CAPILLARY SEPARATIONS

1. Introduction

Capillary scale separations, most typically implemented in chromatography and electrophoresis, generally offer higher performance indices than their larger scale counterparts. Capillary chromatography and electrophoresis are typically used for the analyses of complex samples, containing a large number of analytes. In chromatography, analytes are separated based on their differential

partitioning between an immobile stationary phase and a mobile fluid phase. In electrophoresis, different charged analytes are separated based on the different velocities with which they move in an electric field. Micellar electrokinetic chromatography (MEKC) and capillary electrochromatography (CEC) are hybrid techniques where separation by differential partitioning and movement under the influence of an electric field are both involved.

Though chromatography and electrophoresis differ in how separation is achieved, many common principles apply to both. In both techniques, the sample is introduced at the head of the column as a narrow zone and the analytes separate as they move to the exit end. The effluent analyte bands are detected as peaks using suitable detectors. The parameters of the separation process, such as elution/migration time, efficiency (how narrow peak is while eluting at a given time, often given as the number of theoretical plates), resolution between adjacent peaks, etc. are all defined similarly. Some aspects are different between them, however; both similarities and differences are further discussed later in this article.

2. Basic Chromatography Principles

As the analyte is partitioned between the mobile and the stationary phase, an equilibrium or distribution constant K can be defined

$$K = \frac{C_S}{C_M} \tag{1}$$

where C_S and C_M are the concentration of the analyte in the stationary and the mobile phase, respectively. The longer time the analyte spends in the stationary phase, the longer is its retention time, t_r, the total time the analyte spends in the separation process. The mobile phase moves through the column with a constant velocity; all unretained analytes move at the same velocity, eluting at the so-called dead time, t_0. All other analytes, which are retained to any extent by the stationary phase, elute later, the time increasing with increasing K. Figure 1 shows a model chromatogram with two analyte peaks with corresponding retention times t_{r1} and t_{r2} and an unretained molecule (eluting at t_0). Since t_r is a function of the mobile phase flow rate, the retention volume, V_r, the volume of the mobile phase necessary to elute the analyte, which is independent of the flow rate, is sometimes used instead of t_r:

$$V_r = t_r Q \tag{2}$$

where Q is the volumetric flow rate of the mobile phase. Similarly, the dead volume or holdup volume for an unretained analyte, V_0, can be defined from equation (2) by substituting t_0 for t_r.

The retention factor, k', used to describe the movement of the analytes through the column, is also commonly used to describe retention and it is readily

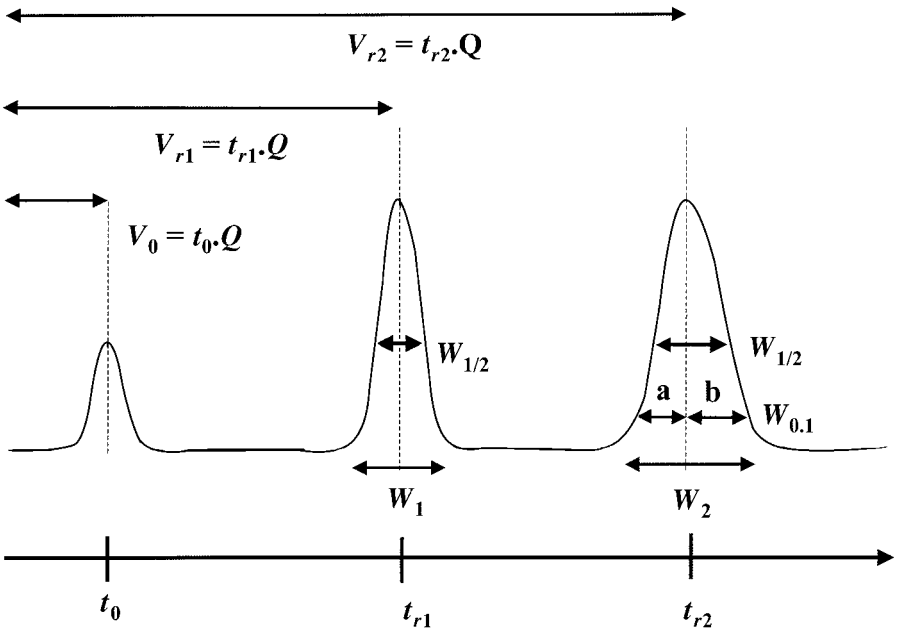


Fig. 1. A model chromatogram. The parameters shown are described in the text.

computed as follows:

$$k' = \frac{t_r - t_0}{t_0} = \frac{V_r - V_0}{V_0} \tag{3}$$

Parameters to compute k' can be easily obtained from a chromatogram such as Figure 1. For practical reasons, best chromatograms result with retention factors in the range of 2–20. If k' values are too low, analytes are not well separated; they all elute near the dead time; if k' values are too high, analysis time increases and the ability to detect an analyte also suffers because a broad wide peak of the same total area as an otherwise sharp narrow peak results.

The difference in retention factors or retention times is not the only factor that governs how well two or more analytes separate. Band broadening occurs during the separation process. This phenomenon was originally explained by Martin and Synge (1) by the "plate theory". Subsequently, the "rate theory" of chromatography advanced by van Deemter and co-workers (2) became more commonly used. This theory describes analyte band broadening in terms of the peak variance, σ^2.

In chromatography, several processes contribute to the final zone width, such as resistance to mass transfer, eddy diffusion (in packed columns), longitudinal diffusion, and extra-column effects. The overall peak variance is the sum of the individual variances:

$$\sigma^2_{\text{tot}} = \sigma^2_{\text{res}} + \sigma^2_{\text{eddy}} + \sigma^2_{\text{long}} + \sigma^2_{\text{extra}} \tag{4}$$

For ideal (Gaussian) analyte peaks, the width of the peak is directly related to the variance, σ^2. The variance per unit column length is the plate height, H:

$$H = \frac{\sigma^2}{L} \tag{5}$$

where L is the column length. Capillary separation systems excel in their separation abilities relative to their larger counterparts in that small plate heights, typically expressed in micrometers (μm), are observed. The total number of theoretical plates in a column, N, which ultimately governs the quality of the separation, is hence given by

$$N = \frac{L}{H} \tag{6}$$

For idealized Gaussian peaks, experimentally N can be readily calculated from the width at half height of the peak, $w_{\frac{1}{2}}$:

$$N = 5.54\left(\frac{t_r}{w_{1/2}}\right)^2 \tag{7}$$

For capillary gas chromatography (GC), the most mature and perhaps the most widely practiced of all separation techniques, the number of theoretical plates achieved in a typical separation system can be in millions.

True Gaussian peaks, are, however, rarely observed in practice. Foley and Dorsey (3) describe a more realistic approach to calculate N that is applicable to asymmetric peaks. Here the ratio, b/a, of the front and the tail half-widths, $w_{0.1}$, of a peak measured at 10% of the peak height, is used. For a symmetric peak, the value of b/a is unity, for a tailing peak the value will be >1, for a fronting peak the value will be <1. The parameter N is then calculated as:

$$N = \frac{41.7(t_r/w_{0.1})^2}{1.25 + (b/a)} \tag{8}$$

As band broadening increases, plate height, H, increases. In the classical van Deemter equation, H, is described as a function of mobile phase flow velocity:

$$H = A + B/v + Cv \tag{9}$$

where A, B, and C are coefficients pertaining to eddy diffusion, longitudinal diffusion and resistance to mass transfer, respectively, and v is the velocity of the mobile phase.

The eddy diffusion term, of importance only in a packed column, arises from flow inhomogeneities in a packed-bed structure. The coefficient A is directly proportional to the diameter of the packed particles, d_p, and the quality of the packing, λ:

$$A = 2\lambda d_p \tag{10}$$

This term is zero in open tubular columns, as used in capillary GC. Longitudinal diffusion is related to diffusive broadening of analyte zones in the mobile phase and the B-term in the van Deemter equation is directly related to the diffusion coefficient of the analyte in the mobile phase, D_m:

$$B = 2\gamma D_m \tag{11}$$

where γ is related to the packing structure. The shorter t_r is, the less opportunity there is for this type of diffusive broadening; thus the second term in equation (9) is inversely proportional to the mobile phase velocity. This term applies to both packed and open tubular columns; in the latter case, γ is unity.

The third term in equation (9) relates to the rate of transport of the analyte molecules between the stationary and mobile phase. As the mobile phase velocity through the column is increased, it has less and less time to equilibrate with the stationary phase. Some molecules are left behind in the stationary phase that would not have been if equilibrium were achieved. This nonequilibrium condition persists along the column length and results in band broadening that increases with increasing mobile-phase velocity. The coefficient C is proportional to the square of diameter of the packed particles in packed columns or conversely, square of the column diameter for open tubular capillaries and the effective thickness of the active layer on the stationary phase. It is also inversely proportional to the diffusion coefficient of the analyte, both in the mobile and stationary phases.

2.1. Resolution. Resolution, R_s, provides a quantitative measure of separation between two analytes and is given by equation:

$$R_S = \frac{2(t_{r2} - t_{r1})}{w_1 + w_2} \tag{12}$$

where t_r and w are the retention times and base widths of respective analyte peaks. Sometimes it is useful to relate R_s to the retention factors k'_1 and k'_2, selectivity factor α, defined as $\alpha = k'_2/k'_1$, and N:

$$R_S = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{1 + k'_2}\right) \tag{13}$$

R_S increases with increasing N and α. Using a longer column, decreasing the particle diameter in packed columns, and optimizing the mobile phase velocity can all increase N. All of these are more easily changed in a capillary-based system relative to their large bore counterparts. The parameter R_s is also advantageously increased by increasing the selectivity factor, α, through changing the stationary phase, or by changing the composition/nature of the mobile phase in liquid chromatography (LC).

3. Basic Capillary Electrophoresis (CE) Principles

Whereas chromatography is carried out both in larger bore columns and in the capillary format, for a variety of reasons there is no larger bore equivalent of CE.

In CE, analytes are carried along a capillary in which an electric field is present along the length of the capillary. The analytes separate because they exhibit different mobilities (speeds) due to differences in their size and charge. Every charged particle (ion) in an electric field, E, experiences an electric force, which causes it to accelerate, countered by the friction generated between the particle/ion and the surrounding medium. As a result, the particle/ion moves through the capillary with a constant velocity, v_i, proportional to the applied electric field E:

$$v_i = \mu_i E \tag{14}$$

where μ_i is the electrophoretic mobility of the particle. For spherical particles, a shape approximated by most solvated ions, μ_i can be estimated from

$$\mu_i = \frac{z_i \cdot e}{6\pi \cdot \eta \cdot r_i} \tag{15}$$

where z_i is the formal integer charge on the ion, e is the electron charge in coulombs, η is the viscosity of the medium and r_i is the radius of the solvated ion.

In chromatography, the movement of the sample constituents takes place due to the movement of the mobile phase, in turn accomplished by a pressure gradient. In CE, bulk flow of the separation medium (often called the background electrolyte, BGE) can occur due to electroosmosis (see below) but is not necessarily essential for the separation of the analyte components, because these can move without bulk electroosmotic flow. However, in most cases, electroosmosis is present and plays an important role in the separation process.

CE is most often conducted in fused silica capillaries. The capillary wall contains silanol groups, $-$SiOH, which are deprotonated when the pH of the electrolyte is >2, thereby inducing a net negative charge on the inner capillary surface. Typically, solvated cations such as those of alkali metals are present in the double layer near the capillary wall. As an electric field is applied, these positively charged ions move to the negative end of the capillary. The viscous coupling of the water molecules surrounding the ions and those in the bulk solvent in a narrow bore capillary causes the entire liquid to move; this is termed electroosmosis or electroosmotic flow (EOF). In large bore tubes, this phenomenon does not occur because rather than moving in a unified front, the liquid simply moves along the wall and recirculates by moving in the opposite direction in the center. Additionally, as the tube bore increases, the electrical resistance decreases and extensive joule heating prevents the application of a sustained high electric field.

The sign and magnitude of the EOF is obviously dependent on the sign and magnitude of the charge on the capillary wall, usually expressed as the zeta potential, ξ. As depicted in Figure 2, the flow profile in EOF is flat, significantly different from the parabolic profile common to pressure induced flow. The flat flow profile minimizes axial dispersion and allows the high separation efficiencies observed in CE. The magnitude and direction of the EOF can be derived from the Smoluchovski equation:

$$V_{\text{eof}} = \mu_{\text{eof}} E = \frac{\varepsilon \cdot \xi \cdot E}{\eta} \tag{16}$$

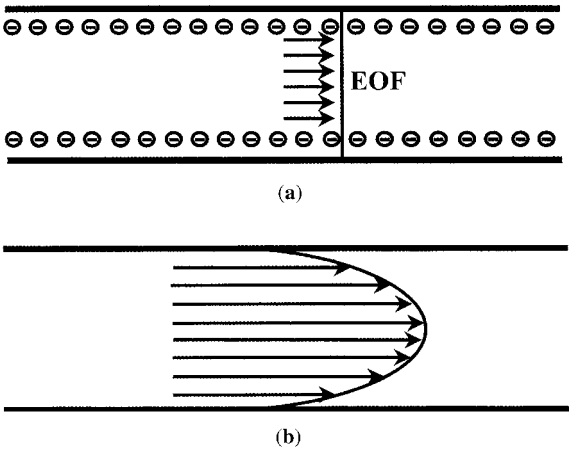


Fig. 2. Flow profiles generated in (**a**) capillary electrophoresis, and (**b**) chromatography.

where ε is the dielectric constant of the electrolyte, ξ is the zeta potential, η is the electrolyte viscosity and E is the electric field applied. The magnitude and direction of the EOF can be modified by adjustment of the electrolyte composition, addition of solvents that change ε or η, or by changing the zeta potential, ξ. The native charge of the fused silica capillary is negative and the EOF is therefore directed from the positive to the negative electrode. However, modifying the wall by any of a variety of techniques can be used to reduce, eliminate, or even reverse the EOF.

The overall velocity of an ion in the fused silica capillary is a combined function of its electrophoretic mobility, μ_i, and the electroosmotic mobility, μ_{eof}. The separation is similar to a boat race along a river where individual analytes represent the individual boats and their speed relative to the river is controlled by μ_i and the bulk flow of the river is represented by μ_{eof}. One can have a race either along or against the river flow, which will control the overall time necessary for the competitors to go from the start to the finish point. In CE, the EOF can be either in the same direction as the electrophoretic movement of the analytes or against it, it can increase or decrease their overall progress to the detection point. When the EOF is zero (much like a completely still river, true zero EOF is difficult to attain), the effective velocity of an analyte is simply given by equation (14).

Note that the uncharged analytes have no electrophoretic mobility and only move along with the EOF. This is similar to the behavior of an unretained analyte in chromatography. However, in CE, a neutral analyte may or may not arrive at the detector, depending on the direction of the EOF. Neutral analytes can be separated by a CE variant, MEKC, described later.

