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A Survey of Separative Techniques

Thomas H. Maugh II

Summary. Separative science has recently undergone numerous advances. This article discusses many developments and trends in liquid, ion, gas, and countercurrent chromatography, field-flow fractionation, and electrophoresis.

The accompanying articles describe six areas of separation science in which major advances have recently occurred. These areas, however, are not the only ones in which progress is taking place. The entire field of separation science is evolving at a surprising rate, considering that separation is the oldest and most basic technique employed in the laboratory. The following sections discuss some of the areas where this evolution—if not revolution—is occurring most rapidly. These examples are meant to be representative rather than exhaustive.

Liquid Chromatography

High-performance liquid chromatography (HPLC), born only 20 years ago as high-pressure liquid chromatography (LC) is, with gas chromatography, one of the two most commonly used separative techniques today. Despite the maturity implied by such wide usage, it is still a rapidly evolving area. Two important current trends are the use of HPLC for separation of macromolecules, particularly those of biological origin, and the use of supercritical fluids as eluants. These are reviewed elsewhere in this issue. An equally important trend is a reduction in column size and a concomitant increase in analytical speed.

The first steps in this direction involved the use of short columns, perhaps 3 to 5 cm long. These columns were developed, in part, as a result of the use of guard columns—short, inexpensive, disposable columns fitted between the injector and the analytical column to trap extraneous materials that would foul the expensive analytical column. Investigators observed that these short columns could have as many as 5000 theoretical plates, an efficiency that is adequate for most uses. Short columns reduce analysis times and cut down on the use of expensive high-purity solvents, albeit at the cost of selectivity. Some of that selectivity can be restored, however, by using smaller particle sizes for packing the column, and 3-μm particles are becoming more common. Analyses that took 10 minutes 5 years ago can now often be done in 1 minute or less.

Solvent use can be further reduced and sensitivity increased by using columns with smaller bores. A standard HPLC column has a diameter of 4.6 mm. New columns are now available with diameters of 2, 1, or even 0.5 mm. A 2-mm column cuts solvent use by 80 percent, while microbore columns, those with diameters of 1 mm or less, cut it by at least 95 percent. The microbore columns are no more selective than conventional columns of equal length, and analysis times are equivalent. They can, however, be fabricated at reasonable costs in much longer lengths that produce very high efficiency. Their small size also allows use of exotic, expensive packing materials, such as diamond dust or finely divided noble metals, and even more expensive solvents: deuterated solvents, for example, might be used when it is necessary to analyze the collected sample by nuclear magnetic resonance. Some investigators are using columns with diameters as small as 50 μm, and it seems likely that these will eventually be commercially available. These ultramicrobore columns can, in some cases,

The author is a member of the Research News staff of *Science*.

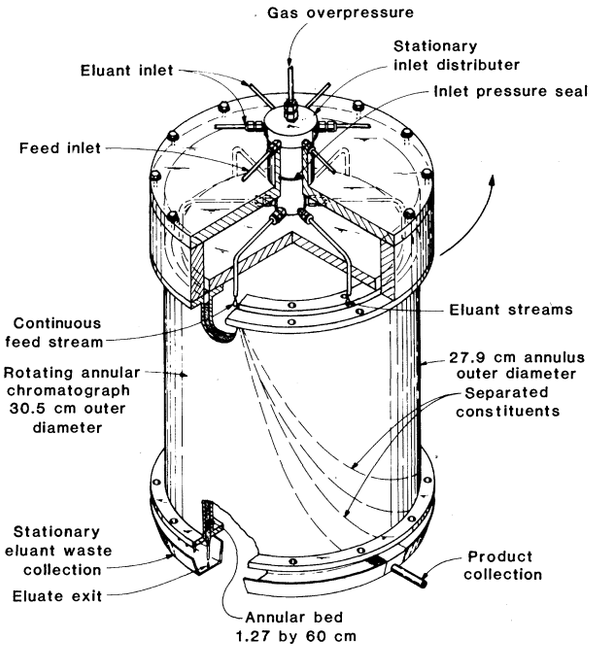

Fig. 1. Continuous annual chromatograph, developed at Oak Ridge National Laboratory. [Courtesy of C. D. Scott]

provide exceptional concentration of the sample, and eluted peaks can be fed directly into a mass spectrometer or other instrument with low interference from the solvent.

Microcolumn HPLC presents some instrumental problems. In particular, it requires accurate pumps and small flow cells for the detector so that column performance is not degraded. In general, this means that a dedicated chromatograph must be used with microcolumns, although there are some commercial instruments that can handle a variety of bore sizes.

Detection is a more substantial problem. New designs of flow cells and electronic refinements have increased detection sensitivity by an order of magnitude during the past 2 to 3 years, but detection remains the weakest link in LC. One recent response to the need for faster detection systems is the use of diode array spectrometers that allow simultaneous analysis of the eluant at many different wavelengths. Another new approach is electrochemical detection, such as oxidation of susceptible species, to identify small samples. Work on other detection techniques may prove to be one of the most fruitful areas of research.

