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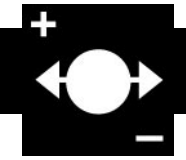
PREPARATIVE ELECTROPHORESIS



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Introduction

The development of electrophoresis in the early part of the 20th century proved to be an extremely important tool for the separation of biologically important molecules such as amino acids, peptides, proteins and DNA. There are four main types of electrophoresis, known as zone electrophoresis, step-field electrophoresis, isoelectric focusing and isotachophoresis. This review discusses the development of zone electrophoresis and step-field electrophoresis for preparative applications.

Electrophoresis instruments use mobility differences in the presence of an electric field to separate mixtures into individual components. The mobility differences between individual species are proportional to the net charge to size ratios of the species. Therefore, separations are most effective for solutes with large differences in this ratio. Additional separation mechanisms, such as molecular sieving using gels, have since been incorporated in the modes of electrophoresis to increase the range of applicability. At present, there are a wide range of electrophoresis instruments and methods which are used routinely in the biotechnology industry for analytical measurements. In contrast, the use of preparative electrophoresis is far less extensive.

The major distinction between analytical and preparative electrophoresis lies in the size and processing of samples. In the case of preparative electrophoresis, sample sizes are generally much larger (mg to g) in comparison with analytical electrophoresis (ng to μg) depending on the availability of the species of interest. Such large samples must not be too crude because irreversible adsorption of some unwanted species can render the system inoperable. The samples also require the collection of fractions after separation, which is often not the case with analytical separations. Thus, after separation, it is essential that the solutes can be easily removed from the buffer solution if they are to be prepared as pure compounds. The constitutions of buffer solutions must therefore be carefully chosen, preferably with volatile components, which facilitate collection of the species of interest.

Preparative electrophoresis systems require scaling up from the respective analytical systems and modifications to the instrumentation have been made which attempt to contend with complications arising from the scale-up. For instance, an inherent problem with electrophoresis is thermal convection caused by the flow of ions in the presence of an applied electric field. As electrophoresis systems are scaled up for preparative applications, convection problems and heat dissipation in the system become more significant owing to the decrease in the relative surface area. Anticonvective media such as filter paper, agarose, starch, glass powder or polyacrylamide have been used to limit these convection processes. The use of anticonvective media, however, has led to other problems, such as adsorption, endosmosis and diffusion. These combined factors have therefore prompted the development of a number of different designs of preparative electrophoresis systems that are suitable for continuous or batch-wise separations of multicomponent mixtures. Two basic strategies have evolved which are collectively termed preparative free-flowing electrophoresis or preparative gel electrophoresis. Both strategies exploit the same basic electrophoresis process for the separation, although both approaches have been used in a variety of configurations.

Preparative Free-flowing Electrophoresis

In the case of free-flowing electrophoresis, there is no anticonvective medium. Separation of samples therefore occurs in free solution and sample zone broadening, as a result of convection, tends to be more pronounced than in the analogous gel method. A support, such as paper, may be used for the buffer to flow across and is cooled to maintain maximum thermal stability in the system. One method of reducing convection processes in the system is to perform the investigations in a cool room (e.g. at 5°C). Such temperatures also ensure that there is no degradation of heat-sensitive samples.

Early reports of free-flowing electrophoresis were first made by Barrolier who devised a continuous separation process. A system was developed in which an electric field was applied perpendicularly to the direction of the buffer flow. Sample was introduced as a constant stream into the top of the system which travelled in the same direction as the buffer. As in the case of other electrophoresis systems, differences in the charge to size ratios of the solutes caused mobility differences in the presence of an electric field. This, in turn, brought about movement of the solutes perpendicularly to the pumped buffer flow, leading to a lateral separation between the two electrodes. Separators at the end of the support facilitated collection of the sample streams into pure fractions (**Figure 1**). The system was found to be suitable for a series of highly mobile dyes, but convection in the system made it more difficult to separate more complex samples. A more drastic approach to suppress thermally induced density gradients contributing to convection has been to perform electrophoresis experiments in space. The microgravitational fields in orbiting spacecraft provide a suitable environment for reduced convection in electrophoretic processes.

