Capillary Electrophoresis

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Electrophoretic separations carried out in capillary tubes offer the possibilities of rapid and automated analyses of small volumes of complex mixtures with unprecedented resolution and sensitivity. Some emerging developments of this new instrumentation are reviewed.

MANY MOLECULES EXIST IN SOLUTION AS CHARGED SPECIES (ions), either with positive charges (cations) or negative charges (anions). When an external electric field is applied to a solution of charged species, each ion moves toward the electrode of opposite charge. The rate of motion rapidly reaches a constant value such that the frictional drag just balances the electric force. Under these steady-state conditions, the velocity of the ion is directly proportional to the electric field strength. The proportionality constant is called the electrophoretic mobility. Different molecules have different mobilities depending on their average charge, size, and shape, as well as on the properties of the solvent medium. This effect forms the basis for electrophoretic separation techniques. Thus, when a narrow zone of a mixture of molecules is injected into a migration channel and an electric field is applied, the mixture separates into various zones that migrate at different rates depending on their mobilities. Beginning in the 1930s, with the elegant moving boundary experiments of Tiselius (*1*) on the separation of human serum into some of its constituent proteins, electrophoretic techniques (*2*) have joined liquid chromatography and ultracentrifugation as the three most widely used methods for separating and analyzing mixtures of biomolecules.

Free-zone electrophoresis generally suffers from poor resolution, which is caused by convective disturbances arising from Joule heating by the current that passes through the migration channel. The most common way to counteract this effect is to use some solid support, such as paper, starch gel, agarose, cellulose acetate, or polyacrylamide gel as part of the migration channel. These solid supports have the further advantage of acting as a sieve in some cases. Impressive results have been obtained by this means in an enormous variety of biomedical applications. For example, two-dimensional (2-D) gel electrophoresis, combined with computer-image analysis, can display several thousand protein spots at one time (*3, 4*), so that this method has the greatest resolving capacity. Also noteworthy is the use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) because of its ability to separate many peptides and proteins on the basis of molecular weight (*5*). In this procedure, the protein mixture is denatured, and disulfide bonds are cleaved by heat in the presence of excess SDS and a reducing agent. Under these conditions, most polypeptides bind with the negatively charged surfactant, SDS, in a constant weight ratio. This process yields molecules of similar shape and constant charge density, resulting in similar free electrophoretic mobilities. Separation is then accomplished by the sieving action of the porous polyacrylamide gel and is based on size (molecular weight) differences.

Some very large biopolymers, such as various DNA fragments, exhibit nearly size-independent mobilities, a phenomenon thought to be caused by their reptile-like mode of migration (*6*). One way to address this problem is to change the magnitude and direction of the electric field in time. Schwartz and Cantor (*7*) have used this method to study different DNA molecules, including the intact chromosomal DNA molecules of yeast and several protozoans. They used an electrode geometry that allowed alternate application of two transverse electric fields that are spatially inhomogeneous. The idea is to force these large DNA molecules to turn corners periodically in the gel matrix, thereby introducing a size dependence in their migration rates. Carle, Frank, and Olson (*8*) have advocated an alternative procedure of periodically inverting the electric field. By tuning the frequency of field inversion from 10 to 0.01 Hz, they have been able to resolve selectively DNAs in the size range from 15 to greater than 700 kilobase pairs. Recently this technique has been used to recognize abnormally sized DNA fragments resulting from deletions in the Duchenne muscular dystrophy locus (*9*). In these methods, the electric field is inhomogeneous, so that DNA migrates with a mobility and a trajectory, both of which depend on the sample loading position in the gel. This effect makes it difficult to compare multiple samples across a gel. To overcome these limitations, Chu, Volrath, and Davis (*10*) have developed a different electric field manipulation scheme in which multiple electrodes are arranged on a closed contour and clamped to predetermined potentials. DNA molecules up to 2 megabase pairs in size are separated, independent of position in the gel, with this contour-clamped homogeneous electric field (CHEF), which alternates between two orientations 120° apart.

