.

STATIONARY PHASES FOR TLC

The same stationary phases that are used in column chromatography can be used in TLC.

The stationary phase consists of a finely divided powder (particle size 10 to 50 μm). It can be an adsorbent, an ion exchanger, or a molecular sieve, or it can serve as the support for a liquid film. An aqueous slurry of the powder is prepared, usually with a binder such as plaster of paris, gypsum, or poly(vinyl alcohol) to help it adhere to the backing material. The slurry is spread on the plate in a thin film, typically 0.1 to 0.3 mm, using a spreading adapter to assure uniform thickness. Adapters are commercially available. The solvent is evaporated off and adsorbents are activated by placing in an oven at 110°C for several hours. Commercially prepared plates and strips on plastic are available.

Adsorbents are used most frequently.

The most commonly used stationary phases are **adsorbents**. Silica gel, alumina, and powdered cellulose are the most popular. Silica gel particles contain hydroxyl groups on their surface which will hydrogen bond with polar molecules. Adsorbed water prevents other polar molecules from reaching the surface, so the gel is activated by heating to remove the adsorbed water. Alumina also contains hydroxyl groups or oxygen atoms. Alumina is preferred for the separation of weakly polar compounds, but silica gel is preferred for polar compounds such as amino acids and sugars. Magnesium silicate, calcium silicate, and activated charcoal may also be used as adsorbents. Adsorbents are sometimes not activated by heating, in which case the residual water acts as the stationary phase.

Thin-film **liquid stationary phases** can be prepared for separation by liquid–liquid partition chromatography. The film, commonly water, is supported on materials such as silica gel or diatomaceous earth, as in column chromatography. Either

silica gel or diatomaceous earth may be silanized to convert the surfaces to nonpolar methyl groups for reversed-phase thin-layer chromatography.

Ion exchange resins are available in particle sizes of 40 to 80 μm, suitable for preparing thin-layer plates. Examples are Dowex 50W strong-acid cation exchange and Dowex 1 strong-base anion exchange resins, usually in the sodium or hydrogen or the chloride forms, respectively. An aqueous slurry of six parts resin to one part cellulose powder is suitable for spreading into a 0.2- to 0.3-mm layer.

Size exclusion thin layers can be prepared from Sephadex Superfine. The gel is soaked in water for about 3 days to complete the swelling, and then spread on the plate. The plates are not dried, but stored wet. The capillary action through these molecular sieves is much slower than with most other thin layers, typically only 1 to 2 cm/h, and so development takes 8 to 10 h, compared to about 30 min for other stationary phases.

MOBILE PHASES FOR TLC

Use the same guidelines as for column chromatography.

In adsorption chromatography, the eluting power of solvents increases in the order of their polarities (e.g., from hexane to acetone to alcohol to water). A single solvent, or at most two or three solvents, should be used whenever possible, because mixed solvents tend to chromatograph as they move up the thin layer, causing a continual change in the solvent composition with distance on the plate. This may result in varying R_f values depending on how far the spots are allowed to move up the plate.

The developing solvent must be of high purity. The presence of small amounts of water or other impurities can produce irreproducible chromatograms.

QUANTITATIVE MEASUREMENTS

The powerful resolving power of two-dimensional thin-layer chromatography has been combined with quantitative measurements by optically measuring the density of chromatographic spots. This can be done by measuring the transmittance of light through the chromatographic plate or the reflectance of light, which is attenuated by the analyte color. Or, fluorescence intensity may be measured upon illumination with ultraviolet radiation. Full spectrum recording and multiple wavelength scanning (with diode arrays) capabilities are commercially available.

HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY (HPTLC)

High-performance TLC uses finer particles for fast and efficient separations using smaller samples.

The power of thin-layer chromatography has been enhanced by consideration of chromatography principles to improve the speed and efficiency of separation and by the development of instrumentation to automate sample application, development of the chromatogram, and detection, including accurate and precise *in situ* quantitation as mentioned above. The use of a very fine particle layer results in faster and more efficient separations. The particle size has a narrower distribution range, with an average size of 5 μm, instead of the average 20 μm for conventional TLC. Mechanical applicators permit reproducible application and reduction in the diameter of the starting spots. Smaller volume samples are used compared with conventional TLC, about one-tenth, and separation times are reduced by a factor of 10. In addition to precoated silica gel layers, a range of chemically bonded phases, similar to those used in normal- and reversed-phase high-performance liquid chromatography, are available.