Pump technology is also improving. Newer pumps are nearly pulse-free, improving separation and reliability. One microprocessor-controlled pump, furthermore, can now often replace the two or three pumps that were previously required for producing gradients or other unusual elution patterns. The microprocessor has also had a major role in automating the optimization of separations. Finding the correct solvent combinations and gradients for separation of unknown mixtures was previously a trial and error process that could require days or weeks. New software first introduced in 1982 by the DuPont Company and now widely copied allows the microprocessor to "learn" from each of a series of trial elutions and thereby optimize the separation procedure literally overnight. HPLC has progressed to the point, says one investigator, where formerly difficult problems are now easy and the impossible ones merely take a little longer.

Flash chromatography. Another technique for rapid separations is termed flash chromatography. It was first reported in 1978 by W. C. Still of Columbia University, but has only recently gained acceptance. Flash chromatography is best described as a hybrid of open-column and medium-pressure LC. The key to its implementation is the use of relatively large (40-μm) sorbent particles with a narrow distribution of sizes. The sorbent is packed in a conventional thick-walled glass column with a stopcock. After the sample is applied to the top of the column, a special adaptor is fitted that allows solvent flow and a pressurization of 2 to 5 psi to give a velocity of about 5 cm per minute. In this fashion, 10 to 25 mg of sample can be purified in just a few minutes much more cheaply than is possible with preparative HPLC. A 13-cm column typically has sufficient efficiency to separate, say, an intermediate in a chemical synthesis. The technique has been used in organic syntheses, in the pharmaceutical industry, and for small biomolecules.

Preparative liquid chromatography. While many investigators have been working with smaller columns, a few have been moving in the opposite direction. The sharp separations characteristic of analytical HPLC also make it useful for producing larger quantities of pure materials. At least four companies now manufacture preparative liquid chromatographs that are capable of producing as much as 10 g of sample at a time. Nonetheless, this approach has its limitations. The large columns are expensive to purchase, and they require substantial quantities of ultrapure solvents. They are thus most suitable for products with a high intrinsic value, such as polynucleotides, polypeptides, and the products of genetic engineering.

A more substantial problem develops at still larger sizes. There is a sharply increased tendency for channeling of the eluant so that it bypasses portions of the sorbent bed. Perhaps the best approach to this problem has been developed by Waters Associates. After the column has been filled, a constant radial compression is applied so that channels cannot form. Waters Associates has developed columns 13 to 22 cm in diameter and 53 cm long. These have flow rates of 3 to 20 liters per minute and can separate 1 to 10 kg of sample per hour. Two or more columns can be connected in series if greater separative power is required.

Typically, these columns use larger sorbent particles than analytical columns. This eases engineering problems and reduces the pressure required, making it possible to use less sophisticated, more reliable pumps. Extensive solvent recycling is necessary to reduce operating costs, but it may be possible to use less pure solvents. One potential benefit of process-scale chromatography is that the large quantities of sorbent required will make it economically feasible to produce custom-tailored resins that could improve certain types of separations.

Continuous chromatography. Even large-bore liquid chromatographs require batch processing, a procedure that is generally not desirable from either an economic or an engineering standpoint. This problem can be overcome with continuous chromatography, which is now used to a limited extent in industry. In particular, UOP, Inc., has developed a series of processes in which the adsorbent bed moves in a direction opposite to that of the solvent flow. In this manner, samples can be continuously injected at one point and products continuously removed at other, fixed points. Such systems are used, for example, for recover-

ing *n*-paraffins from light naphthas, *p*-xylene from other C_8 hydrocarbons, and *n*-olefins from mixtures of *n*-olefins and *n*-paraffins.

A somewhat different and potentially more useful approach, known as continuous annular chromatography, is being developed by a team at Oak Ridge National Laboratory. The sorbent, typically an ion-exchange resin, is contained in a ring or annulus that rotates slowly (Fig. 1). The feed mixture is introduced through one or more fixed feed points, as is the eluant. The pressurized downward flow combines with rotation to force each component of the sample into a readily calculated helical path that culminates at a fixed product withdrawal location. The investigators have separated a synthetic mixture of copper, nickel, and cobalt complexes that models the carbonate leach liquor of the Caron process for recovering nickel and cobalt from laterite ores.

They have also developed a continuous process for separating zirconium and hafnium, metals used in the nuclear industry. Zirconium ore contains about 3 percent hafnium, but the two are difficult to separate; current technology uses a complex series of liquid extractions. The Oak Ridge group has used their prototype annular chromatograph to produce zirconium containing less than 0.01 percent hafnium and hafnium containing less than 1 percent zirconium. They estimate that a continuous annular chromatograph 450 mm in diameter and 1 m long could produce 2000 kg of zirconium per year at a competitive cost. They are now applying the technique to the separation of other materials, including biomolecules.

