the sensitivity of gold stain can be further enhanced by incubation with a silver lactate solution such that as little as 400 pg of protein per band can be detected. Although nylon or charged nylon membranes possess the greatest protein-binding capacitites (450 vs. 80 $\mu g\,cm^{-2}$ (NC/PVDF)), staining of nylon membranes is very problematic. Anionic organic dyes as well as colloidal gold and silver are not useful for staining nylon membranes due to extremely high backgrounds. However, colloidal sols are especially useful for the detection of proteins on nylon membranes. On nylon membranes the positively charged colloidal iron particles bind to negatively charged SDS-denatured proteins and protein staining can be intensified using potassium ferricyanide, which gives deep blue-stained bands with low backgrounds. India ink and a modified silver stain have been reported to have been used to stain charged nylon membranes.

A less frequent, but none the less useful method for the visualization of protein bands on NC and charged nylon membranes involves protein iodination *in situ* with chloramine T/potassium iodide, followed by formation of a purple complex between the bound iodine and starch.

Autoradiographic Detection

Electroblotting of proteins radiolabelled with ^{14}C or ^{35}S permits more efficient autoradiography since the gel matrix is no longer present to quench the β-emissions. The minimum level of ^{14}C or ^{35}S that can be detected in 24 h is about 400 $dpm\,cm^{-2}$. While fluorography is necessary to detect ^{3}H on polyacrylamide gels, ^{3}H exposure can be detected directly on electroblots using autoradiography, although 2×10^4 $dpm\,cm^{-2}$ is required for detection in 24 h. The efficiency of detection for all isotopes is enhanced if fluorography is employed (100 dpm and 500 $dpm\,cm^{-2}$ for $^{14}C/^{35}S$ and ^{3}H, respectively).

Future Developments

PAGE, in particular 2D-PAGE, remains the method of choice for the separation of complex protein mixtures. This has necessitated the development of highly sensitive protein visualization protocols incorporating both nonradioactive and radioisotopic imaging methodologies. The development of methods for the transfer of polypeptides from gels to membranes where they are readily accessible to react with stains, specific antibodies, group-specific ligands and detailed structural characterization, including amino acid microsequencing and mass spectral analysis, has permitted the identification of previously unidentified proteins. Further developments are likely to take place in low background staining polyacrylamide formulations and modified membrane support matrices in which proteins may be bound either covalently or which form reversible covalent bonds. Such proteins can be easily and selectively eluted for more detailed biochemical studies. Future advances are likely to take place in the development of more sensitive and group-specific dyes/stains and increased speed and sensitivity of detection systems such as the enhanced bioluminescent and chemiluminescent systems, as well as the development of faster and more sensitive photographic detection film.

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Detectors for Capillary Electrophoresis

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Detection is a particularly critical issue in capillary electrophoresis (CE) because of the extremely small cell volumes available. Considerable effort has gone into overcoming this limitation and a bewildering

variety of methods has been described, ranging from the straightforward adaptation of existing chromatography detectors to less obvious and highly experimental techniques. The path has not been smooth, but many obstacles have turned out to be less serious than anticipated. Optical methods have proved very useful despite the short pathlengths involved. Electrochemical detection methods, intuitively considered incompatible with the applied high voltage and long neglected in favour of optical means, are now readily implemented. However, the development of detection methods is still in flux and it may take several more years before maturity is reached and different methods have found their established roles for particular applications.

The Detection Challenge

The internal diameters of the capillaries employed in CE range from 100 μm down to about 5 μm and a single analyte zone is approximately 1 mm long. Because the detection volume has to be smaller than the peak volume available, detection volumes range from about 1 pL to 1 nL. In high performance liquid chromatography (HPLC), in contrast, detection volumes of at least 1 μL are available. One would therefore expect sensitivities for CE to be several orders of magnitude lower than those in HPLC and, as a consequence, the detection limits to be much inferior. However, in CE the sample does not experience significant dilution before it reaches the detector, as is the case in HPLC, because of the flat flow profile in CE. Therefore, the sensitivities are not in fact as significantly degraded in comparison with HPLC as might be expected. Nevertheless the issue of detection limits is still critical in CE and detector sensitivity is not always adequate. Preconcentration by electrostacking is sometimes advocated, but this method is only possible for samples with low ionic strength and generally leads to poor precision unless an internal standard is employed.