As in chromatography, the plate height, H, or the number of theoretical plates, N, is a measure of the efficiency of the electrophoretic separation. Here, H is defined as

$$H = \frac{\sigma^2}{L_{ef}} = \frac{2Dt}{L_{ef}} = \frac{2DL_t}{\mu_i V} \tag{17}$$

where L_{ef} is the effective capillary length (the length from the injection to the detection), L_t is the total capillary length, V is the voltage applied and D is the diffusion coefficient. The van Deemter equation is also applicable. But there is no stationary phase or packing in CE, only the longitudinal diffusion term (B-term) is relevant. However, other processes, not occurring in chromatography, contribute to the total zone variance:

$$\sigma_{tot}^2 = \sigma_{dif}^2 + \sigma_{joul}^2 + \sigma_{inj}^2 + \sigma_{int}^2 \quad (18)$$

where $\sigma_{dif}{}^2$ is the variance due to the diffusion (B-term), $\sigma_{joul}{}^2$ is the variance caused by joule heating in the capillary, $\sigma_{inj}{}^2$ is the variance caused by processes associated with the injection of the sample plug and $\sigma_{int}{}^2$ is the variance caused by the analyte interaction with the capillary wall (unwanted chromatography). Extensive description of all phenomena contributing to the zone broadening in CE is given in the volume by Foret and co-workers.

Concepts such as number of theoretical plates, N, or resolution, R_s, apply to CE in much the same as chromatography.

4. Sample Preparation for Chromatography and Capillary Electrophoresis

Sample preparation or extraction prior to the actual separation and analysis is most often required in complex samples. Sample preparation is sometimes more challenging than separation/analysis and is often tedious and time consuming. It is particularly important in trace analysis applications (pesticides in fruit/vegetables, polychlorinated biphenyls in soil or sediments, to name a few) where the analyte must first be extracted/separated from the matrix (see TRACE AND RESIDUE ANALYSIS). Currently, several methods are used for sample treatment prior to chromatographic analysis, such as soxhlet extraction, liquid–liquid extraction, supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), solid phase extraction (SPE), solid phase micro extraction (SPME), headspace analysis, and purge-and-trap methods. For air samples, a variety of sample collection devices including adsorbent tubes, filters or denuders are used.

In liquid–liquid extraction (see EXTRACTION, LIQUID–LIQUID) the analyte(s) of interest are extracted from the sample (usually aqueous) into an immiscible extractant phase. Liquid–liquid extraction or soxhlet extraction are the most commonly prescribed analyte extraction method in most United States Environmental Protection Agency (US EPA) and Association of Official Analytical Chemists (AOAC) methods. The main disadvantage of liquid–liquid extraction is the significant consumption of organic solvents (often toxic). SFE (see SUPERCRITICAL FLUIDS), ASE and microwave assisted sample extraction have become popular replacements. Reviews that describe comparison of different extraction procedures as well as description of specialized applications are available (4–8).

For extraction of liquid samples, SPE has become popular. A sample containing the analytes is passed through the solid packing material contained in a SPE-tube or embedded in a disk. In the more common mode of using SPE, analytes of interest are retained on the packing while most matrix components are

unretained. Analyte preconcentration factors as high as 100 or more can be obtained. A prewash is used sometimes to remove some unwanted retained material, the analytes of interest are then selectively washed from the SPE phase using a suitable eluting solvent. The eluate is analyzed by any compatible method. In the other approach, unwanted matrix components are retained while the analytes pass through. No analyte preconcentration is possible in this mode. SPE is a convenient, inexpensive, and time saving sample preparation mode for many liquid samples; it significantly reduces the volume of organic solvents used. For complex samples, SPE is often used after SFE or ASE.

Solid phase microextraction (SPME) is a miniaturized form of SPE, particularly useful for the analysis of gaseous samples. A fused silica fiber is coated with an absorbent such as carbowax, polydimethylsiloxane, etc. The fiber is exposed to the sample for a fixed period (usually 2–30 min, with stirring for liquid samples) during which the analytes of interest are taken up by the coating. Commonly, analysis by capillary GC is used; analytes are desorbed from the fiber thermally in the GC injection port. A specially designed LC or CE interface can be used for coupling to these instruments. SPME requires no solvent, the fiber is reusable and can be used for concentration of a variety of analytes and is thus particularly attractive.

Headspace and purge-trap methods are used for analyzing volatile analytes from a liquid, often an aqueous sample, most typically for analysis by capillary GC. Headspace sampling is a static method for the collection of a volatile analyte. The liquid sample is placed in a vial enclosed by a septum. After the equilibrium between the phases has been reached, the vapor in the headspace is withdrawn into a syringe for injection into a GC. The gaseous phase contains analytes in proportion to their concentration in the liquid phase. An SPME fiber is also often used to sample the headspace.

In a purge-and-trap procedure, an inert gas is bubbled through the (typically aqueous) sample causing the removal of the volatile compounds into the gaseous phase. The gas is made to flow through a sorbent trap that retains the analytes. Subsequently, the analytes are desorbed from the trap (typically by heating) and injected into a GC.

5. Capillary Gas Chromatography (GC)

In GC, the sample is vaporized and injected onto the chromatographic column. Flowing gaseous mobile phase (carrier gas) transports the sample through the column and separation between analytes takes place based on the partitioning of the analytes between this mobile phase and the immobilized stationary phase on the capillary wall. The first paper on chromatographic principles by Martin and Synge (1) and experimental demonstration of GC (9) started the phenomenal growth of this technique. Originally, packed columns were used, however, as early as in 1957, Golay (10) realized that higher efficiencies can be achieved with capillary open tubular columns. Further practical development awaited practical capillary columns, based on fused silica capillaries (11). Presently, an estimated 80% of GC analyses are carried out in open tubular capillaries. The high efficiency of open tubular columns is due to the absence of the

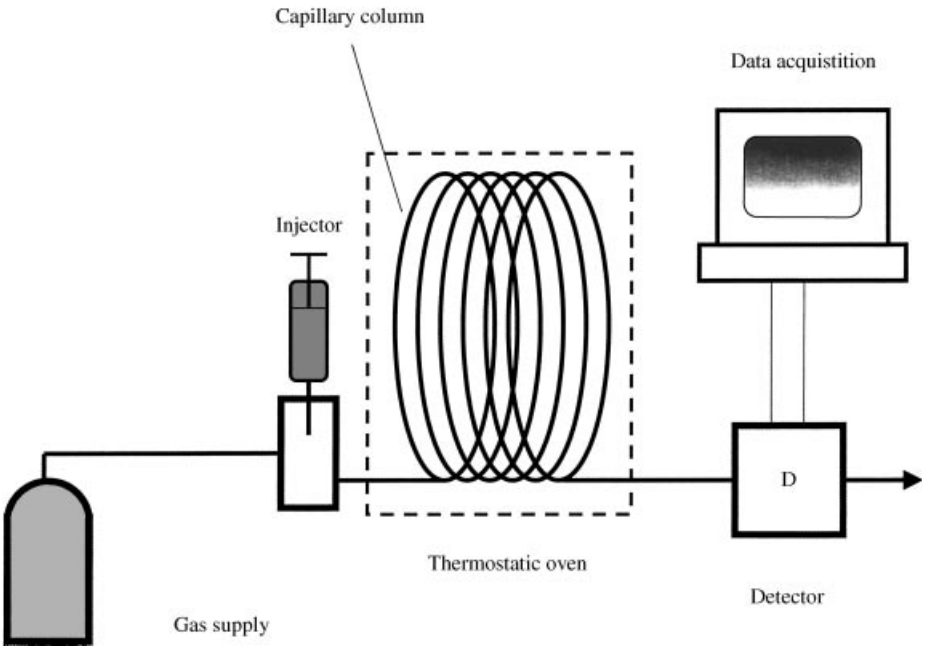


Fig. 3. Schematic diagram of a typical GC system.

column packing and the short radial travel distance to the wall. Capillary columns have much less flow resistance, and hence it is practical to use very long capillary columns. This permits a 2–3 order of magnitude increase in theoretical plate numbers relative to packed columns. On the other hand, due to the limited amount of the stationary phase on the inner-capillary wall, the capacity of the open tubular columns is considerably lower than that of packed columns. They cannot be used for analysis of large sample volumes (ie, $<$500 or 1 μL for gas and liquid volume, respectively).

5.1. Instrumentation. A typical GC is depicted in Figure 3. It consists of a pressurized source of a carrier gas (typically a gas cylinder), an injector, a capillary column, a controlled temperature oven and a detector. A typical GC will also contain several pressure regulators and/or flow controllers, since it is vital to control the carrier gas flow rate.

5.2. Carrier Gas. The carrier gas transports the sample through the column. It does not interact with the analytes. Occasionally, the choice of the carrier gas is dictated by the detector used. Common carrier gases used are He, N_2, and H_2; sometimes Ar, CO_2, and air (especially in portable instruments) are also used. Carrier gas flow rates typically range from 1 to 25 mL/min in capillary GC.

5.3. Sample Injection. *Microsyringe Injection.* Direct injection of a liquid sample with a microsyringe, although commonly used with packed columns, is less commonly used in capillary GC since overloading often occurs. The sample is injected with a microsyringe through an injection port that is heated above the boiling point of the least volatile analyte in the sample.

Split Injection. Split injection is one of the most common injection techniques in capillary GC, since it allows injection of samples independent of the solvent choice, at any column temperature and causes minimal band broadening. The vaporized sample is mixed with the carrier gas and a portion of the sample is introduced into the column, while the majority of the sample is vented out of the system. The ratio of the amount of the sample injected to that vented (split ratio) usually range between 1 and 10 and 1 and 1000. Split injections can exhibit poorer quantitative reproducibilities compared to other alternatives due to: (*1*) a pressure pulse is associated with sample injection that can alter the flow of the gas in an irreproducible way, (*2*) discrimination against less volatile analytes can occur. Advances in instrumentation and the use of automated injectors in recent years have ameliorated much of these problems.

Splitless Injection. This injection mode was devised to allow trace analysis, and allows injection of relatively large sample volumes (1–10 μL) on the columns with a bore $\geq$ 300 μm. The sample is introduced onto the column over a relatively long period of time (several seconds) creating a broad initial zone and then this zone is focused. This focusing relies on *cold trapping* or *solvent effects*. In *cold trapping*, the column inlet is maintained at a temperature of $\sim$80°C lower than the elution temperature of any analyte and not $<$15°C below the solvent boiling point. The analytes then focus at the cooled column inlet due to its increased retention ability. Focusing due to *solvent effect* occurs when the column inlet is cooled to a temperature of at least 20–30°C lower than the boiling point of the solvent causing it to condense at the column inlet. Provided that the solvent liquid film has sufficient retention power to delay migration of the analytes and sufficient wetting of the stationary phase, broadening of the sample zone is minimized.

In *retention gap injection mode*, a significant length of uncoated and deactivated fused silica capillary is situated prior to the analytical column. This is referred to as the *retention gap*. The temperature during injection is sufficiently low so that the analytes that are not retained in the retention gap are trapped at the beginning of the analytical column. After the injection is accomplished, the temperature is raised and analytes start to migrate through the column. The retention gap technique is used also for injection of large sample volumes. It can be used advantageously with highly reproducible loop type injectors.

Programmed Temperature Vaporization (PTV). A PTV based injector can be used in different modes. In *split injection* with a hot or cold vaporization chamber, the sample is introduced as a liquid into the cold vaporization chamber, which is subsequently rapidly heated to the temperature of the highest boiling analyte. This minimizes both the pressure wave and discrimination against less volatile analytes. In *solvent elimination mode*, the sample is introduced to the cold injector at a temperature close to the boiling point of the solvent, which is vented out of the system by the carrier gas. Thereafter, the injector is heated to the sample vaporization temperature and the sample is introduced onto the column. This technique can be used to inject large sample volumes if the analyte is much less volatile than the solvent. The *cold solvent splitless mode* is used in a similar way as hot splitless injection and relies on *cold trapping* and *solvent effect*. The precision and accuracy attainable by a *PTV* based injector

is significantly superior to that attainable in classical split and splitless injection modes.

Cold On-Column Injection. In this mode, a sample is introduced into the column directly as a liquid and is subsequently vaporized. The technique requires a special narrow bore syringe terminus, which fits the inner diameter of the capillary column. This technique eliminates discrimination of less volatile analytes and sample decomposition. It is easy to implement and automate; small sample volumes, 0.2–2 μL, are typically used. One caveat is that since the sample is injected directly on the column, nonvolatile residues in the sample can contaminate the column inlet, causing problems with subsequent analyses.

5.4. Controlled Temperature Oven. A thermostated oven is used in GC, which requires exact control of the column temperature. Modern gas chromatographs are typically equipped with a programmable temperature oven that quickly and reproducibly changes the temperature as a function of time. The upper temperature limit is at least 350 °C.

5.5. Detectors for Gas Chromatography. The detector is one of the most important components of a separation system; it may or may not be integrated with the rest of the instrument. An accurate and precise detection of analyte peaks is required for both qualitative and quantitative analysis with any separation system. Ideally the detector should be sensitive, have linear response to the analytes over a wide range, be stable and reproducible, and have a short response time. No detector meets all of the ideal criteria above. In reality, a detector is chosen based on the particular analytical problem. GC detectors, almost all of which are available for use with capillary systems, can be broadly subdivided into ionization detectors, detectors measuring bulk physical properties, optical detectors, and electrochemical detectors.

Ionization Detectors. Flame Ionization Detector (FID). In this detector, the effluent from the chromatographic column is made to burn in a hydrogen-air flame. As organic compounds burn in such a flame, ions and electrons are produced that in turn generate a current between electrodes (placed in the flame) with a potential applied across them. The FID detector responds approximately linearly to the number of carbon atoms the analyte molecule contains and in this sense, the detector is more or less a mass sensitive detector for most organic analytes. It is not sensitive to the gases such as CO_2, SO_2, NO_x or H_2O, etc. and is useful for the analysis of samples containing organic compounds. The FID is the most widely used detector in GC because of its good sensitivity, a wide linear response to a variety of organic compounds and affordability. Unfortunately, it does destroy the sample.

Thermionic Ionization Detector (TID). The flame thermionic detector (FTD), thermionic detector (TD), nitrogen/phosphorus detector (NPD) all refer to the same device generally similar in design to the FID. An electrically heated (600–800°C) thermionic bead (a ceramic or glass bead typically doped with Rb) catalytically decomposes N and P containing organic compounds in the presence of an H_2/air plasma. The resulting ions are collected by electrodes, as in the FID, generating a current. The detector does not respond to hydrocarbons. In one mode, no hydrogen is used and the detector responds only to nitro compounds and chlorophenols. The detection mechanism remains poorly understood. It is a destructive detector.

Photoionization Detector (PID). In a PID, the molecules in the effluent stream are ionized by intense ultraviolet (uv) radiation from a discharge lamp (typically 10.3 eV). The effluent passes between two electrodes with applied potential and the electric current resulting from the passage of ionized molecules is measured. The detector is easy to operate and provides higher sensitivity for alkenes and aromatic compounds than the FID. The choice of the discharge wavelength governs what compounds will respond. It is nondestructive and often used in series with an FID.