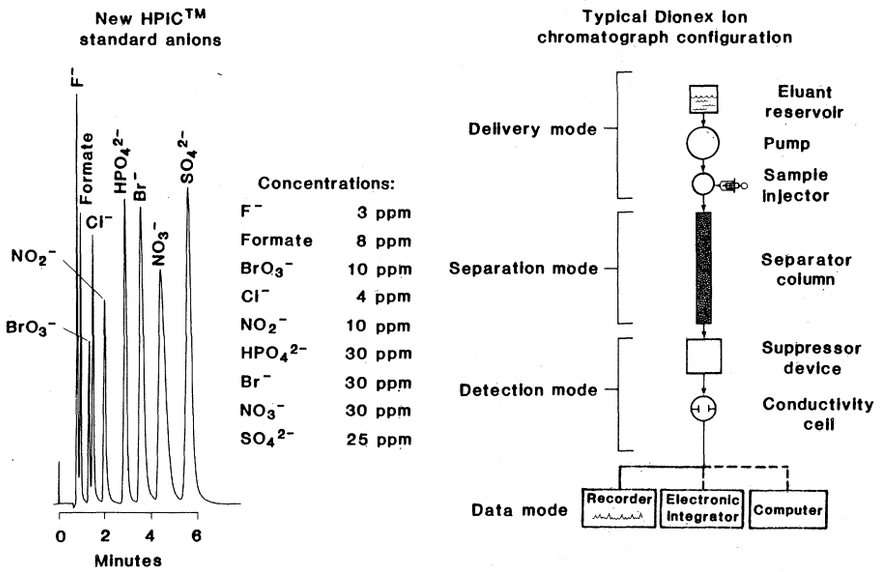

Fig. 2. Schematic diagram of an ion chromatograph (right) and illustration of the separation of anions by ion chromatography. [Courtesy of Dionex Corporation]

Ion Chromatography

One of the fastest growing separative techniques is ion chromatography (IC). Since it was first reported in 1975 by Hamish Small, Timothy S. Stevens, and William C. Bauman of Dow Chemical, IC has become one of the most commonly used techniques for analyzing inorganic ions. New refinements have greatly expanded the number of ions, particularly organic ions, that can be analyzed by IC.

Ion chromatography is actually a variant of conventional ion-exchange HPLC. It was developed not because inorganic ions are particularly difficult to separate by conventional LC but because it is difficult to detect them as they leave the column, since most do not contain chromophores. Small reasoned that they could be detected conductimetrically if their conductivity could be separated from the normally much larger conductivity of the eluant. He designed a system with two columns to achieve this.

The separator column contains a pellicular sorbent that has only about 1 percent of the capacity of conventional ion-exchange resins and no interior pores. This permits elution of the sample with a dilute solution of a weakly ionic species. The separator column is followed by a short suppressor device with a polarity opposite that of the separator resin (Fig. 2). Its most important function is conversion of the conductive eluant to a less conductive form. If the sample is eluted with sodium hydroxide or sodium bicarbonate, for example, the sodium cations are exchanged for protons in the suppressor device. This forms a species, either water or carbonic acid, with low conductance, so the conductivity of the sample can be readily measured.

Among the more common ions for which IC is used are the halides, sulfate, sulfite, nitrate, nitrite, and phosphate, as well as more exotic species such as azide, arsenate, thiosulfate, and phosphite. A recent study reported more than 60 ions that can be measured by IC. It has been used to monitor ions in such diverse environments as brine, soil, milk, kraft black liquors, diesel exhaust, nuclear fuel reprocessing streams, ambient aerosols, fertilizers, boiler waters, air exhaust scrubbers, plating baths, blood, urine, and foodstuffs.

The range and usefulness of the technique have been expanded by some new developments. Perhaps the most important of these is continuous regeneration of the suppressor device. This is accomplished with hollow-fiber exchange membranes. The eluant flows through the cores of the fibers for suppression while a strong electrolyte is pumped past the exterior of the fibers to regenerate them. Leakage of the regenerant ions into the fiber cores is prevented by the Donnan exclusion effect, which creates an ionic barrier. Both anion and cation suppressors can now be continuously regenerated in this manner.

The range of samples has been expanded with another new technique called mobile-phase IC. This technique is analogous to reversed-phase chromatography in conventional HPLC and makes it possible to use the differing hydrophilicities of alkyl side chains to increase the separation of such materials as aliphatic amines, aliphatic and aromatic sulfonates and sulfates, quaternary amines, and such hydrophobic inorganic ions as fluoborate, thiocyanate, iodide, and perchlorate.

Ion chromatography also has certain limitations. One is the need for corrosive reagents, particularly those used for regenerating the suppressor. Another is the potential peak broadening and dilution of the sample by the suppressor column. Several companies, notably Wescan Instruments, Inc., have attempted to get around these problems by eliminating the suppressor device. One way to do this is by using ions of low intrinsic conductance, such as phthalate ion, combined with sophisticated electronics that make it possible to detect conductivity changes as low as 1 part in 30,000. Sensitivity by this technique is in the

parts per million range (compared to parts per billion for dual-column IC), but can be improved by a concentrator column. Between 15 and 20 percent of IC is now done by single-column techniques.

Other detection techniques are also beginning to be used, some of which can eliminate the need for the suppressor device. Elution with phthalate ion makes possible indirect photometric detection, since elution of transparent ions causes a decrease below the background absorbance. Refractive index is a universal detection technique, but its sensitivity is less than that of dual-column IC. Electrochemical (primarily amperometric) detection can be used with some easily oxidized species such as cyanide and sulfide. Electrochemical detection is highly sensitive and extremely selective. There are also now available new ultraviolet detectors that reach down to 200 nm or less, making possible the spectrometric detection of ions such as nitrate, nitrite, bromide, iodide, bromate, iodate, and thiosulfate that absorb in this region.