The very fine particles used in HPTLC slows the movement of the mobile phase after a relatively short distance. To overcome this limitation, a "forced-flow"

technique has been employed, using a pressurized chamber. The mobile phase is delivered with the aid of a pump at a constant velocity through a slit in a plastic sheet covering the stationary phase. For details, see the studies by Kalász and coworkers [*J. Chromatogr. Sci.*, **18** (1980) 324; *Chromatographia*, **18** (1984) 628].

Modern thin-layer chromatography can be complementary to HPLC. It allows the processing of many samples in parallel, providing low-cost analysis of simple mixtures for which the sample workload is high. The TLC plates acts as "storage detectors" of the analyte if they are saved.

21.6 Electrophoresis

Large molecules, such as proteins, migrate in an electric field based on their charge-to-mass ratios, but also interact chromatographically with the support.

Electrophoretic methods are used to separate substances based on their charge-to-mass ratios, using the effect of an electric field on the charges of these substances. These techniques are widely used for charged colloidal particles or macromolecular ions such as those of proteins, nucleic acids, and polysaccharides. There are several types of electrophoresis, **zone electrophoresis** being one of the most common.

In zone electrophoresis, proteins are supported on a solid so that, in addition to the electric migration forces, conventional chromatographic forces may enter into the separation efficiency. There are several types of zone electrophoresis according to the different supports. The common supports include starch gels, polyacrylamide gels, polyurethane foam, and paper. Starch gel electrophoresis has been a popular technique, although it is now somewhat superseded by the use of polyacrylamide gels, which minimize convection and diffusion effects. A block or "plate" of starch gel is prepared, and the sample is applied in a narrow band (line) across the block about midway between the ends, which are contacted with electrodes through a connecting bridge. When current is passed through the cell, the different components of a mixture move with velocities that depend on their electric charges, their sizes, and their shapes. As electrophoresis proceeds, the negatively charged components migrate toward the anode and the positively charged components migrate toward the cathode. The result is a series of separated bands or lines of sample constituents, such as visualized by a stain.

Very complex mixtures can be resolved with zone electrophoresis. For example, the starch gel-electrophoretic separation of plasma proteins reveals 18 components. A densitometer can be used to measure the intensity of the colored zones and thereby obtain quantitative information. Capillary gel electrophoresis is a powerful variant of this (discussed below).

Mobility is affected by the applied voltage and the pH (which influences the charge on the analyte).

The migration rate of each substance depends on the applied voltage and on the pH of the buffer employed. The applied voltage is expressed in volts per centimeter. It is up to 500 V in low-voltage electrophoresis and can be several thousand volts in high-voltage electrophoresis. The latter is used for high-speed separation of low-molecular-weight substances. Macromolecules have lower ionic mobilities and are less amenable to high-voltage separations.

Proteins do not migrate at the pH of their isoelectric point.

Zone electrophoresis is used largely in clinical chemistry and biochemistry for separating amino acids and proteins. These contain amino and carboxylic acid groups that can ionize or protonate, depending on the pH. At a certain pH, the net charge of an amino acid is zero and it exists as a **zwitterion** (see Chapter 8) that exhibits no electrophoretic mobility. This pH is the **isoelectric point** of the amino acid.

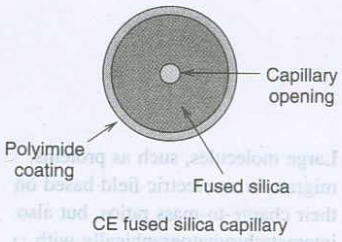
In Chapter 25, we describe the use of gel electrophoresis for separation of nucleic acids in DNA sequencing.

21.7 Capillary Electrophoresis

A relatively new separation technique that is capable of separating minute quantities of substances in relatively short time with high resolution is **capillary electrophoresis** (CE). It offers the ability to analyze a nanoliter (10^{-9} L) of sample, with over 1 million theoretical plates and a detection sensitivity of injected components at the attomole (10^{-18} mol) level or less!

The basic setup for the technique is illustrated in Figure 21.18. The instrumentation requirements (except for perhaps the detector) are actually very simple, and the system is easy to use. The separation medium is a fused silica capillary tube (e.g., 25 to 75 μm i.d., 25 to 100 cm long) containing an appropriate electrolyte. A small volume of sample is introduced into one end of the capillary (see below) and then each end of the capillary is inserted in an electrolyte buffer solution (usually the same as in the capillary tube). Platinum electrodes immersed in each solution are connected to a direct current (dc) high-voltage source, capable of delivering currents up to ca. 250 μA at voltages ranging from 1000 to 30,000 V.