Because of the small detection volumes, on-column detection schemes are required to avoid band broadening, rather than detector cells attached in an off-column arrangement as is the case in chromatography. A unique property of detection in electrophoresis, which is not shared with chromatography, is the fact that there is a dependence of the peak area (expressed on a time basis) on migration velocity. However in practice this is usually of no concern. Detection methods may be grouped according to whether a bulk property of the solution (such as conductivity, refractive index) or a specific attribute of the analytes (such as optical absorption or fluorescence, redox activity or membrane permeability) is monitored. Detectors used in the first case tend to be more universal, but generally suffer from the presence of a large background signal against which small changes have to be distinguished. This often leads to poor signal-to-noise (S/N) ratios and hence relatively high detection limits. The exploitation of specific interactions is generally better in this regard, but each method is usually only applicable to a certain class of analytes. Some of the specific detection methods also allow additional information on the analyte to be gathered, which may be desirable as migration times can never be taken as absolute proof of identity. These detectors may be termed 'information rich', and include for example mass spectrometers, photodiode arrays and voltammetric detectors.

Important general characteristics of detectors are their sensitivity, dynamic range, and linearity. The term sensitivity generally denotes the gradient of the calibration curve but the precision of the measurement (S/N ratio) has to be considered as well for a complete evaluation. Often, the term sensitivity is used to indicate the lowest concentration that may be detected (limit of detection, LOD) and these parameters are of course interrelated. In CE detection limits are sometimes quoted in terms of the detectable mass or number of moles, as impressive figures in the pico- or atto-gram or -mole range can be given because of the small sample volumes used. However, the standard concentration limits are much more useful and meaningful. The dynamic range is encompassed by the detection limit and by a maximum where a loss of sensitivity occurs. Wide dynamic ranges are desirable as they simplify sample preparation, but they often go hand-in-hand with relatively poor precision. The upper concentration limit in capillary electrophoresis is generally determined by the ionic strength of the background buffer (typically 1–10 mmol).

The choice of detector is guided by the requirement of the application in terms of detection limit, selectivity and information requirements but to a large degree also by commercial availability, cost, robustness and ease of use. Some features of the major detection methods are summarized in **Table 1**.

Optical Methods

Optical detection methods are more widely employed than any other detection means. Commercial CE instruments with optical absorption detectors were introduced in 1989 and are available from a variety of instrument manufacturers. The detectors employed have often been adapted from devices used in HPLC and this may be part of the reason for the prevalence of the ultraviolet (UV) absorption detection method.

Table 1 Main detection methods for capillary electrophoresis

Method	*Features*	*Detection limits*[a] *(mol L^{-1})*
UV/Vis absorption	Readily available commercially	10^{-7}
Indirect UV/Vis absorption	Compromise with poorer detection limits for nonabsorbing species such as most inorganic ions	10^{-5}
Fluorescence	Good detection limits but most species require derivatization; available commercially	10^{-9}
Laser fluorescence	Elaborate; excellent detection limits; available commercially	10^{-11}
Conductometry	Good for small ions; available commercially	10^{-6}
Amperometry	Simple, but only possible for electroactive ions; not available commercially	10^{-8}
Mass spectrometry	Provides information on peak identity; expensive; interfaces available commercially	10^{-8}

[a]The values given should be considered as rough guides only, as these are often very much dependent on species and instrumental set-up. UV/Vis, ultraviolet–visible.

Fluorescence-based detectors are not as widely used but are also on the market.

To carry out on-column detection the usual polyimide protection coating has to be removed from the column by burning, by dissolution with hot sulfuric acid, or by mechanical scraping, to form a window into the capillary. The material is fairly brittle, so that care has to be taken to avoid breakage once the protective cladding has been removed. Fused silica capillaries are transparent even below 200 nm, so that the near-UV range is readily accessible.