Gas Chromatography

Gas chromatography (GC) is a mature technology in which one would expect to see few major changes, if any. Nonetheless, in 1979 Ray D. Dandenau and E. M. Zenner of the Hewlett-Packard Company reported on the use of flexible fused-silica capillary columns for GC. This development has extended the use of GC dramatically.

Actually, the capillary column for GC was first described in 1957 by Marcel J. E. Golay of the Perkin-Elmer Corporation. The unique characteristic of the capillary was not that it had a small internal diameter, but that the tube was open rather than packed like large-bore columns. The impedance to flow of the mobile phase was thus very small. Short columns could be operated at high mobile-phase velocities to provide fast analyses, or long columns could be operated at their optimum velocities to provide high efficiencies and, consequently, high resolving power.

Capillary GC did not become popular, however, because techniques for injecting small quantities of sample were limited, techniques for detecting those small samples were even less well developed, and the columns themselves were unsatisfactory. Metal columns were difficult to coat with certain stationary phases, some samples adhered strongly to the metals, and the metals frequently catalyzed the rearrangement or degradation of many sample components. Nylon

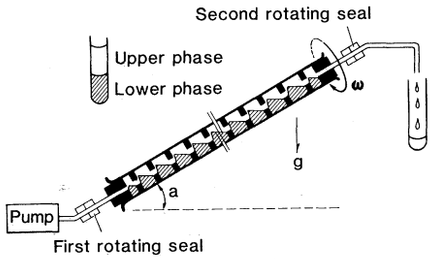

Fig. 3. Mechanism of rotation locular countercurrent chromatography. [Courtesy of Y. Ito]

tubes overcame many of these problems, but they could not be used for temperatures much above 120°C.

In 1960 D. H. Desty introduced a new method to draw glass capillaries that were considered almost ideal for GC. Even these, however, had their drawbacks. They had poor wettability, and, since they were made in the form of rigid coils, the ends had to be straightened before they could be attached to the chromatograph. Not only was this technique clumsy, but the heat required often vaporized the stationary phase from the ends of the column.

Dandenau and Zenner drew on technology developed in the optical fiber industry to produce flexible fused-silica columns by coating the exterior of the freshly drawn capillaries with a film of heat-resistant polyimide. This film prevents the hydration of strained siloxy bonds, the process that makes the normally flexible glass turn brittle. Investigators have since shown that this technique can be used on soft glass and Pyrex capillaries. The fibers produced in this manner are naturally straight, but are sufficiently flexible that they can be tied in a loose knot or formed into coils that fit readily into the oven of the chromatograph.

The glass capillaries have proved to be a very good support for a variety of stationary phases. Perhaps the most important of these are the "bonded" or "stabilized-liquid" phases. These stationary phases are analogous to the reversed-phase packings that have been used in HPLC for many years, but they have been applied in GC only within the last 2 years. Unlike a conventional stationary phase for GC, in which a polymer is used to coat the interior of the capillary, the bonded phases are polymerized in place.

The bonded phases are very stable and exceptionally resistant to solvents and high temperatures. They can thus be washed to remove adsorbed impurities and programmed to higher temperatures to aid elution. The resistance to solvents also makes it possible to inject larger sample volumes, extending the dynamic range of GC for analysis of trace materials. The use of larger samples is also abetted by the recent thick-film bonded coatings, which are about 5 μm thick compared to 0.2 to 0.5 μm for conventional films. The increased thickness makes it possible to use samples that are five to ten times as large as normal. Such columns should be especially useful for GC-FTIR (Fourier transform infrared), where a somewhat larger sample is required.

These changes, combined with microprocessor control of the chromatograph, have made GC far more reproducible, opening the door to qualitative identification of unknowns based on absolute retentions and retention indices. A lot of work on this use was performed 15 years ago, but the chromatograms were simply not reproducible enough. Now they are. Furthermore, the columns and technology are so much better than they were even 5 years ago that much of the application work may have to be repeated. In flavor or pheromone chemistry, for example, many of the polar trace components of great potential importance (such as fatty acids) were probably never eluted from the older capillary columns.

Microprocessors have also had a significant impact, especially in detection. One problem with GC detection has been a drifting baseline as the temperature is increased, caused by bleeding of the stationary phase. This was formerly compensated for by using two columns, one for reference. The microprocessor, however, can be programmed to compensate for the baseline drift of each column, thereby making one-column operation possible.

Microprocessor control is also a key feature of the new mass detectors for GC. These detectors are less expensive ($30,000 to $50,000) and potentially more versatile than mass spectrometers. They are particularly valuable because they are all-purpose detectors that register every component of the sample, they can be used as selective detectors to identify only specific classes of chemicals, and they can be used for identification of sample components by comparison to spectral libraries.

Advances are also being made at the end of the column where the sample is loaded. Two of the more important new techniques are cold, on-column injection and programmed-temperature injection. In the former, small quantities of sample are injected directly into the capillary, so the head is not heated. This process is gentle on the sample, and is particularly good for thermally labile and other sensi-

tive compounds, including free acids, amines, mercaptans, and drugs.