Electron Capture Detector (ECD). The ECD is a structure-selective ionization detector, in which a stable background current is set up between a radioactive source emitting electrons (a β-source, typically ^{63}Ni or ^{3}H) and a positively charged collector electrode. As the column effluent passes in between the electron source and the collector electrode, compounds containing atoms with high electron affinity, notably halogens, capture some of the electrons. The decrease in the background current is measured. The ECD is highly sensitive and often used for trace analysis of halogenated pesticides or polychlorinated biphenyls, drugs, etc. The linear response range of the ECD is limited. The detector requires ~5% CH_4 for proper operation, which is typically added at the column exit. The response does depend on the temperature of the detector chamber, which can be heated as high as ~400°C.

Pulsed Discharge Detector (PDD). The PDD utilizes a pulsed DC discharge in helium as an ionization source. In the electron capture mode, the PDD is similar in sensitivity and response to a conventional radioactive ECD, and can be operated at temperatures up to 400°C. For operation in this mode, He and CH_4 are introduced just upstream from the column exit. In the helium photoionization mode, the PDD is a universal, nondestructive, high sensitivity detector. The response to both inorganic and organic compounds is linear over a wide range. Response to fixed gases is positive (increase in standing current), with an MDQ in the low ppb range. In this mode, the PDD is a serious competitor to the FID especially when a flame and use of hydrogen are unwanted.

Detectors Measuring Bulk Physical Properties. Thermal Conductivity Detector (TCD). The TCD is the only common GC detector based on a bulk property measurement. It is nondestructive and very affordable but not very sensitive, even with He or H_2 as carrier gas, which provides the best response (the thermal conductivity of these gases are 6–10 times higher than most organic vapors). The detector responds to the difference in thermal conductivity of the carrier gas and an effluent analyte, as both flow though a thermostated chamber containing paired sensing elements, typically heated metal filaments.

Optical Detectors (see SPECTROSCOPY, OPTICAL). The Flame Photometric Detector (FPD). is used for the detection of phosphorous- and sulfur-containing species. Analyte molecules are decomposed and excited by a hydrogen-rich flame. The excited species subsequently emit characteristic band spectra centered at 526 nm for P and 392 nm for S.

In the *Atomic emission detector*, the column effluent is introduced into a microwave-energized helium plasma or discharge. The analyte molecules are destroyed and their atoms are excited by the energy of the plasma. The light that is emitted by the excited atoms have lines characteristic of the individual elements. The emitted light is measured with an array-type spectrometer.

Multiple element detection is possible because several wavelengths can be monitored. The detector is expensive but it is universally applicable and allows simplification of chromatograms containing large amounts of peaks.

In *Chemiluminescence detectors*, the analyte molecules are decomposed in flame to form NO or SO_2. These react with ozone to make products in an excited state; as they return to the ground state, they luminescence.

Electrochemical Detectors. Among the electrochemical detectors, the *Hall electrolytic conductivity detector (HECD)* is the most prominent and is available for use with a capillary GC. Halogenated compounds in the column effluent are catalytically reduced to haloacids by mixing them with hydrogen in a heated nickel reaction tube. The reduced compounds flow into a electrolytic conductivity cell, where they dissolve in a solvent (typically *n*-propanol). The conductivity of the latter is monitored.

5.6. Columns. Columns represent perhaps the heart of a chromatographic system; after all, the actual separation takes place on the column. Columns used in capillary GC today are fused silica or glass capillaries ranging in inner diameter from 50 to 700 μm. The use of polyimide coating on the outside of capillaries ensures their durability, and allows coiling in a reasonably small diameter to place in the chromatographic oven. Other external protective coatings can be used; aluminum coated columns are sometimes used in very high temperature work. There are several types of open tubular capillary columns, such as wall coated open tubular (WCOT), porous layer open tubular (PLOT), or support coated open tubular (SCOT) columns. WCOT columns are capillary tubes coated internally with a thin layer of stationary phase that functions as a virtual liquid. In PLOT columns, the column inner surface consists of a porous layer that is formed either by chemical treatment of the capillary interior, or by coating with a layer of porous particles. In SCOT columns, the inner surface of the capillary is covered with a thin layer of a support material on which the stationary phase, a virtual liquid, is attached. The efficiency of these capillary columns decreases with increasing stationary phase thickness and increasing column diameter. Thicker the stationary phase and larger the bore, greater is the sample capacity. SCOT columns in particular allow the largest amount of samples to be injected.

5.7. Stationary Phases. Stationary phases provide means for the analyte separation. The requirement for a suitable stationary phase is low volatility and thermal stability, chemical inertness and suitable values of k' and α for the analyte mixture to be separated. In the early years of GC, a large number of stationary phases were developed. To compare stationary phases, regardless of some variations in operating conditions and instruments, Kovats (12) developed a scheme of retention indices, *RI*, that is still used and bears his name. Retention indices of a series of *n*-alkanes are used as standards for calculation of the retention index of the analyte according to

$$RI = 100N_C + 100\left[\frac{\log t_r'() - \log t_r'(N_C)}{\log t_r'(N_C+1) - \log t_r'(N_C)}\right] \tag{19}$$

where t_r' is the adjusted retention time ($t_r' = t_r - t_{\text{air}}$), N_C is the carbon number of the *n*-alkane eluting immediately before and $N_C + 1$ is the carbon number of the *n*-alkane eluting immediately after the analyte.

The *RI* of any analyte can be calculated from its retention data, and can be presented on a uniform scale, being generally independent of small instrumental variations. Further, the RI system has the advantage of being based upon readily available standards, which cover a wide boiling range.

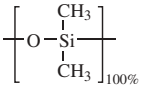
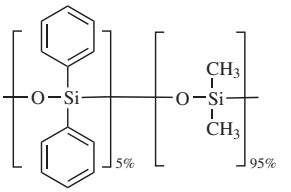
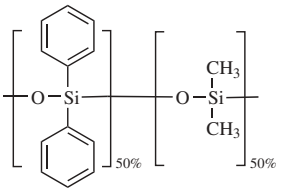
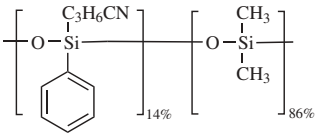
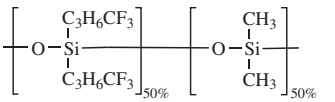
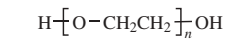
To evaluate the performance of capillary columns, Grob and co-workers (13,14) developed a standard test method for capillary columns. This test provides quantitative information about several important parameters of the tested column, such as separation efficiency, adsorptive activity, acid–base character and the stationary phase thickness. Manufacturers and end-users have both used this test.

However, the number of stationary phases in common use has decreased rapidly over the years. Presently, only a limited number of stationary phases is used for the vast majority of applications. The selection of suitable stationary phase is important for the success of the particular application and the "like dissolves like" principle is often applied. The polarity of the stationary phase is the most important parameter in this respect. The most common stationary phases in use are based on polydimethylsiloxane (PDMS). The PDMS itself is a general purpose, nonpolar stationary phase. As polar functional groups such as –CN, –OH. –phenyl, etc, substitute the methyl groups in PDMS, the stationary phase becomes more polar. Table 1 shows the most commonly used stationary phases and their structures.

5.8. Hyphenated Techniques. *GC–MS.* Coupled or hyphenated techniques are among the most powerful in analytical chemistry. Capillary GC is often coupled to a selective detector such as a mass spectrometer (MS, see MASS SPECTROMETRY; ANALYTICAL METHODS, HYPHENATED INSTRUMENTS) or Fourier transform infrared spectroscopy (FTIR), that can provide additional, especially structural, information. Direct introduction of the effluent from a capillary GC into a MS without special interfaces is possible. The effluent from the GC is fed directly to the ionization chamber of the MS. The ions created from the analyte molecules in the ionization chamber are passed through the mass analyzer and detected. The mass spectrum for each chromatographic peak can be compared with the MS spectra library to aid identification. Most often, an electron impact ionization source is used to obtain reproducible spectra for MS library search. The MS unit should be sufficiently fast to provide mass spectra of all peaks eluting from the chromatographic column with sufficient resolution. Mostly quadrupole and magnetic sector mass spectrometers are used in GC–MS systems. Such instruments have been used for the identification and quantitation of thousands of myriad components present in natural and biological systems, including odor and flavor components, water pollutants, or drug metabolites and pharmaceuticals. Extensive reviews on recent developments on GC–MS are available (15–19).

GC–FTIR. Like GC–MS, GC–FTIR provides a potent means for separation and identification of analytes in complicated mixtures. In this technique, the end of the GC column is connected to a narrow bore gold-coated light pipe in which the infrared (ir) absorption spectrum is acquired. Alternatively, the analyte can be trapped on a disc that has been cryogenically cooled and the absorption spectrum then acquired on the solid sample. The ir spectral data (see INFRARED TECHNOLOGY; SPECTROSCOPY, OPTICAL) are stored in memory and compared

Table 1. **The Commonly Used Stationary Phases**

Stationary phase	Applications	Structure
polydimethylsiloxane	general purposes, amines, phenols, solvents, waxes.	
poly[diphenyl(5%) dimethyl(95%)] siloxane	alcohols, alkaloids, halogenated compounds,	
poly[diphenyl(50%) dimethyl(50%)] siloxane	alcohols, drugs, steroids, pesticides, sugars, nitroaromatics	
poly[cyanopropylmethyl(14%)dimethyl(86%)]siloxane	alcohol, alcohol acetates, drugs, fragrances, pesticides	
poly[trifluoropropyl(50%)dimethyl(50%)]siloxane	drugs, environmental samples, chlorinated compounds nitroaromatics	
poly(ethylene glycol)	free acids, flavors, fragrances	

to an on-board spectral library as in MS. Several extensive reviews on recent developments of GC–FTIR are available (17–22).

Multidimensional GC. In two-dimensional (2-D) GC or multidimensional GC, one or more peaks eluting from a first GC column is separated on a second GC column offering a different selectivity, resulting in very high resolving power. Typically, the effluent from the primary column is injected in pulses to the secondary column. This switching can be performed using a high speed switching valve or be based on differential pressure in the interface between the columns.

In comprehensive 2-D chromatography, the eluent from the primary column is periodically injected onto a high speed secondary column. The separation on the second column need not be totally complete before a subsequent injection. Since no peaks elute naturally during the dead time for a given run, the dead time is used for elution of peaks from the previous injection. This uses column two run time more effectively. Data can be adjusted accordingly prior to plotting. This technique resembles the fast scanning mass spectra in GC–MS. It is particularly useful in cases where a number of structurally analogous compounds or

isomers coexist that mass spectrometry cannot differentiate. Multidimensional GC has been widely applied in the analysis of petroleum products that often contain closely structurally related isomers, in the analysis of traces of structurally analogous chlorinated substances in the environment and the analysis of food and fragrances. Reviews on applications of multidimensional GC are available (21–24).

5.9. Applications. Capillary GC is and has been applied for the analysis and characterization of an enormous number of compounds in various areas, with the primary focus on volatile, volatilizable and semivolatile compounds. The main application areas include (*1*) industrial chemical analysis, such as analysis of mixtures of acids, alcohols, aldehydes, amines, esters, phenols, and other aromatics (25–29); (*2*) analysis of industrial solvents and their mixtures as well as analysis of permanent gases and hydrocarbons in various industries, natural and synthetic gases, refinery gases, sulfur containing gases, permanent and noble gases and freons, gasoline and naphtha, etc (30–34); (*3*) analysis of flavors, fragrances, antioxidants and preservatives in food, beverages, perfumes, and cosmetic products (35–38); (*4*) analysis of pharmaceutical formulations including antidepressants, antiepileptics, anticonvulsants, opiates, barbiturates, alkaloids, etc (39–46). Capillary GC is particularly useful for the analysis of volatile and semivolatile analytes in environmental samples (47–54); many US EPA approved methods for the analysis of aromatics, halocarbons, PAHs, organochlorine pesticides, fungicides and various other pollutants in water, soil, air and waste are based on capillary GC (55).

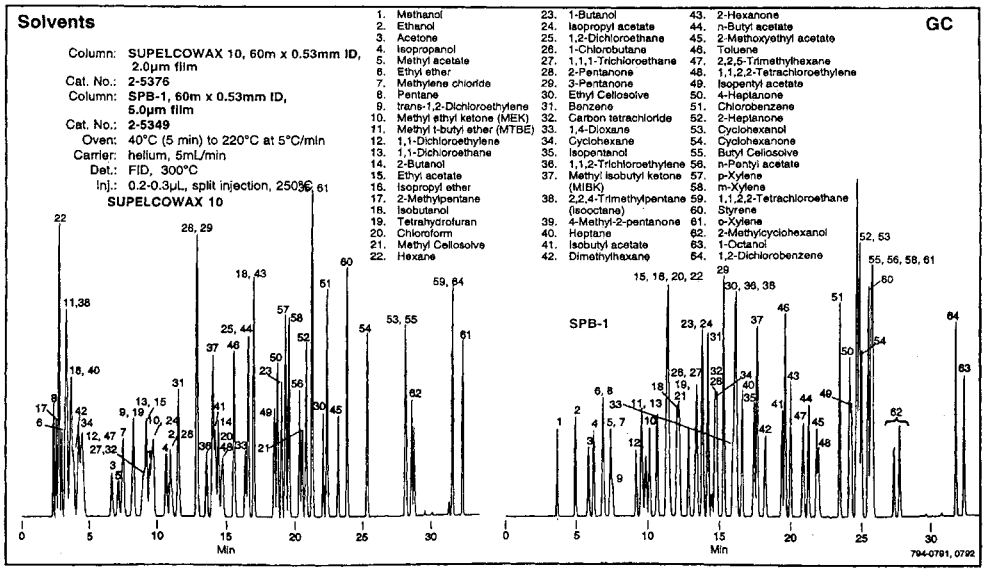
Figure 4 shows some selected applications of gas chromatography. Specific application notes in these and other areas are available from vendors of chromatographic equipment (56–59). Further information on recent advances on GC can be obtained from reviews, eg, the biennial reviews in *Analytical Chemistry* (60–62).

6. Liquid Chromatography

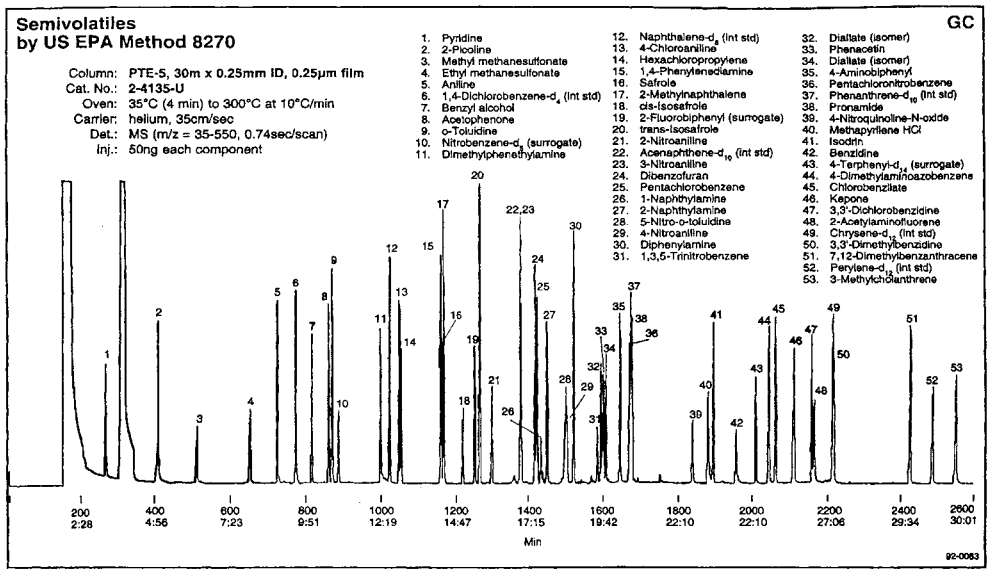
As the name implies, in LC, a liquid mobile phase is used. In contrast to GC, the choice of the mobile phase affects the analyte retention. There are also many more stationary phases available, permitting much greater latitude in developing a separation method. However, a greater choice can also sometimes mean greater complexity.