A detector, for example, a UV absorbance detector, through which the solution flows, is placed near or at one end of the capillary. A focused beam is passed through the capillary and may be collected by an optical fiber coupled to a photomultiplier tube. The short pathlengths (10 to 100 μm) involved make sensitive detection a challenge. But the small peak volumes, often less than 1 nL, lead to very low detection limits, even with moderately sensitive detectors (i.e., the solute is concentrated in a very small volume). The use of laser sources, especially for fluorescence detection, has pushed detection limits to zeptomoles (10^{-21} mol)! A capillary electrophoresis instrument is shown in Figure 21.19.



HOW DOES CE WORK? THE POWER OF ELECTROOSMOTIC FLOW

Electroosmosis is the bulk flow of solvent (solution) through an electric field. All analytes flow in the same direction, with the positive ones migrating the most rapidly and the negative ones least rapidly.

The capillary is made of fused silica. The surface of the internal walls contains ionizable silanol groups (SiOH). The capillary is filled with a buffer. Above about pH 2, the silanol groups ionize to produce a negative charge on the capillary surface, call the **zeta potential**. This attracts cations from the buffer solution to create an electrical double layer along the walls (Figure 21.20). When a high dc voltage is applied, the mobile-phase positive charges in the diffuse outer double layer migrate in the direction of the cathode. Because the ions are solvated, the buffer fluid is dragged along by the migrating charge, creating a solution flow of the bulk solvent up to several hundred nanoliters per minute (depending on the pH, buffer concentration, and other factors that affect the zeta potential). This is called **electroosmotic**

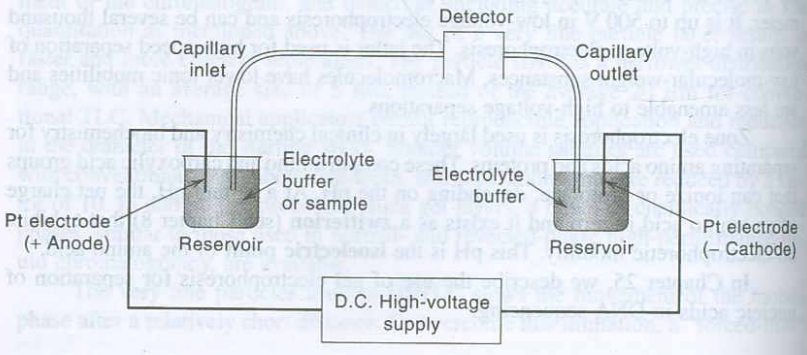


Fig. 21.18. Capillary electrophoresis system.

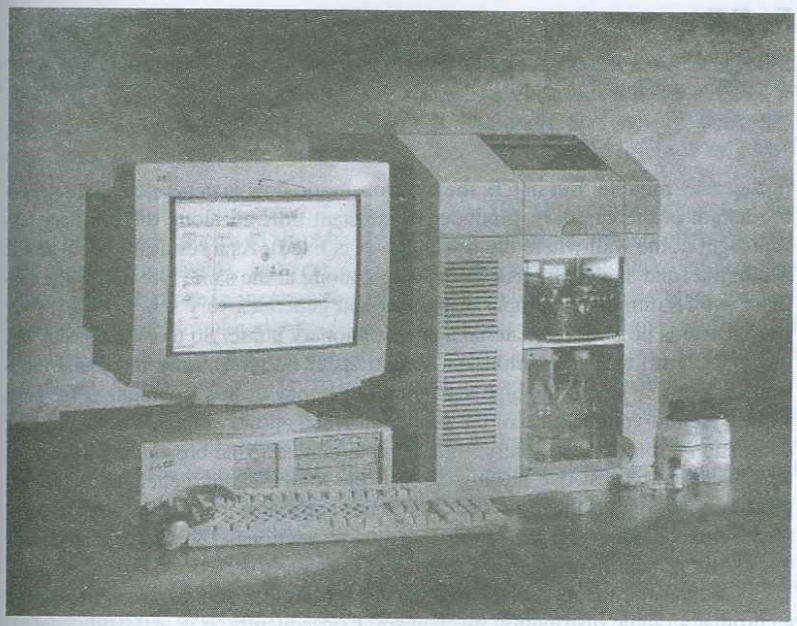


Fig. 21.19. Capillary electrophoresis instrument. (Courtesy of Agilent Technology.)

flow (EOF). Meanwhile, the analyte molecules are subjected to **electrophoretic mobility**, that is, the cations are attracted toward the anode and the anions toward the cathode. But the solution flow toward the cathode results in unidirectional flow of all analytes, regardless of the charge. The smaller, more positively charged ions migrate most rapidly and will be detected first; and the larger, more negatively charged ones will migrate the most slowly. Neutral molecules migrate at the electroosmosis flow rate, since they are not accelerated or retarded by the electric field, and are unresolved.