Despite these impressive advances, modern electrophoresis is a collection, for the most part, of manually intensive methodologies that cannot be run unattended and that cannot be readily automated. Casting of gels, application of samples, running of gels, and staining of gels are time-consuming tasks prone to irreproducibility and poor quantitative accuracy. The use of capillaries as the migration channel in electrophoresis holds the promise of putting electrophoretic separations on the same instrumental footing as high-performance liquid chromatography (HPLC). Thus capillary electrophoresis, also called capillary zone electrophoresis (CZE) and high-performance capillary electrophoresis (HPCE), would have the advantages of rapid and automated analyses of multiple samples, accurate quantification, and improved reproducibility. In addition, the capillary electrophoresis technique provides a simple solution to

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the problem of Joule heating that results in poor resolution. The microchannel of a capillary has such a high surface-to-volume ratio that heat is efficiently dissipated by transfer through the capillary walls. Such heat removal nearly eliminates convection so that the peaks approach the theoretical limit (*11*) of being broadened only by diffusion (from high concentration to low concentration). In favorable cases, about 1 million theoretical plates have been realized (*12*), making capillary electrophoresis the electrophoretic method of highest efficiency.

After the work of Pretorius and co-workers (*13*) demonstrated that electroosmosis acts as a pump, Virtanen (*14*), as well as Mikkers, Everaerts, and Verheggen (*15*), pioneered CZE. Jorgenson and Lukacs (*11*) soon followed with their own contributions and were largely responsible for popularizing this technique (*16*).

As shown in Fig. 1, a typical CZE system consists of a fused silica capillary (inside diameters range from 20 to 200 μm and lengths range from 10 cm to greater than 100 cm) whose ends are placed in electrolyte reservoirs that also contain electrodes. A power supply furnishes high voltages, typically 20 to 30 kV. A detector completes the system. The tube is initially filled with a buffer or electrolyte that is the same as that containing the sample. Sample injection is made either electrokinetically, by using electroosmotic flow, or hydrostatically, by using pressure, vacuum, or gravity. Sample volumes are in the nanoliter to picoliter range and detection volumes can be as small as 30 pl or less.

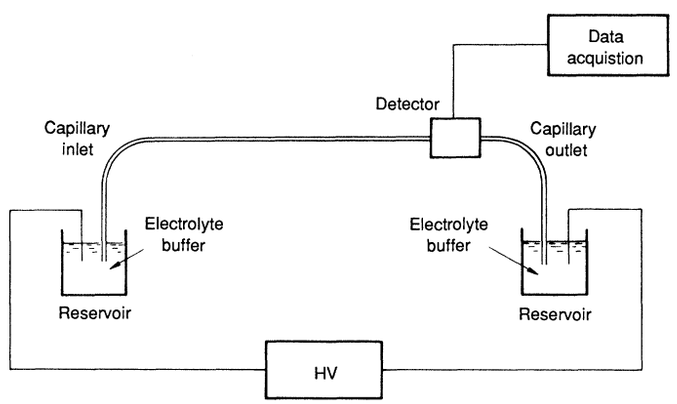

Fig. 1. Generic diagram of a capillary electrophoresis system.