Tswett (63) described the first LC separation at the turn of the century: Various plant pigments were separated on a glass column packed with finely ground chalk. It was not until 1960s, when the technology for production of uniform spherical particles of diameters $\leq$10 μm became available that LC became a high efficiency separation method and high-performance liquid chromatography (HPLC) was born. In the intervening years, it has evolved into a separation method of such importance that pharmaceutical or biotechnology industries will cease functioning without it.

The introduction of microcolumn LC is generally attributed to Horvath and co-workers (64,65) in 1967. However, later several research groups published papers that are considered key publications in this area (66–74). The term



(a)



(b)

Fig. 4. Selected chromatograms showing some application areas of gas chromatography, conditions are described in text. (**a**) Separation of organic solvents. (**b**) Separation of semivolatiles by US EPA Method 8270. Reproduced by permission of Supelco, Bellefonte, PA.

microcolumn LC is sometimes used interchangeably with capillary LC. Some commercial instrument makers also use another term, "microbore" LC, to refer to 1- or 2-mm bore columns. The distinction as to where normal bore LC ends and capillary LC begins with "microbore" LC somewhere in between, is rather vague. Most users would not consider 1-mm bore columns as belonging to a capillary format.

As in GC, capillary LC offers better separation performance than its larger bore counterparts. However, it has not yet become as widely used as capillary GC. While capillary GC relies only on open tubular columns, there are few practitioners of open tubular capillary LC (OTLC). While OTLC was reported early on (75,76) and studies continue (77), the big difference is the orders of magnitude lower D_m value in the liquid phase. Either D_m must be increased by increasing temperature (78), or the column bore must be reduced to single digit micrometers (79).

For the most part, capillary LC has established a niche for itself where the ability to analyze minute sample volumes, limited consumption of mobile phase, and high resolution are important. It is also the capillary format of LC that is best coupled with mass spectrometric detectors, although current interfaces allow easy connection of conventional HPLC to MS as well.

The popularity of LC stems from its ability to accomplish almost any separation, given appropriate sample preparation. This is especially true for nonvolatile thermally unstable species, such as amino acids, proteins, carbohydrates, drugs, antibiotics, pesticides as well as myriad inorganic and ionic species.

6.1. Instrumentation. A typical liquid chromatograph is depicted in Figure 5. The mobile phase(s) is(are) contained in glass or other inert reservoir(s)

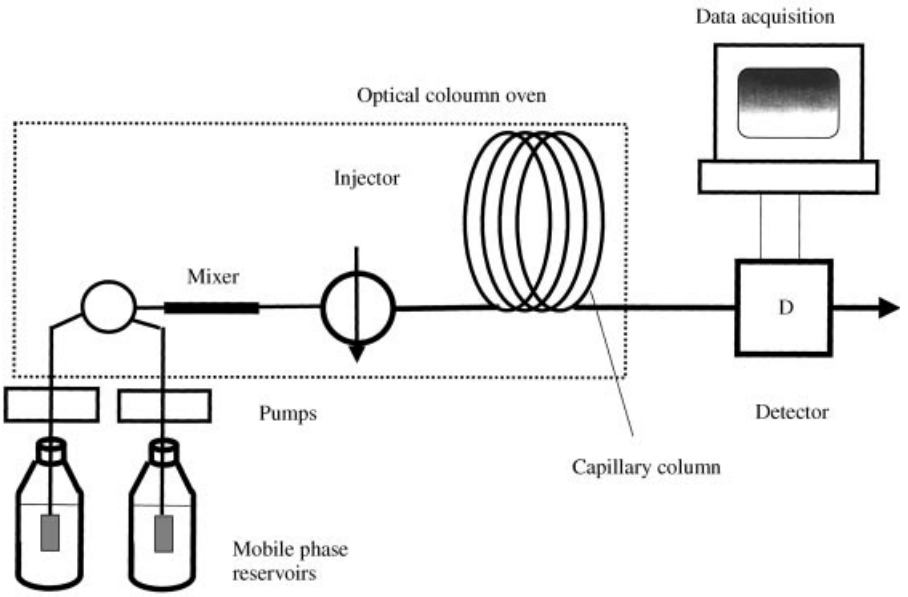


Fig. 5. Schematic diagram of a typical capillary LC system.

and are typically sparged/degassed and filtered through on-line filters prior to entering the pump. With reciprocating pumps, if more than one solvent is used, typically they are blended by a proportioning valve system before entering the pump inlet. The chromatographic pump (see PUMPS) must be capable of pumping at the desired flow rate at very high pressures. Operation at pressures of 1000–2000 psi are common in conventional HPLC. In capillary LC systems, the operating pressure is typically at least as high. The current trend for both conventional and capillary system is to use smaller diameter packing particles and higher pressures to improve sample throughput. The pumped eluent then passes through an injector to the column and to the detector. A short "guard column" is sometimes used ahead of the analytical separation column to protect the latter from particles in the sample or very strongly retained components that do not readily elute and thus poison the column. In capillary LC, it is essential that band dispersion be kept to a minimum. As such, connections associated with the insertion of a guard column produce unwanted dispersion and such a column is often not used. When a column is poisoned, this occurs at the head of the column. One great advantage of a fused silica separation column is that it is always possible to cut and discard a little portion of the column at the top.

6.2. Mobile-Phase Delivery. A detailed review on types of pumps can be found elsewhere (see PUMPS). Capillary LC is sometimes conducted with conventional scale pumps and injectors, most of the flow is discarded or recycled using a splitter. This arrangement cannot take advantage of some of the principal benefits a capillary LC system has to offer. Syringe pumps of 1–10-mL capacity are most commonly used in capillary LC. Use of multiple solvents for gradient elution, etc require multiple syringes. An exception is capillary ion chromatography (IC, a subclass of LC) where eluents can be electrodialytically generated in-line; the syringe is merely used to pump pure water (80). The pump will normally be equipped with a three-way valve arrangement (ruby and sapphire ball and seat check valves are typically used) that allow it to pump at high pressure through the column and then refill from an eluent reservoir. Low-flow single- or multiple-head reciprocating pumps (most commonly used in conventional bore HPLC), with similar check valves at the inlet and the outlet are also used in microcolumn LC, however, considerable innovation and ingenuity goes into making pumps operating in this mode to be pulse free (81).

6.3. Injectors. In early years, injectors that can inject volumes small enough for capillary LC were not available. With the advent of such injectors, approaches of the yesteryears, such as moving injection technique (82,83), static split (84) or pressure pulse driven stopped flow injection (85) have fallen by the wayside. Virtually all injectors in use with microcolumn LC today are two-position rotary-type injectors. For injection volumes less than 1 μL, internal loop injectors (consisting of a slot-in-a-disk inside the injector) can be used and are available down to injector volumes of 20 nL. It is inconvenient to change injection volumes with these valves (it must be disassembled), however, it is possible to use commercially available low internal volume six-port injectors containing external loop for injection volumes $\geq$1 μL. For smaller injection volumes, the concept of partial loop filling injection can be used with these valves. This technique conserves sample and provides flexibility in the choice of sample volumes to be loaded into the injection valve without changing loops. The

precision of either type of injector is usually better than 1%, which allows for accurate and reproducible sample injection and quantitation.

6.4. Detectors. All detectors that are used in capillary scale LC can also be used in CE, which will not be separately discussed. With the exception of refractive index and conductivity detectors, bulk property detectors are not commonly used. Many detection principles allow on-column detection and thus eliminate extraneous dispersion in transit to the detector. Some GC detectors can be used with capillary LC, but the practice is not common.

Absorbance Detectors. The uv-vis absorbance detector is the workhorse in both capillary and conventional HPLC (see SPECTROSCOPY, OPTICAL; PHOTODETECTORS) and is available in fixed and variable wavelength, fast-scanning, and photodiode array (PDA) forms. Typically a narrow beam of monochromatic light (white light for a PDA) is focused on a window made in the capillary immediately after the column frit (on-packing absorbance detection has also been reported but is not common). Light passes radially through the tube and is then focused or dispersed on the detector. The radial path is short and alternatives such Z-cells and light-pipe based cells of longer path are commercially available.

Fluorescence Detectors. Either monochromatic light from a powerful continuum source (Hg- and Xe-lamps) or increasingly, a laser (see LASERS), is used for excitation. A window is made in the capillary as for absorbance detection above. The light emitted by a fluorescing analyte is measured usually perpendicular to the excitation beam. Filters or monochromators are used to filter the desired excitation and emission wavelengths. Fluorescence detectors, especially Laser-induced fluorescence (LIF) detectors are very sensitive, with concentration LODs in the range of 10^{-9}–10^{-12} M. The disadvantage is that the analyte must be fluorescent; otherwise a derivatization with fluorogenic agent must be carried out in advance. Such derivatizations are often carried out postcolumn in conventional scale HPLC but is more difficult to implement in capillary LC or CE because of strict requirements to limit band dispersion.

Electrochemical Detectors. Electrochemical detectors rely on measurements of electrochemical properties of the analytes (see ELECTROANALYTICAL TECHNIQUES). They can be subdivided into several groups, such as amperometric, polarographic, coulometric, or conductometric detectors. Amperometry at a single graphite fiber was one of the earliest techniques used for detection in capillary IC (76). Electrochemical detectors permit high sensitivity (often to 10^{-9} M) and respond to several groups of organic compounds typically analyzed by HPLC. However, careful selection of the mobile phase is necessary and electrodes can be easily poisoned from the analytes or the sample matrix. In a conductivity detector, electrical resistivity is measured; strictly speaking, it is not an electrochemical detector because no chemistry is carried out. These detectors are easy to miniaturize, are particularly robust in behavior and are discussed further below.

Conductometric Detectors. Conductivity detectors are mainly used with IC in which ionic analytes are separated by ion exchange. Although a conductometric detector is in principle a bulk property detector, typically IC is used in a "suppression" mode where all the ionic species originally present in the mobile phase are selectively removed, making the detector essentially selective to ionized analytes. Typical sensitivity is similar to the uv absorbance detectors ($\sim 10^{-7}$ M). Conductometric detectors of many types have been described

(86,87), the current trend is toward contactless conductivity detectors, see Zemann for a review of capillary scale conductometric detectors (88).

Refractive Index (RI) Detectors. The RI detector measures the difference in refractive index of the analyte zones and the mobile phase. The advantage of this detector is its universal response to virtually any compound. There are few RI detectors for use with capillaries. A laser-based microinterferometric backscatter detector developed by Bornhop (89) is an exception, permitting detection of a RI change of the order of 10^{-7}. The RI detectors must be carefully temperature stabilized to attain good sensitivity. For recent developments in this type of detector and a general review of detectors used in capillary LC/CE, see Swinney and Bornhop (90).

Evaporative Light Scattering Detector. In this detector, the column effluent is nebulized and the mobile phase is evaporated. Nonvolatile analyte particles are detected by laser light scattering. The detector provides universal response to all nonvolatile compounds and is sensitive. Commercially available detectors can be adapted for use with capillaries and can also be used with supercritical fluid chromatography (SFC). Nonvolatile (eg, buffer) components cannot be present in the mobile phase. In one variation of this, the evaporated particles are used as condensation nuclei and single molecules can be detected (91).

6.5. Columns for LC. Although many different styles of columns, eg, drawn packed capillaries (pack a large bore glass capillary with particles and subsequently draw it to the desired diameter using a glass drawing machine—incompatible with thermally sensitive packing), wall-functionalized open tubular columns as in GC, etc have been described, the mainstay today is packed fused silica capillaries with the most popular column bore being ~300 μm, albeit packed columns in 50–500-μm bore capillaries are commercially available. Packed microcolumns are prepared by slurry packing. A frit is made at one end of the capillary to retain the packing material. This frit is usually made from glass wool, metal or porous silica. The particles are suspended in a suitable solvent, having a density similar to that of the particles to avoid particle sedimentation. The reservoir with the slurry is agitated or sonicated to achieve a homogenous mixture and the capillary is packed under pressure or vacuum. The particle size, uniformity, and the homogeneity of the packed bed are the major factors contributing to the column performance. Hundreds of column packings are commercially available for conventional HPLC; most major types are available as capillary columns, at least on a semicustom basis. The one exception is capillary IC, no commercial columns are available at this time.

6.6. Stationary and Mobile Phases. In LC both the stationary and mobile phase play an important role in the analyte partitioning mechanism. In virtually all present-day columns, the stationary phase consists of spherical support particles, ranging in diameter of 1–10 μm, which are often chemically modified by covalently bonded functionalities. The particles themselves are composed of silica, alumina, zirconia, carbon, and poly(styrenedivinylbenzene) (PSDVB) or related polymers. In the past 5 years, a tremendous amount of effort was devoted to making organic-modified silica solid supports to overcome temperature and pH problems.

The type of mobile phase depends on the chromatographic mode used. In "normal-phase" LC, a nonpolar solvent such as hexane or isopropyl ether are

used, in "reversed phase" (RP) LC, by far the most common mode in which LC is currently practiced, aqueous eluents containing methanol or acetonitrile (occasionally, tetrahydrofuran (THF)) as well as buffering salts/acids/bases are used. Since RPLC is widely used for ionized or ionizable compounds, a proper selection of pH of the mobile phase is important. Phosphate and Tris-based buffers are often used and are optically transparent in most of the wavelength range of interest. In IC, the mobile phase is typically dilute aqueous solution of a strong acid or base.

6.7. Normal Phase LC. In normal phase LC a column is packed with a polar stationary phase, the mobile phase is of a nonpolar or moderately polar nature. The polarity of the analytes plays an important and crucial role in their retention on the stationary phase. The stationary phase contains either unmodified silica or alumina or a chemically bonded stationary phase, typical functional groups being cyano, amino, dihydroxypropyl modified polysiloxane. A wide variety of organic solvents can and has been used for normal-phase chromatography with polarity ranging from fluorocarbons to methanol. In normal-phase chromatography, the mobile phase is typically less polar than any of the analytes. Usually, a binary mixture of two solvents of different polarity, the less polar solvent being most commonly hexane, and a more polar solvent, are blended to achieve the required elution strength; a gradient (eluent changing composition with time) is sometimes used. Normal-phase LC mode is used in $<20\%$ of all LC applications.