In programmed-temperature injection, the sample is injected into a special head as a liquid so that syringe discrimination, a problem with gaseous samples, is eliminated. The sample is then heated rapidly and the desired portion split off into the column. Programmed-temperature injection is good for the same types of samples as cold, on-line injection, but is also good for dirty samples, such as biomedical specimens, where the undesirable components can be shunted away from the column. It also has the advantage that it can be easily automated for handling multiple samples.

A distant cousin of programmed-temperature injection is an older technique called, variously, headspace chromatography, dynamic headspace chromatography, or purge and trap chromatography. The technique is widely used for monitoring trace concentrations of volatile materials in a liquid sample. The sample is placed in a special head and purged with an inert gas to expel volatile compounds, which are trapped and concentrated by an adsorbent. The trap is then heated and backflushed to release the sample to the column. The technique is used to monitor trace organics in water, alcohol in blood, and flavors and fragrances in perfumes, food, and beverages.

In the past, most headspace analyses have been performed on dedicated chromatographs. Recently, however, it has become possible to purchase accessories to perform headspace analysis on a conventional gas chromatograph. Another development is the use of higher temperatures, up to 200°C, in the purge process. Robert G. Westendorf of the Tekmar Company reported earlier this year that high-temperature purging is especially valuable for measuring residual solvents and monomers in plastics, as well as for studying decomposition products. It is also useful for analyzing compounds that produce off-odors and tastes in foods, particularly those that are oil-based, and for studying tobacco and textiles.

Countercurrent Chromatography

The classical liquid-liquid partition device, the separatory funnel, is a good way to separate components of a mixture into immiscible fluids—if the components have markedly different physical properties. As the nature of the solutes becomes more similar, however, a multistep extraction is required. In 1944 L. C. Craig developed a device for discontinuous countercurrent distribution in which as many as 1000 extractions could be performed. This device still has many applications for large-scale separations of polar compounds, particularly natural products, polypeptides, and macromolecules, but is little used in the research laboratory because of the complexity of the apparatus, the formation of emulsions that are sometimes hard to break, the frequent need for excessively large volumes of solvent, and the long separation times.

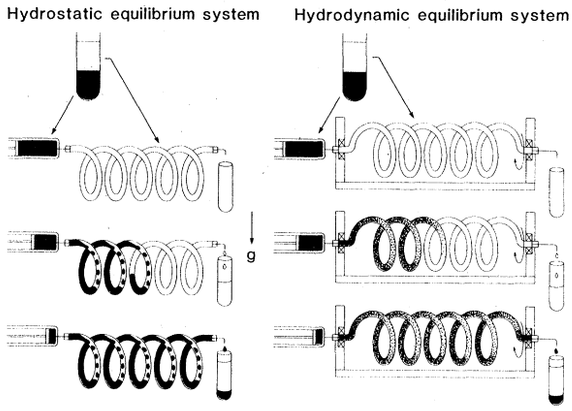

Fig. 4. The most common form of countercurrent chromatography. A coil is used to retain the stationary phase while the mobile phase is continuously eluted. A stationary coil establishes a hydrostatic equilibrium (left) while a rotating coil establishes a hydrodynamic equilibrium (right). [Courtesy of Y. Ito]

Most of these problems can be overcome with a family of techniques known collectively as countercurrent chromatography (CCC). These techniques have been developed and refined primarily by Yoichiro Ito and his colleagues at the National Heart, Lung, and Blood Institute, and several types of equipment have recently become available commercially. CCC yields highly efficient separations (comparable to those achieved with LC), while retaining the good sample recovery rate, the high purity of collected samples, the reproducibility, and the predictability of countercurrent distribution. Equally important, CCC avoids such undesirable complications as loss and denaturation of samples, contamination, and tailing of solute peaks—all of which are caused by the presence of the adsorbent in LC. Its chief limitation is the long time often required for separations, but some newer techniques are becoming available to speed the process.

There are three basic types of CCC, each based on the passage of a mobile phase through an immiscible stationary phase. Perhaps the simplest, at least conceptually, is droplet CCC, in which the stationary phase is retained in a cluster of vertical tubes connected in series. If the mobile phase is lighter, it is pumped into the bottom of the first tube, where droplets form and rise to the surface. A connecting tube carries the mobile phase from the top of the first tube to the bottom of the second, where droplets are again formed. This process can be repeated through as many tubes as is necessary to effect the desired separation. At the end, the eluant is collected and monitored as in conventional LC. Droplet CCC can also be performed in a mode in which the mobile phase is heavier and is introduced at the top of each vertical tube. A major restriction in either case is that the mobile phase must be capable of forming droplets in the stationary phase.

In locular CCC, the stationary phase is segmented in small cells or locules separated by narrow passages (Fig. 3). Typically, the tube or cylinder holding the locules sits at an angle of about 45° so that the stationary phase is retained in the cells. The locular column may remain stationary, but better separation is usually achieved if it is rotated about its central axis.

In the most common and most useful forms of CCC a horizontal or near-horizontal coiled tube is used. The tube is filled with the stationary phase and the mobile phase is pumped through (Fig. 4). Part of the stationary phase is forced out until the system reaches a hydrostatic equilibrium in which part of the stationary phase is retained in each coil. Further pumping then causes only mixing and elution of the mobile phase. Better mixing and more efficient separation can be achieved by rotating the coil about its central axis, producing a hydrodynamic equilibrium in which the mobile phase is broken into much smaller droplets. Still better resolution can be obtained by imparting a further rotation to the column about one or more additional axes.