WHY DOES CE HAVE SUCH HIGH RESOLVING POWER? AGAIN, THE MAGIC OF ELECTROOSMOTIC FLOW

We can get an understanding of the difference between pressure-driven chromatography systems and electroosmotically driven CE systems by comparing the flow profiles (Figure 21.21). In pressure-driven flow, the flow profile is parabolic, with the flow at the center moving at twice the average velocity (**laminar flow**). This results in band broadening, which is a reason GC or LC peaks become broader the

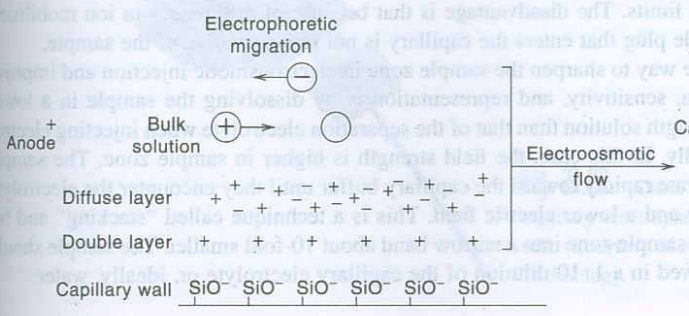


Fig. 21.20. Charge distribution and electroosmotic flow in fused silica capillary.

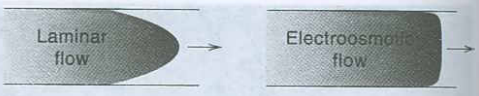


Fig. 21.21. Pressure-driven versus electroosmotically driven flow profiles.

further they migrate. But in CE, the electroosmotic flow is generated along the entire length of the capillary, producing a constant flow all along the capillary (except right at the wall where the double layer is fixed). As a result, the flow profile is pluglike, and analyte molecules are swept along at the same rate across the capillary, which minimizes sample dispersion and generates very sharp peaks.

In CE, there are no eddy diffusion or mass transfer effects, only molecular diffusion broadening. The separation efficiency of CE is 10–100 times that of HPLC.

There is no packing material and no stationary phase. So there is no eddy diffusion (*A* term) and no equilibrium mass transfer (*C* term), only molecular diffusion (*B* term). A key to the high separation efficiency of this technique is the large surface area-to-volume ratio of the capillary, which allows efficient cooling by heat dissipation through the capillary walls. This minimizes band broadening by thermal effects caused by resistive heating. In fact, the Joule heating generated by the application of a high voltage is what limits the applicable voltage in other electrophoresis techniques, and hence the speed of separations. The CE capillary has a thick wall to help dissipate the heat.

CE is not really a chromatographic method.

You have probably noticed that the CE mechanism actually does not include a chromatographic distribution mechanism. Consequently, it is as readily applicable to macromolecules as to smaller ones. Hence, it is valuable for the separation of large biomolecules. Chemical modification of the silica wall or addition of detergents to the background electrolyte is often required to eliminate wall adsorption of proteins.

SAMPLE INTRODUCTION IN CE

The sample, typically a few nanoliters, can be introduced into the capillary by **hydrostatic injection** (gravity, pressure, or vacuum) or by **electromigration**. The sample volume should generally be less than 2% of the total capillary length. For gravity introduction, the capillary sample end is dipped into the sample (which may be as small as 5 μL) and raised for a short predetermined time to allow sample to flow into the capillary. Or, it is inserted into a pressurized vial to force sample into the capillary. Or it is drawn in by suction from the other end of the capillary. After injection, the sample vial is replaced with a buffer reservoir. Alternatively, the sample end is immersed in the sample solution and a relatively low voltage is applied for a few seconds, for example, 2000 V for 10 s. This injects the small volume of sample by electroosmosis.

Reproducibility with hydrostatic injection is on the order of 1 to 2%. The advantage of electroosmosis injection is that more sample can be introduced, improving detection limits. The disadvantage is that because of differences in ion mobilities, the sample plug that enters the capillary is not representative of the sample.

One way to sharpen the sample zone in electroosmotic injection and improve resolution, sensitivity, and representation is by dissolving the sample in a lower ionic strength solution than that of the separation electrolyte when injecting electroosmotically. In this case, the field strength is higher in sample zone. The sample ions migrate rapidly toward the capillary buffer until they encounter the electrolyte boundary and a lower electric field. This is a technique called "stacking" and focuses the sample zone into a narrow band about 10-fold smaller. The sample should be dissolved in a 1:10 dilution of the capillary electrolyte or, ideally, water.