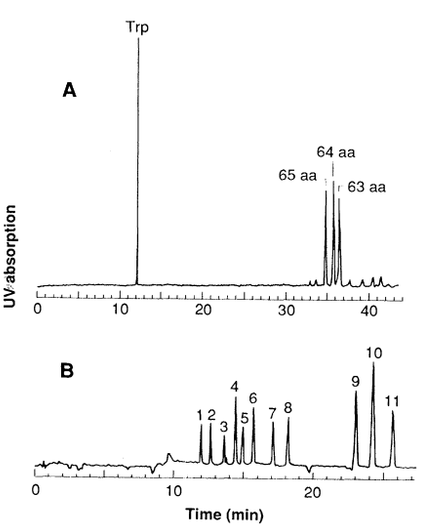

Fig. 2. Capillary electropherograms measured with a UV absorption detector: (**A**) separation of recombinant hirudin and its degradation products (*41*); (**B**) separation of bases, nucleosides, and nucleotides, concentration = 1.25 × 10^{-4}*M*. Sample components were: 1 = uridine; 2 = cytidine; 3 = thymidine; 4 = cytosine; 5 = thymine; 6 = 2′-azidocytidine; 7 = *N*-methylthymidine; 8 = 5-methylcytidine; 9 = thymidine 5′-monophosphate; 10 = 2′-deoxycytidine 5′-monophosphate; and 11 = 3′-azidothymidine (AZT). Electrolyte was 0.2*M* phosphate buffer, *p*H = 7.0, containing 0.2*M* SDS. The applied potential was 12 kV, and the capillary tubing was 75 μm inside diameter and 70 cm long, with a distance of 50 cm to the UV detector (280 nm).

The walls of fused silica capillaries have a negative charge in aqueous solution from the ionization of surface silanol groups. When a voltage is applied, a bulk flow of fluid toward the cathode occurs. This is electroosmotic flow, which is caused by the electrical double layer formed at the wall-electrolyte interface. A positively charged sample component emerges early because both the electrophoretic motion of the ion and the electroosmotic motion of the electrolyte are in the same direction. If the component is negatively charged, but its electrophoretic mobility is less than the electroosmotic mobility, then that component also migrates toward the cathode but at a much lower rate.

When a viscous, incompressible fluid is forced to move through a cylindrical tube by a pressure difference, the flow front has a parabolic velocity profile; that is, the velocity of the fluid vanishes at the walls and increases quadratically with distance from the walls (Poiseuille flow). In contrast, the velocity profile in electroosmotic flow can be shown to be nearly flat (plug flow) because the maximum charge imbalance occurs at the wall-electrolyte interface (*17*). The plug-flow characteristics of electroosmotic flow cause less band spreading than hydrostatic flow. Although the hydrostatic flow can be adjusted to be more rapid, the electroosmotic flow rate is independent of the capillary length for a given applied electric field strength, depending instead on the zeta potential, the dielectric constant, and the reciprocal of the viscosity of the fluid. Thus, for many purposes, the weak, built-in "pump" of electroosmotic flow can be used with advantage in carrying out electrokinetic separations.

Capillary zone electrophoresis is simple, fast, and readily automated. The major challenges appear to lie (i) in the area of detectors, because the small volumes involved demand high detector sensitivity, and (ii) in the area of solute-wall interaction, because slow solute adsorption-desorption kinetics may substantially reduce the resolving power of the method.

In many applications, capillary electrophoresis may offer incremental but substantial improvements over other analytical techniques. However, when the sample amount is extremely limited, capillary electrophoresis may possess unparalleled advantages. For many years, those who studied cells have wanted to assay a single cell for its constituents. Typically, this requires one to work with picoliter samples or less—a volume so tiny as to be unrealized to date. Instead, large numbers of cells are assayed and the analysis reports only average concentrations. Capillary electrophoresis is well suited to the possibility of fulfilling this dream. With very small diameter capillaries, injection volumes of 1 pl or less can readily be handled. The challenge is providing detectors with adequate sensitivity.

Methods for Selectivity

Micelles. Neutral molecules present a special problem for CZE, that is, they do not separate from each other but migrate as a group with the electroosmotic flow. Terabe and co-workers (*18*) and later Sepaniak and co-workers (*19*) and Karger and co-workers (*20*) developed the use of SDS micelles. These micelles have a negative charge, and their electrophoretic motion opposes the electroosmotic motion. Micelles act as a partitioning agent for the neutral molecules that are internalized according to their hydrophobic nature. The separation is based on the differential distribution of the solute molecule between the electroosmotically pumped aqueous mobile phase and the slower moving, electrophoretically retarded micellar phase. This technique is also called micellar electrokinetic capillary