Unlike many other separation methods, says Ito, CCC handles a broad spectrum of samples, ranging from small ions and molecules to macromolecules and particles. The choice of solvent systems is almost limitless, and it is nearly always possible to find a suitable sol-

vent. The maximum capacity of most existing systems is about 1 g of sample, but larger systems are under development. Stepwise and gradient elutions are possible with CCC, and ion-exchange CCC can be accomplished with suitable liquid ion exchangers. CCC performs foam separations with extremely high efficiency. The slowness of the technique, however, generally makes it unsuitable for repetitive analyses.

Like countercurrent distribution, CCC is very good for purification of natural products and polypeptides. Ito and his colleagues have worked extensively, for example, with deoxyribonucleoprotein amino acids and oligopeptides. Some of the natural products on which CCC has been used include glycosides of saponins, xanthones and flavonoids, diterpenoids, sugars, alkaloids, peptides, and lipids. It has also been used to separate prostaglandins, catecholamine metabolites, and most types of plant hormones. It is good for isolating impurities from commercial pesticide samples. Other materials that have been separated include glycosaminoglycans, polynucleotides, cells, and blood samples.

Field-Flow Fractionation

A family of techniques that show great potential, particularly for the separation of high molecular weight materials, is known by the collective name of field-flow fractionation (FFF). The techniques were conceived in the mid-1960's by J. Calvin Giddings of the University of Utah and have been developed and refined since then largely by Giddings, Marcus N. Myers, and Karin D. Caldwell. Instruments to perform FFF are not yet available commercially, but in most cases they can be constructed relatively easily. The variety of potential applications suggests that FFF will be used much more often in the future.

Field-flow fractionation takes advantage of the fact that fluid flowing through a thin, ribbon-shaped conduit does not have a uniform velocity. Its velocity approaches zero, for example, near the walls of the conduit because of viscous drag; it reaches a maximum near the center of the conduit (Fig. 5). Giddings reasoned that if it were possible to place different kinds of molecules in regions with different flow velocities, separation would result from their differential migration through the conduit.

Placement in the different flow regions is achieved by application of an external field or gradient perpendicular to the direction of flow (Fig. 6). In most cases, the species of interest is forced close to the wall so that its elution is retarded. The techniques have been used to separate ionic and nonionic species in aqueous and nonaqueous media. Separations have been achieved over a 10^{15}-fold mass range—from polymers with a mass as low as 1000 daltons to particles 100 μm in diameter.

Experimentally, FFF operates much like chromatography. A small sample is injected at the head of the separation channel and separation occurs as the components are carried along at different velocities. At the end of the channel the components of the sample are detected and collected by conventional means. A graph of detector response versus time, called a fractogram, looks like a chromatogram. Strictly speaking, however, FFF is not chromatography: by definition, chromatography requires that separation be accomplished by distribution of the sample between two phases. FFF has only one phase. Nonetheless, the technique has been called one-phase chromatography or polarization chromatography.

The length L of the channel is generally much greater than the thickness w over which the applied field is acting. Typically, L varies from 30 cm to more than 1 m, w ranges from 0.1 to 0.5 mm, and L/w is at least 10^3. A long L allows multiplication of a weak, field-induced enrichment effect into a complete separation; this multiplication is much like that which occurs in LC or GC. The flow is unidirectional, so that carrier properties (such as density and pH) can be changed continuously to generate programmed carrier systems to enhance separation characteristics. Perhaps most important, FFF is based on physical rather than chemical properties. It is therefore often easy to apply theory and predict when a given molecule will be eluted. Conversely, molecular weight and other properties can be calculated in a straightforward manner from retention time. Hence the techniques can be used for characterization as well as separation.

The particular applications of FFF depend on the nature of the perpendicular field or force. Sedimentation FFF, for example, puts the channel inside a centrifuge that creates a gravitational field. The Utah group has worked with fields up to about 1700 gravities. J. J. Kirkland and W. W. Yau of DuPont have used a somewhat different design that is capable of sustained operation at 50,000 gravities. Sedimentation FFF has been used to separate, among other things, viruses and polymers. It is particularly useful for determining the droplet size distribution of emulsions. In terms of molecular weight, the resolution ranges from about 10^6 (at 50,000 gravities) up to the onset of steric effects, which generally occur for particle sizes around 1 μm. Steric effects occur when the specimen is so large that it protrudes from the slowest streamlines near the wall into ones that move faster. Steric effects can themselves be used to effect separations. In that case, however, the order of elution is reversed and the largest particles are eluted first. Steric effects have been used, for example, to separate silica particles of different sizes and polystyrene latex beads. The technique has also been used, in a field of 3.6 gravities, to separate viable HeLa cells from red blood cells.

In thermal FFF a temperature gradient of as much as 70°C is established between the walls of the channel. It is well known that a strong temperature gradient will cause many components in a mixture, particularly polymers, to migrate along the gradient. Thermal FFF has thus been used to characterize synthetic polymers such as polyethylene, polymethyl methacrylate, polyisoprene, and especially polystyrene.