6.8. Reversed Phase LC. RPLC accounts for the majority of present LC separations. In reversed-phase chromatography, the elution order of the analytes is opposite to the normal-phase chromatography, ie, the most polar analyte elutes first. A nonpolar stationary phase and a polar mobile phase is used. Most commonly the stationary phase is porous silica to which polysiloxane, typically modified with alkyl chains, such as C8 (*n*-octyl) or C18 (*n*-octadecyl, this phase is also called octadecyl silica or ODS and is the most commonly used) are bonded. The hydrocarbon chains are aligned parallel to one another and perpendicular to the particle surface making the surface hydrophobic. The PSDVB and porous graphitic carbon (eg, Hypecarb) are alternatives to silica-based stationary phases and can be used in extremes of pH. Zirconia-based phases have extraordinary temperature stability. Separation of aromatic hydrocarbons with pure water as eluent on polybutadiene or carbon modified zirconia phases have been reported at a temperature of 370°C with sufficient backpressure to keep the water in the liquid state (92). Hypercarb is more retentive than silica based stationary phases (C18) and has been popular in capillary LC. Its high retaining ability contributes to on-column focusing effects and allows for injection of larger sample volume. Aside from the solvent composition of the mobile phase, appropriate buffering and the selection of the optimum buffer, buffer pH is also very important for optimum separation of many compounds, especially those with weakly acidic or basic character.

6.9. LC–MS. The great potential in coupling liquid chromatography with mass spectrometry (LC–MS) has been recognized especially in recent years, because conventional GC–MS methods are not adequate for variety of applications due to limited volatility and thermal lability (see ANALYTICAL METHODS, HYPHENATED INSTRUMENTS; MASS SPECTROMETRY). In developing a successful coupling between a LC and a MS, one needs to deal with the fact that a

significant amount of solvent comes out as the column effluent and all of this must be evaporated off. Many early MS units also had serious problems with nonvolatile components in the eluent. Capillary LC is much more directly compatible with MS than large bore LC in this regard since the total volumetric flow rates are much lower. The LC–MS interfaces in current use include continuous flow fast atom bombardment (CF–FAB), thermospray, particle beam, electrospray and atmospheric pressure chemical ionization (APCI) interface.

In a CF–FAB interface, a liquid stream is mixed with FAB matrix solvent (typ. glycerol) and ions are generated by bombardment of this liquid by accelerated atoms or ions (Cs^+ or Xe).

In a thermospray interface, small droplets of eluent are generated in a heated vaporizer tube. The ionization of analytes is accomplished by solvent-mediated chemical ionization and ion evaporation processes. This interface is compatible with substantial liquid flow rates, up to 2 mL/min, and has been widely used in many areas such as analysis of drugs, their metabolites, natural products, and in environmental analysis.

In the particle beam interface, the column effluent is nebulized pneumatically or by thermospray into a desolvation chamber, where the high mass analytes are separated from the low mass solvent molecules. The ionization of analytes takes place by a conventional electron impact or chemical ionization. This interface is able to generate electron-impact spectra (but limited to MW <1000), which makes it useful in pharmaceutical and environmental analysis.

In the electrospray interface, a high voltage (typ. 3 kV) is applied between the eluent emerging from a capillary needle attached to the column outlet and a counter-electrode, causing the formation of small droplets of the eluent. Solvent is evaporated and analyte molecules are ionized prior to entering the MS proper.

In an APCI interface, the eluent from the column is nebulized by pneumatic or thermospray nebulization and ionized in an atmospheric pressure ion source.

Coupling of LC to MS provides an important tool in qualitative and quantitative analysis of various samples and is useful in several areas such as analysis of pharmaceuticals, biochemical and environmental samples (93–102).

6.10. Applications of Capillary LC. LC is the most popular and widely used analytical separation method today. The application area is enormous and thousands of LC based analyses are in routine use. Major areas for LC application include analysis of pharmaceutical formulations and drugs, food, and consumer products, large molecules of biological importance such as DNA, DNA-fragments or peptides, and proteins (see BIOSEPARATIONS). The LC analysis of mixtures of nonvolatile or semivolatile pollutants supplement the available GC methodologies; approved methods are listed in the EPA list of methods (55), (see also WATER, ANALYSIS).

Virtually all methods developed for conventional LC with large bore columns can be readily implemented to the capillary scale. While capillary LC is not at the moment poised to displace conventional scale LC (as happened with GC), its future is increasingly brighter. It has already found a niche in bioanalysis (103–105), neuroscience, in vivo measurements, protein/peptide research (106–109) including proteomics and chiral separations (110–112), where the sample availability, cost of reagents and compatibility with MS detection benefit from the capillary scale. Developments in microcolumn LC are summarized in

several recent reviews (113–119). In Figure 6, selected chromatograms of microcolumn LC applications are shown.

6.11. Ion Chromatography (IC). IC is an important subclass of LC and is used primarily to determine inorganic and organic anions and cations. In IC,

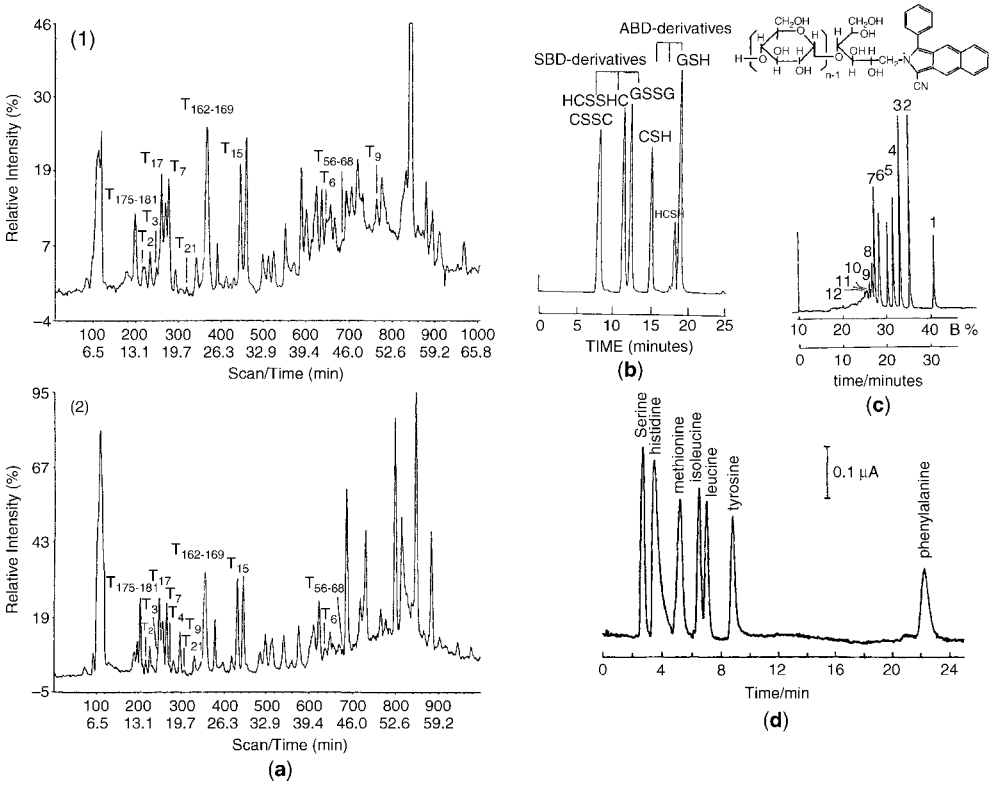


Fig. 6. Selected chromatograms showing some application areas of capillary LC. (**a**) Comparative peptide mapping of enzymatic digests of bovine ribonuclease B with micro-LC-electrospray MS (1) TIC trace of tryptic digest of reduced and (*S*)-carboxymethylated–ribonuclease B, (2) TIC trace of digested reduced and (*S*)-carboxymethylated–ribonuclease B with trypsin and peptide-*N*-glycosidase F. Conditions: capillary column: 33 cm × 0.25 mm id, packed with C_{18}. Mobile phase–Solvent A = 0.1% TFA in water, solvent B = solvent A/acetonitrile (20:80), gradient elution from 0 to 60% solvent B over 120 min. Reproduced from J. Liu, K. J. Volk, E. H. Kerns, S. E. Klohr, M. S. Lee, I. E. Rosenberg, *J. Chromatogr.* **632**, 45 (1993); by permission of Elsevier Science. (**b**) Chromatogram of mixture of thiols and disulfides: Conditions: capillary column 25 cm × 0.32 mm, packed with 5 μm Rosil C_{18}. Mobile phase 150 m*M* H_3PO_4:acetonitrile, gradient elution from 92:8 to 70:30 in 15 min, 70:30 for 5 min. Peaks: cysteine (CSH), homocysteine (HCSH), glutathione (GSH), cystine (CSSC), homocystine (HCSSC), and oxidized glutathione (GSSG). Reproduced from B. L. Ling, C. Dewaele, W. R. G. Baeyens, *J. Chromatogr.* **553**, 433 (1991); by permission of Elsevier Science. (**c**) Chromatogram of oligosacharide derivatives: Individual 3-benzoyl1-2-naphtaldehyde-derivatized components of Dextrin 15. Conditions: Capillary column 90 cm × 0.3 mm id, packed with Capcell-C_{18}. Linear gradient elution with water:acetonitrile. Reproduced from J. Liu, O. Shirota, M. Novotny, *J. Chromatogr.* **559**, 223 (1991); by permission of Elsevier Science. (**d**) Chromatogram of underivatized amino acids. Conditions: Capillary column 15 cm × 0.3 mm id, packed with ODS-3 (3 μm). Mobile phase 50 m*M* phosphate:10 m*M* KBr, pH 7.5. Amperometric detection. Reproduced from K. Sato, J.-Y. Jin, T. Takeuchi, T. Miwa, Y. Takekoshi, S. Kanno, S. Kawase, *Analyst* **125**, 1041 (2000); by permission of the Royal Society of Chemistry.

the separation is based on ion exchange (see ION EXCHANGE; CHROMATOGRAPHY) between the analytes from the sample and stationary ion exchange sites on a column and displacement by eluent ions. The column capacity is significantly lower than conventional ion exchange resins (eg, as used for water softening) to allow reasonable retention times. The columns are typically based on PSDVB based polymeric beads that have been modified to contain strongly acidic ($SO_3^-H^+$), strongly basic ($NR_3^+OH^-$), weakly acidic (COO^-H^+, $HPO_3^-H^+$), or weakly basic ($-NH_2$, $-NRH$, $-NR_2$) functional groups. Chelating functional groups are present in columns used for the preconcentration of transition metals. One of the most interesting classes of columns used uniquely in IC utilizes a surface-agglomerated packing. If a PSDVB bead is mildly sulfonated, negatively charged sulfonate groups are formed on the surface. If one now passes a suspension of very fine (submicrometer size) quaternized, positively charged latex particles atop the surface sulfonated beads, the positively charged latex microbeads agglomerate electrostatically on the surface of the negatively charged beads. This provides for a highly efficient anion exchanger where ions can readily exchange in the top layer.

Conductometric detection is the mainstay in IC; all ions share the property of electrical mobility. In so-called single column IC, the signal arises from the difference in equivalent conductance between the sample ion and the analyte ion. For example, if dilute HCl is being used as the eluent, the detector background is the conductivity of the eluent. As various cations, eg, Li^+, Na^+, K^+, etc elute from the column, the H^+ concentration decreases in a proportionate manner because the metals replace H^+ in the effluent. Because the metal ions have substantially less electrical mobility than H^+, negative peaks in the conductance signal are observed with the elution of each metal. To limit the background conductance, the eluent concentration needs to be limited. To make this work, the column capacity must be limited. These limitations have largely relegated this technique presently to historical importance only. The detection principle is the same as indirect photometric detection commonly used in CE.

The major mode in which IC is used today is the so-called "suppressed" mode. The eluent is a strong base (eg, KOH) or a very weak acid salt of a strong base (Na_2CO_3) for anion separations on an anion exchanger column or a strong acid (eg, HCl) with a cation exchange column for cation separations. In the anion separation system, the separation column is followed by a "suppression" device and then the conductivity detector. The suppressor device above exchanges all cations for H^+. The eluent KOH is thus converted to water (Na_2CO_3 will be converted to H_2CO_3), a very weakly conducting background. In contrast, when Cl^-, NO_3^-, SO_4^{2-}, etc elute from the separation column and pass through the suppressor, they emerge as strongly conducting HCl, HNO_3, H_2SO_4, etc permitting direct detection. Hence, the suppressor "suppresses" the conductivity of the eluent while amplifying the conductance of the analyte. Similarly, in cation separation system, the suppressor is a OH^- exchanger that converts an HCl or HNO_3 eluent to water while analytes Li^+, Na^+, K^+, reach the detector as LiOH, NaOH, KOH, etc. This technique, the mainstay of ionic analysis today, was invented by Small and co-workers in 1975 (120). The original suppressor device was a periodically regenerated resin column; it is still used in this form in a capillary format but the majority of suppressor devices today are based on a chemically or

electrically regenerated membrane or resin-packed device (or a hybrid) (see MEMBRANE TECHNOLOGY).

In an electrically operated membrane suppressor, the eluent ions are removed electrodialytically. The exact reverse of this technique, electrodialytic eluent generation, allows the electrical production of high purity eluents on demand and at specified concentration. Recently, Small and Riviello (121) described "Ion reflux". As applied to IC, this is a new ion-exchange technique where an electrically polarized ion-exchange bed becomes the source of eluent as well as its means of suppression. Using water as the pumped phase, such polarized beds enable the "perpetual" generation and suppression of eluent with little intervention by the user. In one embodiment of ion reflux, continuous eluent generation, ion separation, and continuous suppression are accomplished within a single bed. In another case, where separation is uncoupled from the other two functions, the ion reflux device may be used with existing separators. Indeed, the latter technique holds much promise for capillary IC since eluent generation and suppression can be carried out on a different scales.

Currently, capillary IC instrumentation is commercially unavailable. This makes widespread use impossible. The power of capillary IC is apparent from the chromatogram in Figure 7. Field portable electrical eluent generation based capillary IC has also been demonstrated (122).

6.12. Applications of IC. IC is applied for separation and analysis of small charged analytes in various samples. It is used particularly for the analysis of different types of water samples, such as tap, drinking, rain or wastewater from industrial processes (see WATER, ANALYSIS). Several industries, such as pulp and paper, chemical, pharmaceutical, power generation and the semiconductor industry depend on IC analysis (123–132). Among the compounds mainly analyzed by IC are inorganic anions and cations, small organic molecules such as carboxylic acids or amines, short/long chain surfactants or metalcyano complexes (133–142). Heavy metal speciation by post column reaction detection or element specific detection (124,135,143,144) is an important area. Literature is available for further reading (145).

7. Supercritical Fluid Chromatography

In supercritical chromatography, the sample is injected onto the column and carried through it by a mobile phase consisting of a supercritical fluid, a fluid that is being maintained in the separation system at a temperature higher than its critical temperature T_c and critical pressure P_c. (see SUPERCRITICAL FLUIDS). Properties of supercritical fluids such as density, viscosity, and diffusion coefficient are intermediate between those of liquids and gases. For example, density of the supercritical fluid is high compared to gases; this promotes dissolution of large nonvolatile compounds. On the other hand, its viscosity is close to that of gases, with a proportionately high diffusion coefficient. This makes SFC an interesting alternative to both GC and LC (146). SFC can be advantageously used for compounds not easily analyzed by GC, eg, nonvolatile, thermally labile, or polymeric compounds or compounds lacking chromophores or electrochemical properties that are not so easily detected by conventional LC detectors.