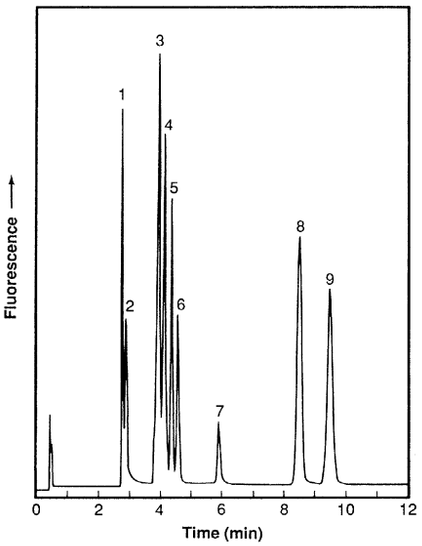

Fig. 3. Capillary electropherogram of a nine-component amino acid mixture with on-column OPA derivatization and subsequent detection by LIF (*42*). Sample components were: 1 = L-arginine; 2 = L-lysine; 3 = L-isoleucine; 4 = L-histidine; 5 = L-serine; 6 = L-asparagine; 7 = L-cysteine; 8 = L-glutamic acid; and 9 = L-aspartic acid. Electrolyte was 0.2*M* borate buffer, *p*H = 7.8. The applied potential was 25 kV, and the capillary tubing was 75 μm inside diameter and 74 cm long, with a distance of 48 cm to the LIF detector.

chromatography (MECC). Micelles have also been used to enhance markedly the separation of charged species [see, for example, the work of Karger and co-workers (*20*) and that of Wallingford and Ewing (*21*)].

Formation of diastereomers. The resolution of racemic mixtures has long been an area of active research. In chromatographic separations, the resolution of a racemic mixture into its component enantiomers is brought about by diastereomeric interaction with either a chiral stationary phase or a chiral mobile phase. Gassmann *et al.* (*22*) and Gozel *et al.* (*23*) have demonstrated that enantiomeric mixtures can also be resolved by CZE. This resolution was accomplished in the case of D- and L-dansyl amino acids through diastereomeric interaction between the amino acids and transition metal–chiral ligand complexes that were incorporated into the separation buffer. For example, by addition of a Cu(II)-aspartame complex to the support buffer, 14 out of 18 amino acid pairs were resolved with typical separation times of less than 12 min (this is a special example of the use of an additive to facilitate CZE separations; see the section on additives and wall coatings below).

Gel-filled capillaries. Several of the separation modes that have been successful in open-bed gel electrophoresis also appear to be feasible in capillary electrophoresis. These include the possibility of performing isoelectric focusing and molecular sieving in capillary tubes, as first demonstrated by Hjertén and co-workers (*24*). Recently, Karger and co-workers (*25*) have advanced this method by developing a procedure to covalently bind the packing material to the fused silica walls of the capillary tubing. This modification has permitted them to carry out capillary SDS-PAGE.

Gel-filled capillary columns offer several advantages over open-bed gel electrophoresis, including higher resolution, reduced analysis time, and increased sensitivity, as well as the ability to use greater potential fields and to use on-line sample detection without the need for staining procedures.

Additives and wall coatings. The large majority of CZE separations to date have been performed by using aqueous electrolyte-buffer systems in untreated fused silica capillaries. Several groups have, however, demonstrated an improvement in the separation of complex mixtures by coating the walls to reduce unfavorable solute-wall interactions and by adding modifiers to the carrier electrolyte to promote the resolution of similar compounds. Walbroehl and Jorgenson (*26*) described the first capillary electrophoretic separation performed in a totally non-aqueous medium consisting of 0.05*M* tetraethylammonium perchlorate and 0.01*M* hydrochloric acid in acetonitrile. With this system, they achieved the baseline resolution of isoquinoline and quinoline. Subsequently, they reported (*27*) the separation of neutral organic molecules using a water-acetonitrile medium containing the hydrophobic tetrahexylammonium cation. This separation medium promotes hydrophobic interaction between the cation and hydrophobic analyte molecules, which effectively charges the otherwise neutral analyte molecules.