An alternative design for free-flowing electrophoresis apparatus was developed by Dobry and Finn: this utilized upward movement of the buffer and samples. In this apparatus, electrodes were separated from the system by means of semipermeable membranes placed perpendicularly to the cylindrical electrophoresis chamber. This caused differences in the direction of migration of the solutes, perpendicular to the direction of the buffer flow, and a range of dyes was successfully separated. On separation of the solutes, a series of outlet ducts at the top of the apparatus enabled collection of the purified species. Unfortunately, this early apparatus also appeared to have problems with convection because an effective cooling system was absent. This inferred the occurrence of a temperature gradient within the system, which would affect slower-moving protein mixtures more significantly.

It was not until recently, however, that the approach of reducing convective processes in free-flowing electrophoresis has been made simpler and more effective. This new approach has been labelled capillary free-flow electrophoresis. A coolant solution is continuously passed through a number of evenly spaced Teflon capillary tubes within the separation chambers and has been found considerably to reduce convection processes in the electrophoresis system

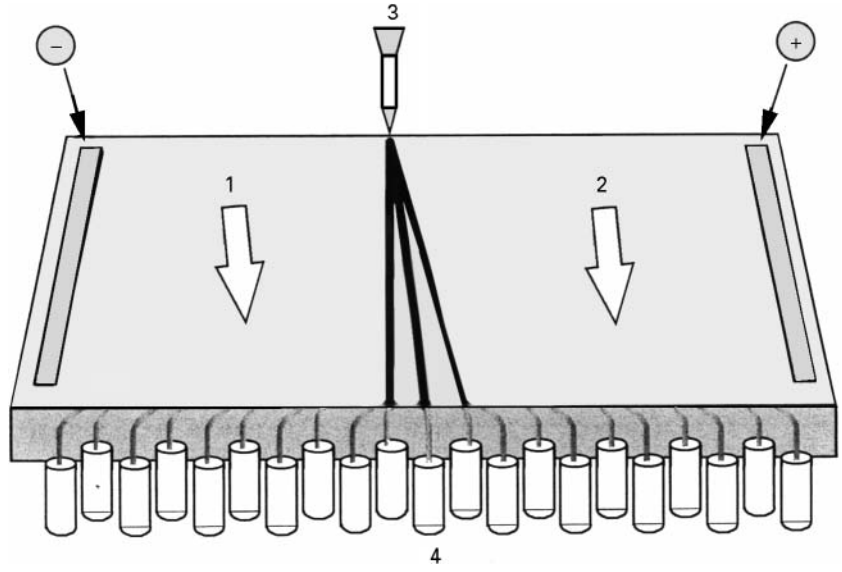

Figure 1 A free-flow electrophoresis system. The direction of buffer flow is indicated by 1 and 2. Sample enters continuously at 3 and purified solutes are collected at the vials at position 4.

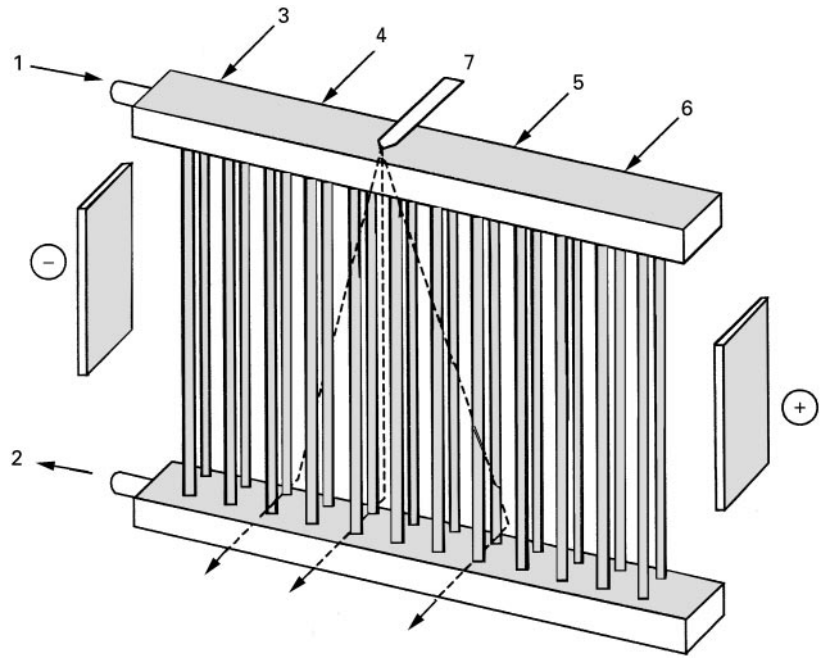

Figure 2 Design of capillary free-flow electrophoresis system. Cooling water enters at 1, passes through a series of parallel Teflon tubes (number reduced for simplification) and exits at 2. Buffer enters at ports 3–6 and sample stream enters at 7.