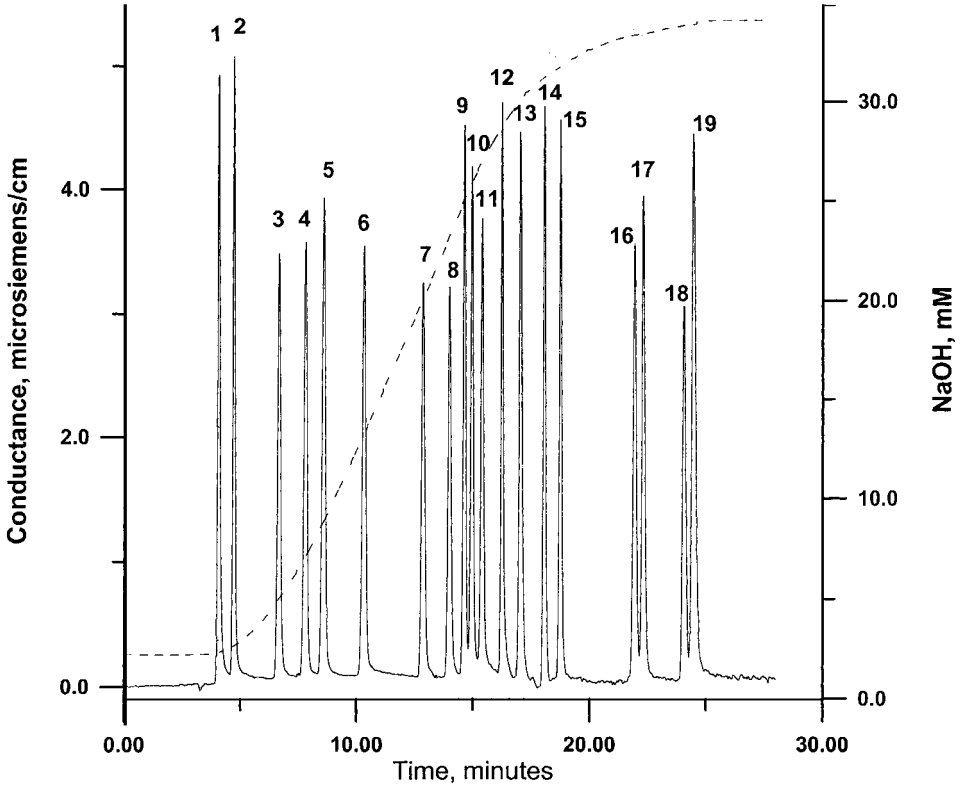


Fig. 7. Capillary ion chromatogram : 56 cm × 0.18 mm column packed with Dionex AS-11 packing. The right ordinate shows the electrodialytically generated gradient (dashed trace) in m*M* NaOH. Peak identities: 1, Fluoride; 2, Formate; 3, Monochloroacetate; 4, Bromate; 5, Chloride; 6, Nitrite; 7, Trifluoroacetate, 8, Dichloroacetate; 9, Bromide; 10, Nitrate; 11, Chlorate; 12, Selenite; 13, Tartrate; 14, Sulfate; 15, Selenate; 16, Phthalate; 17, Phosphate; 18, Arsenate; 19, Citrate. All compounds injected were 50 μ*M* except selenate and sulfate that were 25 μ*M*. Reproduced from Ref. 122 by permission of the American Chemical Society.

Both packed column SFC and open tubular capillary SFC are in use. The open tubular columns usually provide higher efficiencies, longer analysis times, and low sample capacity, while packed columns exhibit lower efficiencies and high sample capacity, analysis time being shorter. The van Deemter equation (eq. 9) is applicable to SFC. The theoretical plot of plate height versus flow rate for SFC yields a curve with broader minima, shifted toward the higher flow rates, compared to LC. In LC, the typical operating flow velocities are usually well above the optimum values, otherwise, analysis time is too long. In SFC, the operating flow velocities closely match the theoretical optimum values and along with the much larger D_m value, this permits higher efficiencies than in LC. For similar reasons, for comparable separations, SFC usually provides shorter analysis times than LC with efficiencies and analysis times approaching those of GC.

7.1. Instrumentation. Figure 8 depicts the scheme of a typical instrument for capillary SFC. It resembles the instrumentation for GC. The chromato-

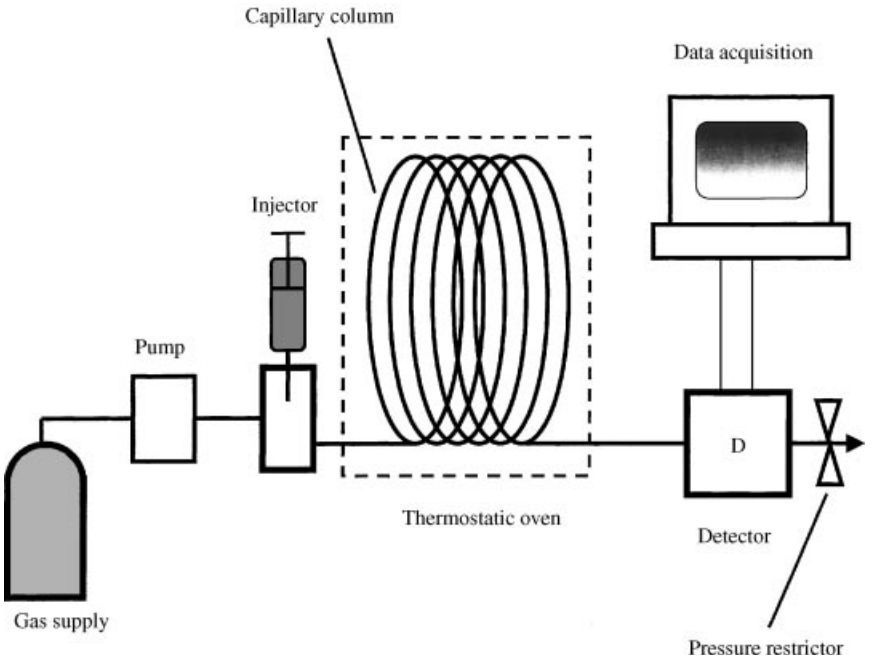


Fig. 8. Schematic diagram of a typical SFC.

graph consists of a gas tank (usually containing CO_2, with or without added modifiers, as the mobile phase), a syringe pump, an injector, a column placed in the thermostatic oven, a pressure restrictor and a detector. The instrumentation for capillary SFC differs slightly from the packed column SFC due to the different pressures generated in the two systems.

7.2. Mobile Phase. As in LC, the mobile phase affects the retention of the analytes. By changing the composition of the mobile phase the selectivity factor, α, can be changed and the selectivity of the separation altered. Carbon dioxide is the most popular and widely used carrier fluid for SFC due to its favorable critical temperature (T_c) and critical pressure (P_c) values, low cost, and low toxicity. Another advantage of CO_2 as a mobile phase is that it does not elicit a response in typical GC detectors such as the FID. Thus, SFC with CO_2 as a mobile phase can be used together with a FID detector, providing sensitive detection for most organic compounds. However, the polarity of CO_2 is quite low, close to that of pentane or hexane, and it does not dissolve more polar compounds. Binary or ternary mixtures of CO_2 and polar additives, such as methanol, water, formic acid or tetrahydrofuran can be used to enhance the applicability of CO_2 -containing supercritical mobile phases to the analysis of polar compounds. Other solvents used for SFC are listed elsewhere (see Table 1 in SUPERCRITICAL FLUIDS).

7.3. Injectors. The type of injector and the size of the injected sample depend primarily on the columns used. For capillary columns, with inner diameter of several tens of micrometers, the sample volume should be sufficiently small so that the column overloading is minimized. Sample splitting techniques described in the GC section are widely used.

7.4. Detectors. SFC benefits from being an intermediate methodology between GC and LC, typically detection can be accomplished by either GC or LC -detectors. The GC detectors, such as the FID, TID, FPD, or ECD can be used with simple supercritical fluids and capillary columns. On the other hand, if analyte contain chromophores or is electroactive, uv–vis absorption or electrochemical detectors for LC can be used, with the modification that the detection cell can withstand high pressure and is placed before the pressure restrictor. In many hyphenated techniques, such as SFC–MS or SFC–FTIR, the two halves are more easily coupled than their LC counterparts (147).

7.5. Columns. The use of packed or capillary columns in SFC is dictated by the needs of the particular analysis. Capillary columns are usually made of fused silica, having an inner diameter from 25 to 100 μm and the length of 1–35 m. The column temperature is maintained using a GC type oven, at temperatures usually not exceeding 200°C. The temperature is not the only parameter, which needs to be kept constant or precisely controlled. Pressure on the column dictates the density of the mobile phase and thus its retention power or retention factor, k', as a consequence of the increase of density of the supercritical fluid with increase of the pressure. An increase in pressure generates greater elution strength of the mobile phase and decreases the analyte retention. This effect can be compared to the temperature effect in GC separations or effect of polarity change of the mobile phase in LC. The pressure can be programmed to increase linearly or asymptotically thus improving the analysis time and peak shapes.

7.6. Stationary Phases. Stationary phases used for open tubular SFC are those routinely used in GC (see Table 1), which are further cross-linked to increase their stability. Polysiloxanes with various percentages of more polar groups are used to coat the inner wall of the fused silica capillaries. Thickness of the coating is usually between 0.1 and 3 μm.

7.7. Applications. Supercritical fluid chromatography (often following supercritical fluid extraction, SFE) has been used, eg, for measuring caffeine in coffee or nicotine in tobacco. SFC has been used for the analysis of fats, oils and food related samples, natural products such as terpenoids or lipids, low molecular weight polymers, fossil fuels and synthetic lubricants, thermally labile large molecules, including biomolecules and synthetic polymers. Figure 9 depicts a few selected SFC applications. Several reviews are available addressing recent instrumental developments and application areas of SFC (148–154). The interesting properties of the mobile phase makes SFC a very attractive technique in principle; however, thus far SFC has not enjoyed the degree of commercial success as originally hoped for.

8. Capillary Electrophoresis

Capillary electrophoresis differs in several important ways from the three chromatographic methods described above. The mode of injection, the mode of sample transport, and the mechanism of analyte separation are all different. CE evolved from the planar electrophoretic methods (see ELECTROSEPARATIONS, ELECTROPHORESIS) performed on paper or a thin gel. First electrophoresis conducted in a capillary was reported by Hjerten (155). Later Virtanen (156) used 0.2–0.5-mm bore

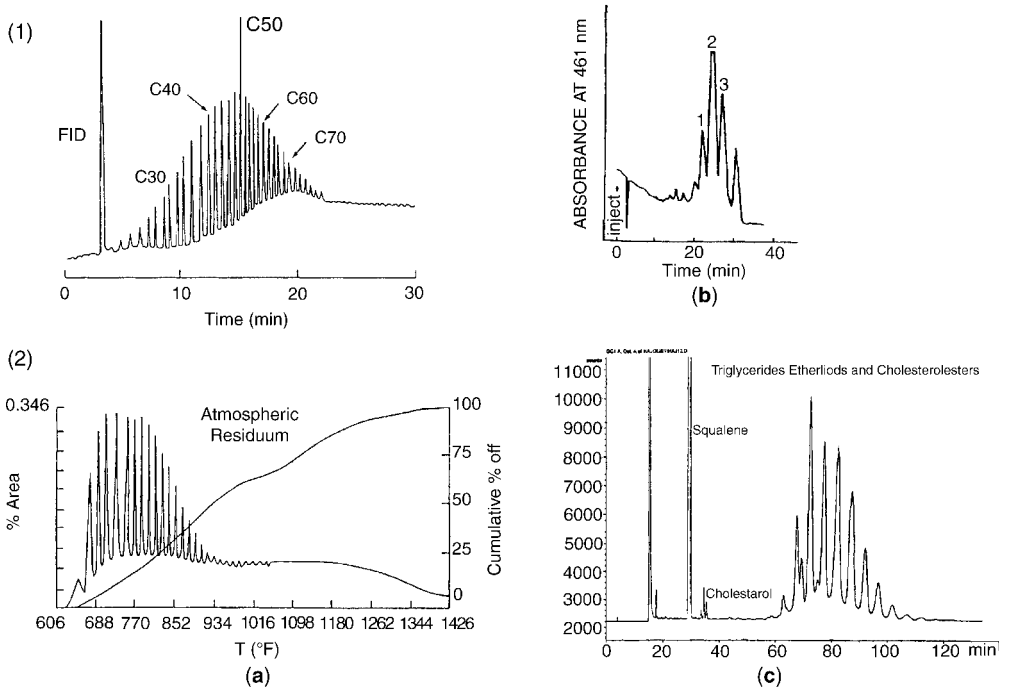


Fig. 9. Selected supercritical fluid chromatograms. (**a**) (1) Chromatogram of polyethylene wax PE740. Conditions: capillary column 10 m × 0.05 mm, statinary phase 0.2 μm film of 5% diphenyl–95% dimethyl polysiloxane. Mobile phase:CO_2 at 100°C, pressure from 2000 to 5000 psi in 20 min, 5500 psi 10 min. (2) Atmospheric residuum of crude oil obtained by removing low boiling fractions, same conditions as (a). Reproduced from H.E. Schwartz and R.G. Brownlee, *U.S. Pat. 4,971,915*. (**b**) Chromatogram of lycopene (2), alpha-(1) and beta-carotene (3) from tomato extract Conditions: capillary column 10 m × 0.05 mm, fused silica SB-phenyl/50, coated with stationary phase 50% phenyl–50% polymethylsiloxane. CO_2 at 45°C with an asymptotic density rise program with a 1/2 rise time constant of 30 min, initial density 0.66 g/m*L*. Reproduced from H. H. Schmitz, W. E. Artz, C. L. Poor, J. M. Dietz, J. W. Erdman, *J. Chromatogr.* **479**, 261 (1989); by permission of Elsevier Science. (**c**) Chromatogram of untreated shark (*Centroscymnus coelolepsis*) liver oil. Conditions: capillary column 25 m × 0.1 mm, fused silica DB-5, film thickness 0.1 μm. Mobile phase: CO_2 at 170°C. Reproduced from C. Borch-Jensen, J. Mollerup, *Chromatographia* **42**, 252 (1996); by permission of Vieweg Publishing.

glass tubes for separation of alkali metals. The use of fused silica capillaries by Mikkers and co-workers(157) and Jorgenson and Lukacs(158) in the early 1980s was a real breakthrough and in particular the latter authors showed the extremely high efficiencies attainable by CE. Since then the use of CE as a research tool has grown rapidly.

In CE, a fused silica capillary is filled with a buffered background electrolyte (BGE) solution, which serves as the separation medium. Unlike the chromatographic methods, in CE, there are no pumps or pressurized fluid sources. The two ends of the capillary are immersed in the reservoirs containing the BGE. Two noble metal electrodes, usually Pt, are placed in each vial as well. The movement of the electrolyte and sample through the capillary is accomplished by

applying a high voltage (HV) (typ. 10–30 kV across a 40–75 cm long, 25–100-μm bore capillary) across the electrodes. Typical electrolytes used are 1–10 m*M* phosphate or borate; with the resulting current being in microamperes.