Fujiwara and Honda (*28*) investigated the effects of both methanol and acetonitrile modifiers upon electroosmosis, electrophoretic mobilities, and the resolution of positional isomers of substituted benzoic acids separated by CZE. Superior separation of these compounds was obtained with an electrolyte-buffer system containing 50% acetonitrile (v/v), resulting in complete separation of all positional isomers of amino-, hydroxy-, and methylbenzoic acids. The authors attribute their improvement in selectivity to suppression of carboxylate hydration upon addition of acetonitrile to the aqueous buffer, resulting in a greater variation of electrophoretic mobilities than in the case of an entirely aqueous separation medium. Roach, Gozel, and Zare (*29*) used CZE in the analysis of the anticancer drug, methotrexate, and its major metabolite, 7-hydroxymethotrexate, in human serum samples. They found that best resolution was obtained upon the addition of 30% methanol (v/v) to an aqueous buffer.

The separation of biopolymers proves to be particularly challenging because of the tendency of these molecules to adhere to the capillary walls, thus causing severe tailing of their zones as well as affecting the electroosmotic flow characteristics of the capillary. Important advances have been made to suppress this effect by the judicious use of wall coatings (*16, 30*), by the use of high ionic strength or extreme *p*H buffers or both (*12, 30, 31*), and by competitive adsorption to a suitable substance added to the electrolyte, such as divalent metal ions in an SDS buffer (*20*).

Future efforts to improve the selectivity of capillary electrophoresis should include the use of additional organic and inorganic additives because these are expected to broaden the scope of the technique as they have already done in HPLC.

Injection Methods

To obtain high resolution in capillary electrophoresis, the volume of the injected sample must be very small compared with the volume of the capillary (*15, 19*), for example, a capillary of 1 m in length and with an inside diameter of 75 μm has a volume of about 5 μl. Thus, 5 to 50 nl is the typical injection volume required to avoid distortion

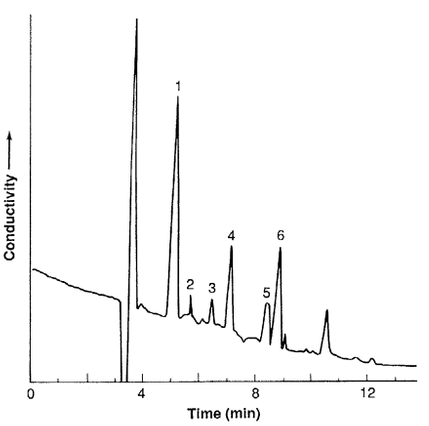

Fig. 4. Capillary electropherogram of a red wine sample (Cabernet Sauvignon) using an on-column conductivity detector (*49*). The peaks are: 1 = tartrate; 2 = malate; 3 = citrate; 4 = succinate; 5 = acetate; and 6 = lactate. Wine sample was diluted 1:39 with 7 m*M* MES-His [2-(*N*-morpholino)ethanesulfonic acid–histidine] electrolyte containing 0.5 m*M* tetradecyltrimethylammonium bromide (TTAB) and 30% methanol to aid separation. The applied potential was 25 kV, and the capillary tubing was 75 μm inside diameter and 67 cm long, with a distance of 66 cm to the conductivity detector.

by overloading (*15, 19*). Injection of these volumes is normally accomplished either by the pumping action of electroosmosis (electrokinetic injection) or by creating a pressure difference between the inlet and outlet of the capillary (hydrostatic injection). Several sample injectors have been reported (*32–34*), including a microinjector suitable for direct sampling of single nerve cells (*35*).

Electrokinetic injection may often be the method of choice because of its simplicity and easy control. Jorgenson and Lukacs (*11*) and Tsuda and co-workers (*36*) remarked that electrokinetic injection may not deliver the same amount of each species because of differences in their mobilities. Huang, Gordon, and Zare (*37*) found that there are two kinds of biases involved. One is from the different mobilities of the species in the sample solution, which results in a distortion of the ratio of peak areas for species having different mobilities. Fortunately, this bias can be removed by knowledge of retention-time ratios. Another bias is related to the electrical resistance of the medium in which the species are dissolved. This bias alters both the electrophoretic and electroosmotic flow rates for different solutions and thus changes the absolute amount injected, but can often be avoided by diluting a small amount of sample in a large volume of electrolyte.

Detection Methods

Several of the detection schemes commonly used in HPLC have been successfully adapted to CZE. The challenge is to detect individual sample components that are present at low concentration in electrolyte volumes that are sufficiently small to maintain electrophoretic resolution.