(**Figure 2**). The coolant tubes have not been found to affect the quality of separations adversely. As in the case of previous free-flow systems, the sample is continuously introduced into the system. Therefore the potential for the purification of large quantities of solutes is available (e.g. 1 g h^{-1}).

A more complex-free flowing electrophoresis instrument was designed by Hjertén to address the problem of convection. An apparatus was constructed in which the buffer flowed through a straight horizontal tube, which was rotated axially at constant velocity. The rotating tube alleviated the effects of gravitational convection and sedimentation. Although this system appeared suitable for analytical separations, convection was still a problem with regard to the quantities used for preparative use. The addition of anticonvective media, such as a density gradient, formed using sugar solutions, or the use of a gel was still required. This instrument was in fact the precursor of the capillary electrophoresis instrument.

Another unique approach to free-flow electrophoresis utilized a fluid endless belt. This design is analogous to a small conveyor belt in the vertical position. Movement of the solutes around the belt reduces convection and sedimentation processes occurring in the system. The sample is introduced as a continuous stream close to the electrode from which the solutes are repelled and are collected from the other side of the system, close to the electrode to which they are attracted. All solutes must, however, be attracted to the same electrode. The solutes travel around the belt several times during the electrophoresis process, moving approximately perpendicularly to the electric field. Solutes with the highest mobilities travel farthest across the belt and the separated solutes are collected at the outlets.

Preparative Gel Electrophoresis

The incorporation of gels as a solid support assists in the prevention of convectional distortion of the analyte zones. Another advantage of using gels is that there may be an additional effect of molecular sieving which can be exploited to enhance the separation of solutes such as proteins. As a result of these considerations, a number of different gel types have been developed for preparative electrophoresis systems.

One of the first designs of a preparative gel apparatus developed by Hjertén used a starch gel in the form of a horizontal slab. One of the main differences between this system and the free-flowing systems was that sample introduction was not continuous. Using this system, separation of milligram quantities of lysozyme and β-lactoglobulin was achieved over periods of about 40 h.

Another type of gel that has been used with success is agarose. Agarose has been found to be a better support than agar, owing to its purity, and displays very little electroendosmosis. Agarose also displays

little adsorption of proteins and therefore migration of solutes is similar to that in free solution.

The greatest number of investigations using preparative gel electrophoresis, however, has been with polyacrylamide-based gels. A significant advantage of using polyacrylamide gels is that pore radii (between 0.5 and 3.0 nm) can be easily controlled by adjusting the acrylamide concentration and the concentration of the cross-linking agent. As with analytical procedures, gel buffer pH and ionic strength as well as electrode buffer pH and ionic strength must be considered before separation.

A popular method of preparative separation is to use polyacrylamide with detergent in the buffer. Ryan *et al*. modified a preparative polyacrylamide gel for use with systems containing the anionic detergent sodium dodecyl sulfate (SDS). As in analytical gel electrophoresis, addition of the SDS allows separation based on the molecular weight of the solutes. The preparative gel method used was essentially that of Laemmli. This type of system has been found to be suitable for a large number of applications listed in the literature.

Early development in the use of polyacrylamide gels for preparative electrophoresis was stimulated by the development of discontinuous or disc electrophoresis by Ornstein and Davis. Disc electrophoresis enables sharpening of sample zones by using variable conditions within the electrophoresis system. Discontinuities in the separation system are achieved by incorporating different buffer compositions or the same buffer composition at different pH throughout the gel and/or by using voltage gradients during electrophoresis. One of the main features of disc electrophoresis is a concentrating step, which ensures that the sample is compressed into an extremely thin band before electrophoretic separation of the solutes. This type of gel usually consists of three components. The largest portion is the separating gel, which is preceded by a spacer gel and then a small mixture of the sample, in either gel or viscous solution. Lewis and Clark described such a system for the preparative separation of components from rat pituitary glands.

Another variant of disc electrophoresis employs the sequential use of two buffer systems at different pH. A pH close to the isoelectric point of one species is chosen to effect separation initially. This enables migration of one species whilst the other species moves very little. After separation of the two species, the pH of the buffer solution is changed, which causes a rapid increase in the mobilities of both species. Both species are continuously eluted from the gel where they can be detected followed by suitable fraction collection. This approach was found to resolve two forms of phosphoproteins successfully.