In flow FFF a hydraulic pressure gradient perpendicular to the eluant flow is established with semipermeable membranes. Membrane permeability sets a lower limit of about 1000 on the molecular weight of compounds that can be separated by this technique. Flow FFF is similar, and complementary, to sedimentation FFF, but in this case it is diffusivity that is the controlling factor. The technique has been used to separate proteins and, in nonaqueous solvents, polystyrene.

Electrical FFF is conducted with electrodes placed outside semipermeable membranes. The electrical field causes differential migration of charged samples. Because the electrodes are kept outside the membranes, any products produced by electrolysis are kept outside the channel also. The electrodes are about 1 cm apart, which produces strong fields with only modest heating. Electrical FFF has also been used for the separation of proteins, but Giddings argues that its full potential has yet to be tapped.

Electrophoresis

Electrophoresis and its variants are the most commonly used techniques for separating and characterizing macromolecules, particularly proteins. The primary form, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), uses an electrical field to force the charged macromolecules

through a gel that separates them by size. It is especially useful for determining approximate molecular weights. Also commonly used is a technique called isoelectric focusing, which is performed much like SDS-PAGE with the exception that a family of ampholytes are electrophoresed first to establish a *p*H gradient. The macromolecules migrate until they reach their isoelectric point—the *p*H at which the positive and negative charges balance. They are thus separated on the basis of composition rather than size.

Many separations are now performed by two-dimensional electrophoresis, in which these two techniques are combined. Two-dimensional electrophoresis was first reported in 1976, but has caught on only in the past 3 years. In it, isoelectric focusing is performed on a thin gel to separate the macromolecules by their isoelectric point. This gel is then placed on top of another gel and conventional SDS-PAGE is performed perpendicular to the original separation to further separate the sample by molecular weight.

Two-dimensional electrophoresis has found increasing use because it can separate large numbers of proteins. Perhaps the best example of its application is the work of Norman G. Anderson and N. Leigh Anderson of Argonne National Laboratory, who are using it to establish maps of the 50,000 proteins in a human cell. Such maps could be used for both the diagnosis and understanding of diseases by identifying the presence of abnormal proteins and the absence of normal ones.

A newer two-dimensional technique is called electrophoretic titration curve analysis. In this case, a *p*H gradient from 3 to 9 is established in a horizontal slab; a trough is then cut into the slab at a right angle to the gradient. The sample is applied to the trough and conventional electrophoresis is performed, again perpendicular to the gradient. This produces a classical titration curve that provides certain useful information about the protein, including its stability and the binding of ligands. Perhaps most important, the curve can be used to predict the behavior of the protein in ion-exchange chromatography.

A new technique of preparative electrophoresis is recycling isoelectric focusing (RIEF), developed by Milan Bier and his colleagues at the University of Arizona (*Fig. 7*). The key to this technique, in which a gel is not used, is the recycling of the solution to be fractionated through a focusing cell and heat-exchange reservoirs. The focusing cell comprises a series of parallel flow chambers separated by monofilament nylon screen elements. These streamline the flow of liquid through the apparatus, avoid loss of resolution due to convection, and allow proteins to migrate from chamber to chamber under the influence of the field. Each chamber is connected to a separate glass channel in the heat-exchange reservoir.

The capacity of the apparatus is determined by the volume of the heat exchanger and the cross-sectional area of the focusing chambers. Usually, only ten compartments are used, but this can be varied at will. Due to the modular design, it is easy to scale up to larger volumes. The *p*H gradient is established by the electric field itself, in the same manner as in analytical gel focusing.

Bier and his colleagues have used the technique to process milligram quantities of antibodies to single band purity in 4 hours. He says that it is also useful for proteins and other materials produced by genetic engineering techniques; it has, in fact, already been used on a trial basis in scaling up for the commercial production of interferon. RIEF can resolve proteins whose isoelectric points differ by as little as 0.05 *p*H unit.

There is a growing trend in electropho-

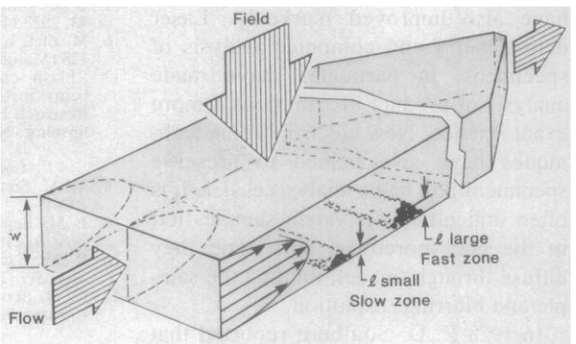

Fig. 5. Field-flow fractionation principle. Solvent close to the walls of the narrow channel moves slowly because of viscous drag, while solvent in the center of the channel has the fastest velocity. [Courtesy of J. C. Giddings]