Optical detection. Optical detectors, based on both ultraviolet (UV) absorbance (*12, 18, 26, 27, 36, 38*) and fluorescence (*11, 16, 22, 23, 29, 39, 40*), are currently the most common means of monitoring CZE separations. In either case, detection is often made on-column in order to avoid loss of resolution caused by detector dead volume. The construction of these on-column detectors is generally quite simple, because most workers in the field use polyimide-clad fused silica capillaries. The polyimide cladding, which renders the capillaries flexible by preventing the hydration of strained siloxy bonds, is simply burned and washed from a small section of the tubing, revealing an on-column flow cell that is transparent throughout the UV and visible spectral regions.

The absorbance detector is obviously the more universal of the two optical detection schemes, as many compounds absorb UV radiation but relatively few fluoresce. The lower limit of detection for CZE with conventional UV absorbance detection depends largely upon the molar absorptivity of the analyte and is on the order of $10^{-6}M$ for strong absorbers. Some striking examples of the power of CZE separation combined with UV detection are shown in Fig. 2. Figure 2A, taken from Lüdi *et al.* (*41*), illustrates the separation of hirudin, a single-chain, 65-residue thrombin inhibitor protein, from its 64- and 63-residue degradation products formed during its recombinant synthesis. Figure 2B [Huang (*42*), repeating work by Ohms (*43*)] shows the separation of several bases, nucleosides, and nucleotides. Recently, Dovichi (*40*) and Yu and Dovichi (*44*) have reduced the limit of detection by roughly two orders of magnitude for the absorbance detector by way of thermooptical absorbance using a 130-mW argon ion laser.

Fluorescence detection typically affords a 100- to 1000-fold improvement in sensitivity compared with the UV absorbance detector because the analytical signal is measured against an extremely low background signal. Under exceptional circumstances, the detection limit can be drastically reduced to about 10,000 molecules or less injected into the column (*40*).

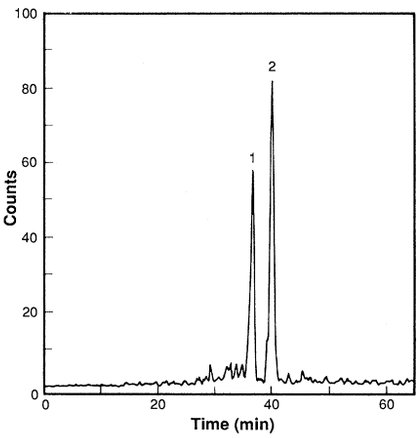

Fig. 5. Capillary electropherogram of guanosine 5′-[α-^{32}P]triphosphate (peak 1) and adenosine 5′-[α-^{32}P]triphosphate (peak 2) adapted from (*55*). Electrolyte was 0.020*M* phosphate buffer, *p*H = 7.0, containing 0.5% ethylene glycol. The applied potential was 20 kV, and the capillary tubing was 100 μm inside diameter and 100 cm long, with a distance of 75 cm to the detector.

On-column detection of fluorescent species separated by CZE has been accomplished with both UV lasers [laser-induced fluorescence (LIF) (*22, 23, 29*)] and UV lamp sources (*11, 16, 39*). The fundamental limitation of fluorescence detection is that relatively few molecules fluoresce. Fluorescence detection has been extended to nonfluorescent analytes by use of derivatizing agents to attach a fluorescent tag to the nonfluorescing sample molecules. Tagging has been accomplished by precolumn (*11, 23, 39*), on-column (*45*), and postcolumn (*46*) derivatization. A nine-component amino acid mixture was separated by CZE with on-column derivatization by using *o*-phthaldialdehyde (OPA) and subsequent detection by LIF [Fig. 3 (*45*)].

An alternative method to detect nonfluorescent analytes is to use indirect fluorescence detection, in which a fluorophore is added to the electrolyte and the nonfluorescing ions are detected through charge displacement of the fluorophore. In this manner, Kuhr and Yeung (*47*) have achieved a detection limit of 50 to 100 attomoles of sample component injected.