Gel Configurations

Since the initial introduction of preparative gel electrophoresis systems in the early 1960s, a range of different instrument designs have also been developed to incorporate gels. Some systems have used glass and others have used Lucite™ (Perspex) for mechanical support of the gel. An advantage using glass is that it has higher thermal conductivity than Perspex, but it has been found that, as the walls become narrower, surface effects from glass become enhanced. In contrast to free-flowing electrophoresis, the use of anticonvective media in these systems allows for magnetic stirrers to be used in the buffer reservoirs. These maintain uniform concentration of buffer components and also prevent bubble trapping. The units have mainly been either cylindrical or slab-like and electrophoresis has been performed in either a vertical or horizontal orientation. In the case of cylindrical gels, gel column heights are important and mainly depend on the diameter of the column used and the sample loading. Improvements in the vertical column cylindrical gel systems have been made by addition of direct cooling. Convection processes have also been reduced by forming the gel around a central cooling capillary. In these cases, elution buffer can be utilized to carry samples to a UV detector before fraction collection and also to cool down the gel as it passes through the central capillary.

A preparative gel apparatus developed by Hediger used a completely sealed system. External buffer reservoirs were used for the electrode and elution buffer. The gel was placed between two adapters in a vertical cylinder. The upper adapter, which was easily movable since it was motor-driven, had a gel-pouring device, which enabled easy preparation of both gradient and nongradient gels.

Slab gels can be run in a vertical or a horizontal position and were initially designed in order to increase the amounts of sample that could be handled in one electrophoretic run. It is not clear whether slab gels offer improved separations over cylindrical gels, owing to the problems associated with cooling and fraction collection in comparison with the column gels.

Hjertén developed a large-scale system that involved a vertical slab gel. This apparatus enables the separation of up to 1 g of material. After elution from the separation chamber, the solutes enter a granular bed of agarose spheres. They are then displaced from the granular bed by a buffer flow, which transfers them to a fraction collector. The system has been used effectively for the separation of a series of proteinases.

The development of an annular-shaped electrophoresis apparatus by Southern enabled a larger

surface area of the gel to be utilized when compared to gels of conventional geometry. The outer electrode is wrapped around the gel and the inner electrode runs on a bobbin on the inside. A rapid flow of buffer passes between a semipermeable membrane and the central electrode to facilitate cooling of the gel. Sample is introduced into a small slot that runs around the periphery of the gel annulus. The material moves from the outside of the gel in concentric circles towards the centre, where it is eluted. Solutes are therefore moving from a large area to a much smaller area, which enables the use of high sample loads. In this case, the fraction volume was kept to a minimum by employing discontinuous elution. The electronics for this system were later modified so that, after electrophoresis was stopped, the current was reversed for a short period of time to remove solutes adsorbed on to the semipermeable membrane surrounding the electrode, before eluting the purified samples from the gel.

Sample Recovery using Preparative Gels

In contrast to free-flow electrophoresis, where sample collection is performed during electrophoresis, preparative gel electrophoresis allows samples to be collected after electrophoresis. Also, most preparative gel electrophoresis is performed as a batch process. There are two methods for recovering the purified samples when using preparative gel systems. Solutes can either be recovered from the gel after the electrophoretic separation, or eluted from the gel during electrophoresis and collected sequentially. In the first case, solutes can be recovered from the gel mechanically (analogous to thin-layer chromatography) or by using electrodialysis. In one example, using polyacrylamide gel columns, samples were cut from the gel using a jig. This method enabled sections to be cut reproducibly from subsequent gels.

The second recovery mode involves electrophoretic migration of analytes from a cylindrical gel using a continuous elution system. As electrophoresis proceeds, the end of the gel is continually flushed with a buffer solution and solutes are transported from the gel. This enables samples to be detected as they leave the system using some form of online detection. After detection, a fraction collector can be used to establish the integrity of the purified samples. An important part of the design of the continuous elution system is a dialysis membrane which is usually placed between the end of the separation gel and the bottom buffer solution. This membrane prevents migration of the solutes into the main buffer solution. For continuous elution systems, elution buffer pH and ionic strength must also be considered.