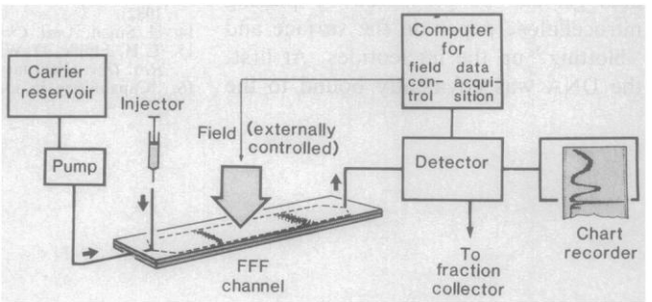

Fig. 6. Schematic view of the FFF column with ancillary equipment. [Courtesy of J. C. Giddings]

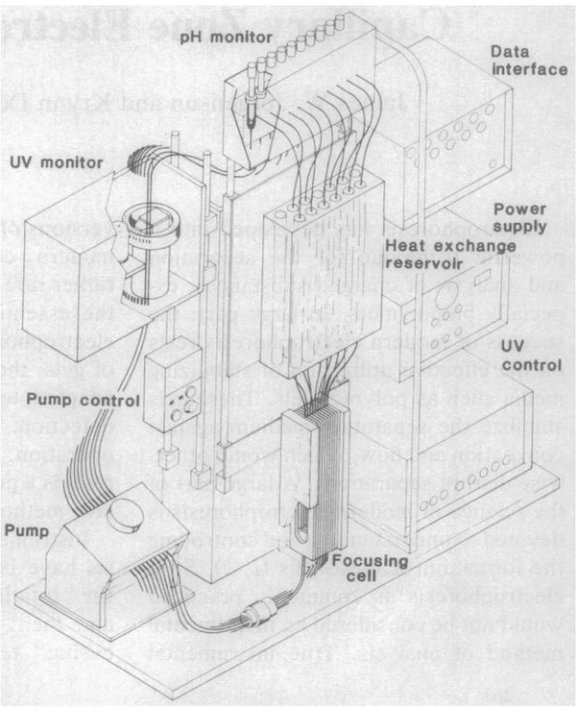

Fig. 7. Schematic diagram of the recycling isoelectric focusing apparatus. [Courtesy of M. Bier]

resis toward the use of thinner gels; these gels, for example, give better retention of nucleotides for DNA sequencing and, because of more efficient cooling, allow higher voltages to be used for faster separations. In the past, gels have typically been 2 mm thick; today, they are often less than 0.5 mm thick. This has been made possible primarily by refinements in preparing the gels. Many more investigators are also now using agarose gels, which are less toxic than polyacrylamide. And finally, it has become possible within the past year to prepare gels with immobilized *p*H gradients.

Detection and analysis of the samples have also improved markedly. Laser densitometry and computer analysis of specimens, in particular, have made analysis of product distributions a more exact science. New electroblotting techniques have also helped to preserve specimens and make analysis easier. It is often difficult to derivatize samples left in the gel; moreover, with time they diffuse through the gel, diluting the sample and blurring resolution.

In 1978 E. O. Southern reported that the distribution pattern of DNA in the final gel can be preserved by placing nitrocellulose paper on the surface and "blotting" up the nucleotides. At first, the DNA was covalently bound to the paper, but subsequent investigators have concluded that simple adsorption is suitable for most purposes. The sample can then readily be derivatized for precise identification, and the distribution pattern preserved for comparison with future gels. The eponymous Southern blot was followed soon after by the Northern blot for RNA and, more recently, by the Western blot for proteins.

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Capillary Zone Electrophoresis

James W. Jorgenson and Krynn DeArman Lukacs

Electrophoresis has developed into a powerful technique for the separation and analysis of charged substances, especially biopolymers. In large part, the success of modern electrophoresis rests on the effective utilization of stabilizing media such as polymer gels. These gels stabilize the separation medium against convection and flow, which would otherwise disrupt separations. A large part of the science of modern electrophoresis is devoted to understanding and controlling the formation of these gels (*1*, *2*). Still, electrophoresis as commonly practiced would not be considered an instrumental method of analysis. True instrumental versions of electrophoresis analogous to modern column chromatography are rather rare. In part, this can be traced to the essential role of stabilizing gels in electrophoresis. Because of the presence of gels, the method has not been easily adapted to on-line sample application, detection, quantification, or automated operation. Instead, modern electrophoresis is a powerful and yet manual-intensive methodology.

Instrumental versions of electrophoresis have been developed. Among these the "rotating tube" system of Hjerten (*3*) and the "transient-state isoelectric focusing" technique of Catsimpoolas (*4*) are particularly notable. These techniques, although novel and quite powerful, have failed to come into routine usage due to their complexity. Capillary isotachophoresis is probably the only instrumental version of electrophoresis to see extensive application, although here, too, acceptance has been slow. The unconventional format of data output in isotachophoresis, coupled with the fact that it appears better suited for separations of relatively small molecules, is the probable reason for its slow acceptance (*5*).

In the course of considerations of causes for zone broadening in zone electrophoresis, it occurred to us that an "open" capillary tube—that is, one containing buffer without stabilizer—offered a unique and simple situation in which to study electrophoresis. In such a system electrophoresis could be studied with minimal interferences, and at the same time causes of zone broadening could be

J. W. Jorgenson is an assistant professor of chemistry and K. D. Lukacs is a graduate student in the Department of Chemistry, University of North Carolina, Chapel Hill 27514.