Capillary electrophoresis is characterized by the very high efficiency due to the plug-like flow profile of the electrophoretic flow (see Fig. 2). Low to tens of nanoliters of sample are typically injected (contents of a single cell have been analyzed, however, in most cases actual sample needed is several μl) and the total consumption of BGE per run is a few microliters (μl) (although in practice a greater amount of BGE is needed to prevent gross alteration of the BGE composition by electrolysis during the run). There are several modes of CE operation and these, together with the instrumental aspects, are discussed below.

8.1. Basic Instrumentation in CE. A simple CE apparatus is depicted in Figure 10. It consists of a HV supply capable of providing a potential difference in the range of ±30 kV, two platinum electrodes, placed in the two electrolyte vials, a fused silica capillary, and a detector.

8.2. Injection Techniques in CE. Although valve-based injection has been used in CE, this is not common. The two most used injection techniques are the hydrodynamic (HD) and the electrokinetic (EK) injection methods. While both differ from injection methods in chromatography systems, both can be fully automated.

Hydrodynamic Injection. In the HD injection mode, the electrolyte vial at the head of the capillary (the tail end is the destination or the detector end) is briefly replaced by the sample vial, and a small portion of the sample is forced into the capillary by applying pressure (or vacuum at the tail end), or by gravity

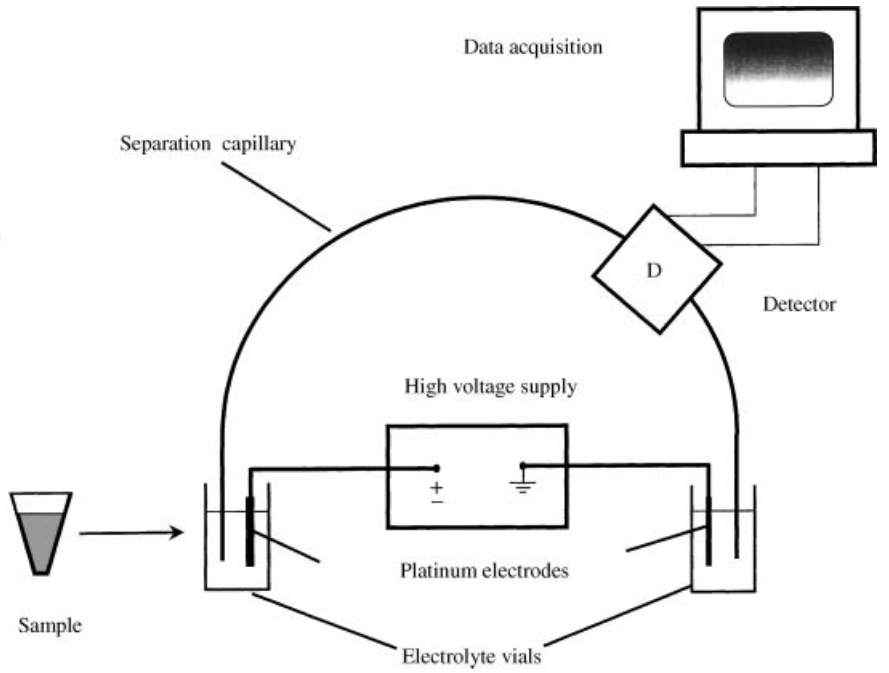


Fig. 10. Schematic diagram of a typical capillary electrophoresis system.

(raise the sample vial with respect to the destination vial). Prior to separation, the sample vial is again exchanged for a BGE vial.

The hydrodynamic injection mode is preferred in CE since the peak areas of the analytes are linearly related to the concentration over a wide concentration range and the injected amount is relatively independent of the sample matrix composition. However, any difference in the viscosity of the sample and the standard will cause a quantitation error. If viscosity difference is significant, the standard addition method (see CHEMOMETRICS) is preferred. In the standard addition method, a small amount of a standard solution of known concentration is added directly to the sample matrix and measurements are made before and after such addition. Most commercial instruments permit good quantitative precision ($\leq$3% RSD) in the HD injection mode.

Electrokinetic Injection. In the EK injection mode, HV of appropriate polarity is applied across the capillary placed in the sample vial and ions from the sample migrate into the capillary. The amount of each analyte introduced is a function of both the mobility of the analyte and the EOF in the capillary.

The EK injection mode is instrumentally simple and allows preconcentration by stacking the analyte ions into a narrow zone (159–161): however, the method has several problems as well. The amount of a particular analyte ion injected in the EK mode increases with increasing electrophoretic mobility, μ_i, therefore ions of larger mobility are injected to a greater extent. This phenomenon, "mobility-induced bias", can in principle be corrected for by correcting peak areas with mobility information that are readily derived from the migration times. A second, more insidious problem arises from differing conductivities between samples and standards. The amount of an analyte introduced is related to the electric field experienced by the sample. In the typical EK injection mode, the capillary is filled with BGE and represents a fixed resistance. If the samples and standards have very different conductivities, the effective electric field experienced by the sample and standard will differ. Under otherwise identical conditions, even when a given analyte is present in equal concentration in the sample and the standard, the actual amount of the analyte introduced in the two cases will differ. Several methods have been proposed to overcome these shortcomings—none are universally applicable.

Flow Injection Approaches. A flow-injection (FI) based sample introduction approach permits repetitive sample injection in CE in a simple manner. The sample is injected in a FI system, typically using the BGE as the carrier and flies by the CE system inlet where a portion of it is injected into the capillary either by hydrodynamic or more typically, electrokinetic, means. The separation of the previously injected sample is not physically disrupted during the subsequent EK injection. Air sensitive or hazardous samples can be injected and the system is easily automated. All limitations of HD and EK injections remain, nevertheless, and sample and BGE consumption are much higher (162,163).

8.3. Detection in CE. As previously stated, detection methods and detectors in CE are essentially identical to those used in capillary LC with one caveat that modifications may need to be made for the high voltage present in the capillaries. In the following, only the special issues unique to adaptation of the detection method in CE are discussed.

Absorbance detection is the most commonly used detection mode in CE. For nonabsorbing analytes, an absorbing electrolyte in the BGE is commonly used. In this indirect detection mode, similar to conductivity detection described for single column IC, analyte elution is detected as negative peaks. Direct absorbance detection is, of course, preferred whenever possible and a large variety of organic substances are generally detected this way using optically transparent phosphate or borate based BGEs. In CE, the detector is always placed near the grounded end of the capillary, to avoid high electric field induced problems.

Conductivity detection is completely analogous to conductivity detection in single column IC, the analyte signal arises from the difference in equivalent conductance between the analyte and the BGE ion of the same charge type. Suppressed conductivity detection has also been demonstrated, in this case, a tubular membrane suppressor is externally grounded electrically. When conductivity measurements must be made within the electric field, either high frequency excitation is used to permit contactless measurement; else, the measurement circuitry is galvanically isolated, eg, by using a transformer.

The grounding and stray current issues also appear in amperometric detection. Detection is normally just at the column outlet. For larger bore capillaries, the current flowing through the capillary itself makes it problematic to detect the much smaller analyte induced current at the working probe electrode. Exact positioning of the working electrode at the outlet of the capillary affects results and reproducible positioning is not trivial. The current flowing through the capillary itself due to the applied HV is inversely proportional to the square of the capillary bore and as such decreases dramatically with decreasing capillary bore. This in turn makes amperometric detection much less of a problem when conducted with smaller bore (≤ 25 μm) capillaries.

8.4. Counter versus Co-current Electrophoretic versus Electroosmotic Flow. Manipulation of the EOF can be used to achieve additional resolution of closely migrating peaks. CZE can be performed in co- and counter electroosmotic flow modes. In the first, the analytes electrophoretic migration is in the same direction as the electroosmotic flow and the overall velocity is thus additive. Separation times under 5 min are not unusual in this mode. This is often used for analysis of small ions and molecules. In the counterelectroosmotic flow mode, the analytes move electrophoretically opposite to the EOF, but are eventually swept to the detector, because in magnitude the EOF is greater and the vector sum of the EOF and the electrophoretic velocity still has the same direction as the EOF. In a system consisting of bare silica capillary at a neutral to alkaline pH, the EOF is from the anode to the cathode, cationic analytes are moving coelectroosmotically and the anions are moving counterelectroosmotically. Counterelectroosmotic electrophoresis is often used for difficult to resolve molecules allowing them greater separation time. In the extreme case, where two analytes move with almost the same electrophoretic mobility, conditions may be adjusted to attain an EOF that is just greater than the higher mobility analyte (in the opposite direction). Under these conditions, even isotopic separation has been attained.

8.5. Factors Affecting the Separation Selectivity in CE. Separation selectivity in CE is mainly achieved by modification of the BGE. Several factors

as described below affect separation efficiency and migration time and are discussed below.

Electrolyte Composition. The BGE composition, especially the BGE ion that is of the same charge type as the analytes, is of vital importance. Analyte peaks in CE be fronting, tailing, or highly symmetrical, based on if the mobility of the analyte is less, greater, or equal relative to the BGE coion. The separation of closely migrating analytes will be best if the mobility of the BGE coion is close to the analyte mobilities; thus a proper selection of the electrolyte coion is important.

pH. Many analytes separated by CE have weak acid–base character and the extent of their dissociation depends on the pH. The effective electrophoretic mobility of an analyte is the product of the mobility of the fully dissociated ion times the fractional extent of ionization. Indeed, the variation of migration time with pH can be used to measure pKa. The choice of pH also affects the EOF. By proper selection of the pH, the separation can be optimized.

Complexation. Complexation equilibria are also commonly used in CE to tailor the separation. In this case, a complexing agent is added to the BGE. The complexing agent interacts with the analytes based on their association constants. A separation of lanthanides, eg, is commonly achieved with a BGE containing α-hydroxyisobutyric acid. Addition of inorganic ions, such as Zn(II) or Cu(II) can be used to tailor the separation of peptides. Guest–host complexation, especially with cyclodextrins, is also frequently used in CE, especially in the separation of chiral isomers, as cyclodextrins are chiral selectors. A variety of compounds, notably amino acids and drugs have been separated (see CHIRAL SEPARATIONS).

Other ways, such as addition of organic solvents, change in the ionic strength of the electrolyte coating the capillary to modify EOF etc, are all used for tuning a separation.

8.6. Separation Modes in CE. Related Techniques. CE is typically carried out in (and what has been described above corresponds to) the zone injection and separation mode, which is therefore often referred to as capillary zone electrophoresis (CZE). There are other modes of capillary electrophoretic separations such as isotachophoresis where the capillary is filled with a high mobility electrolyte (leading electrolyte), which also fills the anode reservoir. The sample is then introduced. A low mobility electrolyte (trailing electrolyte) is now put in; the cathode reservoir contains this electrolyte. As HV is applied, the leading electrolyte moves rapidly toward the anode. The highest mobility component in the sample is drawn toward the leading electrolyte to fill in the conductivity gap left by the quickly migrating leading electrolyte. Progressively, each ion in the sample follows at lower conductivity, just behind an ion of higher mobility and just ahead of an ion of lower mobility. Finally, the "trailing" electrolyte, of lowest mobility, terminates the separation. In this separation mode, the length of each separated band is proportional to their concentration. In capillary gel electrophoresis (CGE), widely used in DNA sequencing, the capillary is filled with a gel (typically polyacrylamide, often referred to as a sieving gel). For this and other related modes, see ELECTROSEPARATIONS, ELECTROPHORESIS.

8.7. Electrophoresis in Sieving Media. DNA Analysis. Electrophoresis in sieving media provides a method for separation of high molecular weight

molecules, such as proteins, nucleic acids, and their fragments based on their size (see also ELECTROSEPARATIONS, ELECTROPHORESIS). While proteins can usually be separated based on the differences in charge and size using CE, nucleic acids, synthetic nucleotides, DNA restriction fragments and higher DNA strains posses very similar charge densities and their separation by CE is difficult. Two modes are in use: capillary gel electrophoresis (CGE) and dynamic size-sieving CE.

In CGE, mostly polyacrylamide gels (PAG) are used. The size of the pores and cross-linking can be varied during the polymerization phase by adjusting the concentration of monomer and cross-linking agent. The PAG polymer is chemically bonded to the capillary wall.

In dynamic size-sieving CE, the capillary is filled with a low viscosity polymer solution such as linear polyacrylamide, agarose, hydroxyethylcellulose, or hydroxypropylmethylcellulose. In these sieving matrices, pores are created by physical interactions and composition can be varied over a large range. The capillary content can be replaced between the runs, thus enhancing the reproducibility and decreasing the sample carry-over between analyses.

In CGE, the separation of the molecules is based on the differences in both charge and size. To separate the molecules based only on their size, a charged ligand, such as sodium dodecyl sulfate (SDS) can be added both to the sample and the separation electrolyte. SDS binds strongly to protein molecules, giving it a constant net charge per mass unit, and the electrophoretic mobility of the molecule under sieving conditions becomes dependent only on its size or molecular weight.

Electrophoresis in sieving media is an indispensable tool for DNA sequencing and the analysis of nucleic acids, their fragments, and synthetic oligonucleotides.

DNA molecules are unique to each living organism and the knowledge of the nucleotide sequence provides valuable information for genetics, clinical biochemistry and molecular biology. In 1990, the Human Genome Project begun (164). The goals of this project were to identify ~30,000 genes in human DNA and determine the sequence of 3 billion base pairs that make up the human DNA. Knowledge about the effects of DNA variations among individuals can lead to revolutionary new ways to diagnose, treat, and someday prevent the thousands of disorders that affect mankind. Fragments almost a 1000 base long, which differ by a single base, have to be resolved for high throughput analysis. It is the high resolving power of high-speed CE that made this possible. Multiplexing of capillaries and separations on a microchip increase the throughput. For instance, multicapillary array electrophoresis systems have been commercially developed and are routinely used for DNA sequencing. Dye-tagged DNA fragments are introduced into the capillary array. Individual base termini are tagged with differently fluorescent dyes, each with their characteristic fluorescence wavelengths. As the separated fragments near the exit, they are irradiated with a spatially scanning laser. Fluorescence in the four colors is individually measured with dedicated photomultiplier tubes or a multichannel CCD detector. The working draft on the sequencing and analysis of the human genome was published in February 2001 (165).

8.8. Micellar Electrokinetic Chromatography. CE methods discussed thus far can only separate charged analytes. In MEKC, first described by Terabe

and co-workers (166), the BGE consists of a surfactant above its critical micelle concentration (CMC, see SURFACTANTS). Surfactants form micelles, spherical aggregates of several molecules of surfactants, above their CMC. In a micelle, the hydrophobic tails (usually alkyl chains) face the interior of the sphere, while the ionic groups extend into the surrounding media. Most MEKC applications use typical anionic surfactants like SDS or cationic surfactants like CTAB. The charged micelles move at a rate different from that of the bulk liquid. Neutral analytes can remain in the bulk liquid or partition to the micelle, the "pseudostationary phase". Differences in this partition behavior for different analytes lead to different effective velocities.