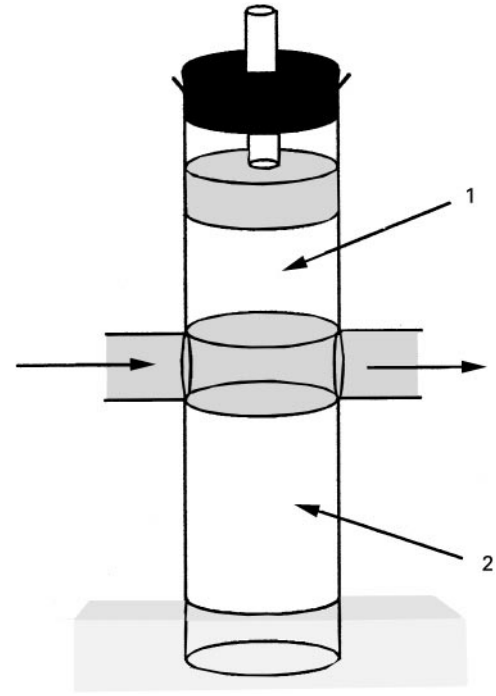

Figure 3 Side-arm apparatus. The top gel is 1 and the bottom gel is 2. The arrows indicate the elution buffer flow collecting solutes.

A variant of the continuous elution recovery system employs continuous elution from between two gel column halves. A dialysis membrane, placed on top of the bottom gel, ensures complete elution of the solutes as they reach the end of the separating gel. The assembly consists of a 2 cm outer diameter glass tube with two side arms through which buffer is pumped (**Figure 3**). A system of this type was effective for the separation of a series of bean leaf proteins.

Another method of collecting solutes, after electrophoresis, has been developed and is known as discontinuous elution. Electrophoresis is stopped for short periods of time whilst solutes which have passed through the gel are collected from a cup at the bottom of the gel. This method is believed to reduce dilution of the samples in comparison with continuous elution systems. After removing the solutes and buffer solution from the cup, the cup is refilled with fresh buffer solution and electrophoresis is reinitiated.

Discontinuous sample collection may be fairly time-consuming owing to the intermittent sample collection process. This was one of the reasons that led to the development of automated preparative gel electrophoresis systems. One such system was designed by Hodson and Latner. After a period of time, electrophoresis in the system is stopped and buffer, containing solutes which have passed through the gel, is collected and the buffer chamber refilled. Up to

300 mg of protein mixtures may be separated using the system, with the potential for further scale-up.

Another discontinuous sample collection system, for use with slab gels, has been developed by Polsky *et al*. An electronic timer was used which controlled the collection of samples and refilling of buffer in the elution chamber. Electrophoresis was terminated during collection of the samples. Polyacrylamide slab gels were found to have much higher sample capacities than agarose gels. Two practical considerations concerning the buffer solution were made during this work because of run times exceeding 100 h. Chloride-free buffers were used to prevent build-up of chlorine gas and sterile solutions were used: these inhibit growth of bacteria in the system. The system was effective in resolving and collecting fragments of genomic DNA.

Buffer Types

In accordance with analytical electrophoresis, a wide range of buffers is also available for preparative electrophoresis. Essentially, the type of buffer used in the system depends on the type of sample to be separated and on the type of separation system used. High ionic strength buffers can cause significant Joule heating in a system, whereas low ionic strength buffers may cause protein aggregation.

Detection

One of the main differences between analytical and preparative electrophoresis is that quantitative detection of solutes is not as critical. This enables cruder detection methods to be used in preparative work. These detection systems can be used in real time with online detection or, in the case of some noncontinuous elution systems, after the separation has finished.

In continuous elution systems, factors which are important in terms of sample detection are the elution buffer flow rate and the elution buffer fraction volume. High elution buffer rates can cause the solutes to become too dilute for the method of detection. Slow buffer rates may cause loss of resolution between the solutes owing to diffusion. One of the most popular forms of online detection is ultraviolet-visible spectrophotometry. Not all solutes absorb or fluoresce in this region of the spectrum, but detection can be achieved in some cases using dye-staining methods. Offline detection includes light scattering, radiography, densitometry, analytical gel electrophoresis and mass spectrometry.

Commercially Available Systems

A number of preparative electrophoresis systems were available in the early 1960s but, owing to lack of demand, are no longer available. This is probably a result of increased competition from other preparative methods such as high performance liquid chromatography.