Conductivity detection. Perhaps the most universal detector reported to date for separations performed by CZE is the conductivity detector (*38, 48, 49*). As is always true of bulk property detectors, the conductivity detector is less sensitive than detectors that respond directly to a physical property of the analyte molecules. Nevertheless, conductivity detection has proven useful in the CZE analysis of several classes of compounds, including metal ions (*36, 38, 49*), organic acids (*15, 38, 50*), and inorganic ions (*38, 51*). The lower limit of detection for the conductivity detector depends upon the difference in mobility between the individual analyte molecules and supporting electrolyte ions and is on the order of $10^{-6}M$ in the more favorable cases. An example of conductivity detection is shown in Fig. 4, which illustrates the detection of a number of carboxylic acids commonly found in red wine.

Electrochemical detection. Electrochemical detection offers excellent selectivity because only a small number of components present in a mixture are likely to be electroactive. As with on-column conductivity detection, electrochemical detection is complicated by the presence of the high electric field. Wallingford and Ewing (*52*) use a porous glass joint to couple two pieces of capillary together. This joint is immersed in the buffer reservoir that contains the ground electrode. The electroosmotic flow that results when the voltage is applied is sufficiently strong to make the fluid flow past the joint and through the other piece of capillary tubing to a carbon-fiber electrochemical detector. This porous glass joint serves to complete the high-voltage circuit before the point of detection, thereby electrically isolating the electrochemical detector. The authors report minimum detection limits of 200 to 400 attomoles for catecholamines separated in a 26-μm inside diameter capillary tube.

Mass spectroscopy (MS). Capillary zone electrophoresis has been successfully coupled with mass spectrometry by way of an electrospray-ionization interface (*53*) adapted to CZE by Smith and coworkers (*54*). Although performance of the interface is reported to vary widely (*53*), depending upon the particular solute and buffer composition, results appear promising, with demonstrated limits of detection in the femtomole range and separation efficiencies exceeding 10^5 theoretical plates in the most favorable cases. The structural information afforded by mass spectrometry combined with the resolving power of CZE have generated a great deal of interest, and CZE-MS will undoubtedly mature to assume a place among the "hyphenated techniques."

Radioisotope detection. The extremely small sample volumes required for capillary electrophoresis, coupled with the selectivity and sensitivity of radioisotope monitoring, could make radiocapillary electrophoresis a valuable analytical technique. Altria *et al.* have described the CZE separation and detection of radiopharmaceuticals containing technetium, a γ emitter with a 6-hour half-life (*55*). Recently, Pentoney, Quint, and Zare (*56*) have designed a simple on-line radioisotope detector for ^{32}P-labeled molecules (^{32}P is a high-energy β emitter with a 14.3-day half-life) separated by capillary electrophoresis. The detector consists of a small semiconducting wafer of CdTe that is positioned near the outlet of the capillary tube and that responds directly to β particles emitted as the labeled sample zones traverse the capillary detection volume. Preliminary work indicates that the lower limit of detection is on the order of $10^{-9}M$ for measurements made "on the fly" with this system. A capillary electrophoresis separation of ^{32}P-labeled guanosine triphosphate (GTP) and adenosine triphosphate (ATP) injected at $5 \times 10^{-8}M$ and detected by on-line radioisotope monitoring is shown in Fig. 5.

Overview

As Joshua Lederberg has written (*57*), "The news of science usually concerns *what* results a scientist has achieved, not *how* he has found them. . . . All too often, the role of instrumentation—from the tedious steps in design, to commercial production, to routine maintenance—is taken for granted . . . . Yet, scientific progress continues to depend on the invention and refinement of tools and techniques." In these rites of passage, capillary electrophoresis is entering its puberty, but has not been initiated into the tribe of well-accepted instrumental techniques. Capillary electrophoresis is just now being commercially developed. Its failure or success will determine whether capillary electrophoresis can fulfill its promise as a separation technique for the analysis of complex mixtures and become another of those "routine methods of choice" that will be used by others, many of whom will be happily unaware of its origins and principles.

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