Thus, the differential partitioning of the nonpolar, uncharged, analytes between the BGE and the hydrophobic micellar interior is the principle of separation by MEKC. This is analogous to reversed-phase LC, where nonpolar analytes are differentially retained on the nonpolar stationary phase. Typically, the electrophoretic velocity of the micelles is opposite in direction and lower in magnitude compared to the EOF. The total elution window ranges from that of an unretained analyte [ethanol, dimethyl sulfoxide(DMSO)], t_{eo}, and a fully retained analyte that elute at the same time as the micelles, t_{MC}, (usually marked by a hydrophobic dye, wholly retained within the micelle). All other neutral analytes will elute within t_0 to t_{MC}. The capacity factor of an analyte eluting at t_i can thus be defined as:

$$\overline{k} = \frac{t_i - t_{eo}}{t_{eo}\left(1 - \frac{t_i}{t_{MC}}\right)} \tag{20}$$

The correspondence to the definition of retention factor in liquid chromatography (eq. 3) is evident. When t_{MC} approaches infinite value, eg, the pseudostationary phase is really stationary, the equation becomes equivalent to the equation 3 for liquid chromatography.

The presence of ionic species brings another dimension to the MEKC separations. Since these species possess their own electrophoretic mobilities and their retention times cover much wider range than that of the neutrals, the simultaneous separation of neutral, anionic and even cationic analytes is possible in MEKC. The type of surfactant, its concentration, temperature, electrolyte pH, and the presence of other additives significantly influence the abilities of the MEKC method to separate target analytes. For example, the use of bile salts, such as sodium cholate or sodium deoxycholate instead of SDS, extends the applicability to the more hydrophobic compounds. The same effect can also be achieved by buffer additives such as urea. The addition of organic solvents alters the retention mechanism by altering the polarity of the aqueous phase and generally increases the span of the separation window. The BGE pH influences the EOF and changes the extent of ionization for weak acids and bases; this affects their effective electrophoretic mobility and therefore alters retention.

MEKC extends the separation range of CE-like methods to uncharged molecules. MEKC can be applied for the analysis of variety of analytes such as amino acids and polypeptides, DNA adducts, flavonoids and steroids, drugs and environmental samples (167–175); a selected example is shown in Figure 11.

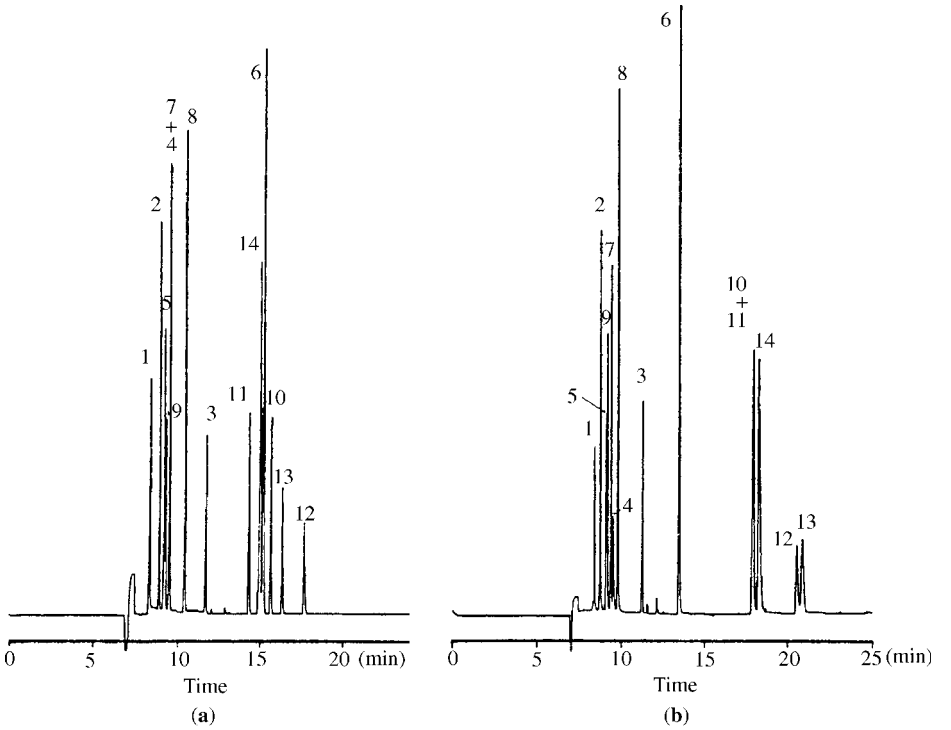


Fig. 11. Selected micellar electrokinetic chromatogram. Separation of 14 active ingredients from a Cold medicine. Conditions: 20 m*M* phosphate-borate, pH 9.0 with (**a**) 100 m*M* sodium cholate, (**b**) 50 m*M* sodium deoxycholate, 20-kV separation voltage, uv detection at 210 nm. Peaks: (*1*) caffeine, (*2*) acetaminophen, (*3*) sulphyrin, (*4*) trimetoquinol, (*5*) guaifenesin, (*6*) naproxen, (*7*) ethenzamide, (*8*) phenacetin, (*9*) isopropylantipyrine, (*10*) noscapine, (*11*) chlorpheniramine, (*12*) tipepidine, (*13*) dibucaine, (*14*) triprolidine. Reproduced from H. Nishi, T. Fukuyama, M. Matsuo and S. Terabe, *J. Chromatogr.* **498**, 313 (1990); by permission of Elsevier Science.

8.9. Capillary Electrochromatography. Capillary electrochromatography combines the high selectivity and versatility of the stationary phases from LC with the high efficiency of electrically driven flow in CE. The column is essentially the same as a packed LC column (although some aficionados believe that CEC columns need to be packed electrokinetically). Since no pumps are involved for the mobile phase delivery, the column can be packed with the particles as small as 0.5 μm, thus providing very high column efficiencies. The eluent is driven through the column electrokinetically, by applying voltage. Extraordinarily high separation efficiencies ($\geq$1 million plates) have been reported.

Will CEC take up some of the applications of LC? Or will the fate of CEC be more like SFC, which is yet to fulfill its originally envisioned market potential? The technique is still not fully mature and reproducibility is often limited. The EOF in CEC, the flow source, depends on several parameters, such as characteristics of the stationary phase and capillary wall, pH, electrolyte composition, ionic strength, temperature, etc. Bubble formation is another vexing problem to which a multitude of solutions have been devised. CEC in monolithic column

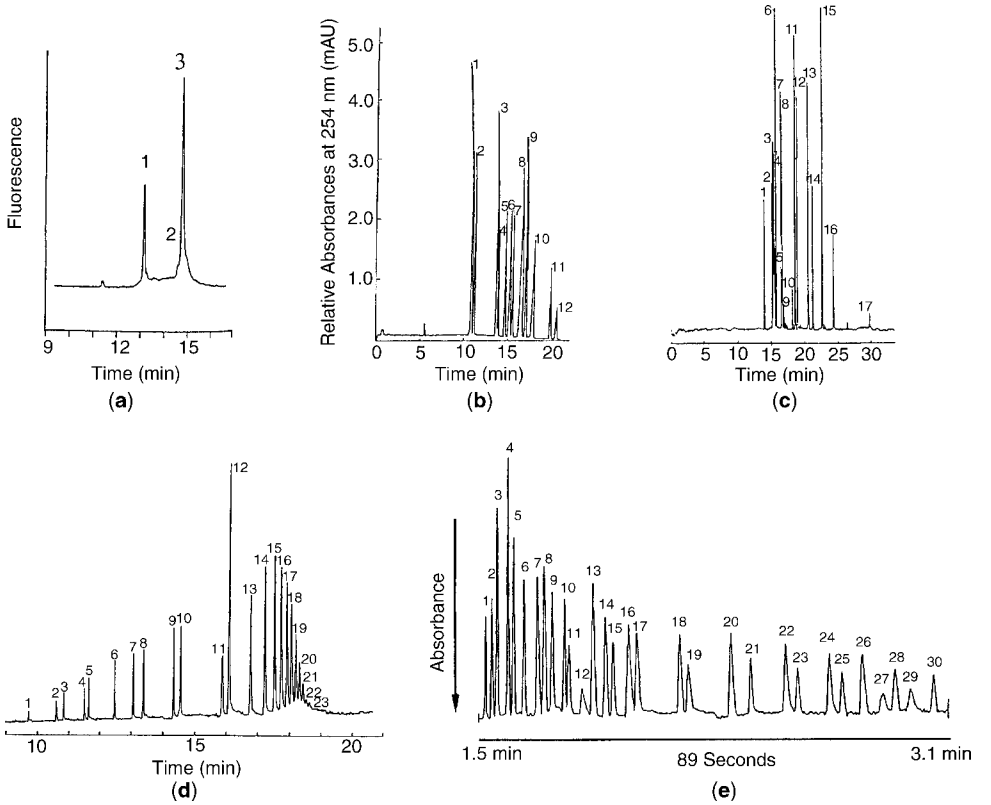


Fig. 12. Selected electropherograms showing some application areas of capillary electrophoresis. (**a**) Electropherogram of major proteins in a single erythrocyte. Conditions: 50 m*M* $Na_2B_4O_7$ electrolyte at pH 9.1 Peaks: (*1*) carbonic anhydrase, (*2*) methemoglobin, (*3*) hemoglobin. Reproduced from T. T. Lee, E. S. Yeung, *Anal. Chem.* **64**, 3045 (1992); by permission of the American Chemical Society. (**b**) Electropherogram of twelve 5'-ribonucleotides. Conditions: 30-m*M* sodium carbonate/bicarbonate electrolyte, pH 9.5, separation voltage 18 kV. Peaks: (*1*) AMP, (*2*) CMP, (*3*) ADP, (*4*) GMP, (*5*) CDP, (*6*) ATP, (*7*) UMP, (*8*) CTP, (*9*) GDP, (*10*) GTP, (*11*) UDP, (*12*) UTP. Reproduced from S. E. Geldart, P. R. Brown, *J. Chromatogr. A* **828**, 317 (1998); by permission of Elsevier Science. (**c**) Electropherogram of 3-(4-carboxybenzoyl)-2quinolinecarboxyaldehyde tagged aminoacids in lysozyme hydrolyzate. Conditions: 50-m*M* 2-[(*N*)-[tris (hydroxymethyl)methyl]amino] ethane-sulfonic acid, 50-m*M* SDS, pH 7.02. Separation voltage 25 kV.Peaks: (*1*) Arg, (*2*) Trp, (*3*) Tyr, (*4*) His, (*5*) Met, (*6*) Ile, (*7*) Gln, (*8*)Asn, (*9*) Thr, (*10*) Phe, (*11*) Leu, (*12*) Val, (*13*) Ser, (*14*) Ala, (*15*) Gly, (*16*) Glu, (*17*) Asp. Reproduced from J. Liu, Y. Z. Hsieh, D. Wiesler and M. Novotny, *Anal. Chem.* **63**, 408 (1991); by permission of the American Chemical Society. (**d**) electropherogram of 1000/base pair DNA ladder on a 3% T, 0.5% C polyacrylamide capillary. Conditions: 100-m*M* TRIS-borate (pH 8.3), 2 m*M* EDTA. Peaks: (*1*) 75, (*2*) 142, (*3*) 154, (*4*) 200, (*5*) 220, (*6*) 298, (*7*) 344, (*8*) 394, (*9*) 506, (*10*) 516, (*11*) 1018, (*12*) 1635, (*13*) 2036, (*14*) 3054, (*15*) 4072, (*16*) 5090, (*17*) 6108, (*18*) 7126, (*19*) 8144, (*20*) 9162, (*21*) 10180, (*22*) 11198, (*23*) 12216 base pairs. Reproduced from D. N. Heiger, S. A. Cohen and B. L. Karger, *J. Chromatogr.* **516**, 33 (1990); by permission of Elsevier Science. (**e**) Electropherogram of 30 inorganic anions. Conditions: 5 m*M* chromate, 0.5 m*M* OFM BT, pH 8. Separation voltage 30 kV. Indirect uv detection at 254 nm. Peaks: (*1*) thiosulfate, (*2*) bromide, (*3*) chloride, (*4*) sulfate, (*5*) nitrite, (*6*) nitrate, (*7*) molybdate, (*8*) azide, (*9*) tungstate, (*10*) monofluorophosphate, (*11*) chlorate, (*12*) citrate, (*13*) fluoride, (*14*) formate, (*15*) phosphate, (*16*) phosphite, (*17*) chlorite, (*18*) galactarate, (*19*) carbonate, (*20*) ethanesulfonate, (*21*) acetate, (*22*) propionate, (*23*) propanesulfonate, (*24*) butyrate, (*25*) butanesulfonate, (*26*) valerate, (*27*) benzoate, (*28*) L-glutarate, (*29*) pentanesulfonate, (*30*) D-gluconate. Reproduced from W. R. Jones and P. Jandik, *J. Chromatogr.* **546**, 445 (1991); by permission of Elsevier Science.

beds is being investigated also as a solution to this problem. The technique is still in its infancy and only time will prove its value. Further reading on CEC is available (176–183).

8.10. CE–MS. Like LC–MS, CE–MS provides identification and structural information (see MASS SPECTROMETRY; ANALYTICAL METHODS, HYPHENATED INSTRUMENTS). Compatibility of the CE BGE to MS, flow rate matching, termination of HV, etc. are important issues in a successful interface.

Sheath-flow, sheathless flow and liquid junction CE–MS interfaces have been developed. The ground (usually cathodic) end of the separation capillary need not to be inserted in an electrolyte vial as long as it is properly biased relative to the HV end. The outlet end of the separation capillary is inserted directly into the interface and the electrical ground of the separation capillary is accomplished either by an externally metallized capillary end, a metallic needle connected to the capillary that functions as the electrospray needle, or via liquid contact with a grounding electrode in the liquid junction interface.

The electrospray ionization mode (ESI) is likely the most popular mode of ionization currently used in CE–MS (and LC–MS). The eluent from the separation capillary is mixed with the hot gas (usually N_2) at the atmospheric pressure. The solvent is evaporated and molecules are ionized prior to entering the MS. FAB–MS, also used in LC–MS, has already been mentioned. Recently matrix assisted laser desorption/ionization (MALDI) has been used off-line for CE separated analytes. Recent reviews on CE–MS provide additional information on instrumentation development and application of CE–MS (184–190).

8.11. Applications. Due to its high resolving power, short analysis times and minute sample consumption, capillary electrophoresis plays an important role in analysis of small samples such as in the life sciences, notably analysis of single cells, human and plant tissues and for monitoring of in vivo processes (191–198). CE can be applied for variety of samples and the range of analytes overlaps application areas of IC and LC. However, it has not significantly displaced either of these for routine quantitative analysis. Despite the advent of MEKC, CE is not competitive in the marketplace with LC or GC for the analysis of neutral analytes. Main application areas of capillary electrophoresis and related techniques are DNA sequencing, analysis of amino acids, bases, nucleosides and nucleotides, proteins and peptides (199–203). High separation efficiency and little need for pretreatment makes it an excellent method for analysis of various enzymatic digests, peptide mapping, analysis of fermentation broths, biological fluids and food samples (204,205).

Another important area of CE is chiral separations, due to the decreased costs of the chiral selectors needed for CE-separation (206–212) (see CHIRAL SEPARATIONS). Further application areas include analysis of drugs, carbohydrates, small organic molecules and inorganic ions (213–226). Figure 12 shows some typical applications of capillary electrophoresis.

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