A large preparative free-flow electrophoresis system was developed at the Harwell Laboratory of the UK Atomic Energy Authority, known as the Biostream separator. This was a large apparatus in which

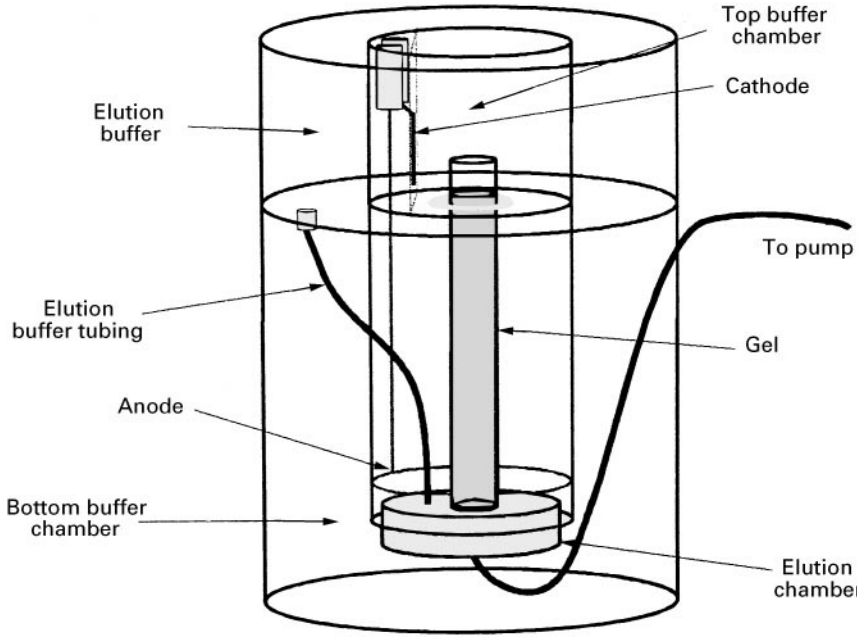

Figure 4 Bio-Rad Mini Prep-Cell.

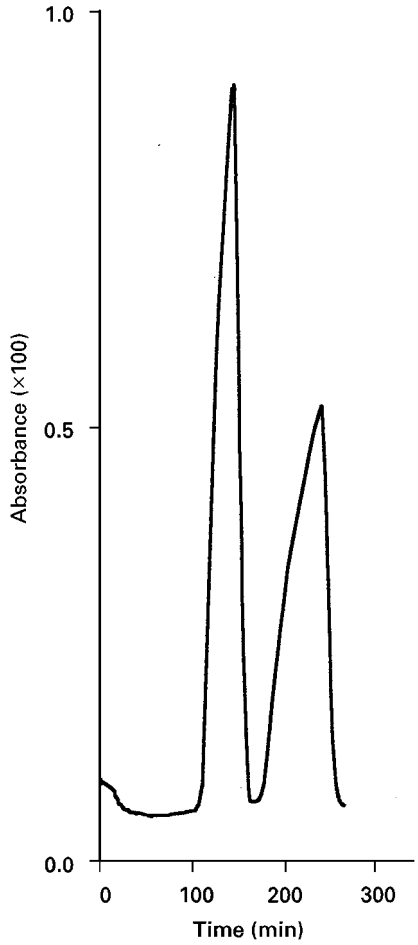

Figure 5 Separation of piperoxan enantiomers using the Mini-Prep Cell.

laminar flow conditions were maintained by rotation of the outer wall of the annulus. Although allegedly being capable of preparing 100 g h^{-1} of protein, it never became readily available on the commercial market.

Bio-Rad Laboratories (Hercules, CA) currently have several preparative gel electrophoresis systems for use with gels (**Figure 4**). These are continuous elution devices based on vertical column gels. One of the larger systems has a ceramic core that is used for cooling the gel.

A capillary free-flow electrophoresis system has been patented by R&S Technologies. The device is believed to be considerably better than previously produced free-flowing electrophoresis systems owing to reduced convection in the system from the capillary cooling.

Applications

As in the case of many of the systems described previously, the main applications for preparative gel electrophoresis systems are large biomolecules such as nucleic acids, enzymes and antigens. Recent developments in preparative gel electrophoresis, however, have extended its applicability to the preparation of enantiomerically pure pharmaceuticals (**Figure 5**). This has been achieved by the addition of a sulfated β-cyclodextrin chiral additive to the buffer of a system employing a vertical column agarose gel in the apparatus shown in **Figure 4**.

Future Developments

The continued development of analytical and preparative instruments can only lead to further improvements in some of the systems previously mentioned. Limitations in design and development of these devices are reducing significantly as engineering and electrical methodologies improve. In contrast to the continuing development, and more widespread use of preparative gel electrophoresis, continuous free-flow electrophoresis instruments offer an interesting prospect for development now that the problems of convection have been more fully addressed by the use of capillary cooling.

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