The basic cell arrangement for absorbance measurement through a capillary is illustrated in **Figure 1**. Generally, besides the light source, there is a monochromator or optical filter to define the wavelength employed, a lens and aperture, and a photodetector. Variations of this arrangement are possible. Most commonly wavelengths in the UV range from about 250 nm down to 185 nm are employed, using different types of sources such as deuterium lamps, but instruments that include the visible range are also available. Variable wavelength as well as fixed wavelength arrangements are in use. It is important to get a high light intensity transmitted onto the detector for best S/N ratio. The usual UV light sources, such as deuterium lamps, are larger than the optical cell and it is only possible to focus a small fraction of the radiation emitted through the cell even with the best available lenses. Ball lenses, mounted directly adjacent to the capillary, are often employed. Apertures are required to minimize the amount of stray light reaching the detector. Optical fibres can be used for transmission of the radiation as this allows efficient electrical shielding of the photodetector and at the same time the distal ends form the optical apertures. Absorbance detectors based on light-emitting diodes (LEDs) and laser diodes have also been demonstrated. These devices give high baseline stability because of the absence of flicker noise present in discharge lamps and allow the construction of battery operated instruments because of their low power consumption. However, these devices are not available for the UV wavelength range.

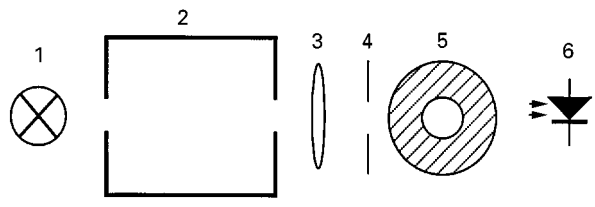

Figure 1 Schematic representation of absorbance detector. 1, Light source; 2, monochromator or optical filter; 3, lens; 4, aperture; 5, capillary; 6, photodiode or photomultiplier tube.

The circular cross-section of the capillary is far from ideal for absorbance measurements because it is not possible to pass collimated light through the interior of the tube without refraction. This means that changes in the refractive index of the solution are a potential source of interference. In practice, however, the only serious limitation appears to be the short optical pathlength, which leads to low sensitivity according to the Lambert–Beer law. For this reason the largest capillary diameters that allow efficient cooling are usually employed in absorbance detection, typically with an internal diameter of 50–75 μm. Different methods of increasing the sensitivity in absorbance detection have been described. These include the use of rectangular capillaries, capillaries bent in a Z-shape to obtain a longitudinal light path, multipass cells by multiple reflections in silver-coated capillaries, and so-called bubble cells formed on the capillary itself. Only the last approach is reasonably easily implemented, and it appears to be the only one that is commercially available (albeit at a cost much higher than that of ordinary capillaries).

The internal diameter of the capillary is widened in the detector region by a factor of about three, thereby increasing the sensitivity by the same magnitude.

A different approach to increase the sensitivity of absorption measurements is the use of thermooptic methods. Here the heat evolved following the absorption of light is sensed indirectly. In the thermal lens method the refraction of a laser light beam is measured, using a relatively simple arrangement. Two light beams perpendicular to each other are employed. One of the beams is of a wavelength that is absorbed by the analytes. The heat evolved through absorption of light leads to a refractive index gradient in the capillary which is monitored by the second beam. A variation on this technique has been reported that uses intensity-modulated light. This leads to a vibration of the capillary that may again be detected with a second probe beam. Ordinary refractive index detection has also been described using the deflection of a laser beam but neither of these two techniques has gained much acceptance.

Most organic analytes possess chromophoric groups that show intrinsic absorbance in the near–UV range, so most methods are based on this wavelength region. It has been demonstrated that for these species it is preferable to use wavelengths that are as short as possible (below 200 nm) for the best sensitivity. Photodiode-array detection is also possible. This technique yields additional qualitative information on the identity of the detected species and allows peak inhomogeneity to be detected. However, the S/N ratio and therefore the detection limit, which is always critical in CE, are degraded because of the reduced integration time available, and the method requires considerable computing power, because of the large amount of data acquired. Ions that do not show absorbance in the UV/Vis range, such as inorganic species or completely saturated organics, may be determined by indirect methods. These methods rely on the displacement of dye molecules of equal charge as the analyte species (to maintain electroneutrality) so that a decrease in absorbance is detected. This is more demanding on the stability of the system than the direct absorbance method and the detection limits are generally higher. However it is the only method employing optical absorbance detectors to be available for most inorganic anions. Chromate is often used as the background ion but other species, some for the visible wavelength range, have also been reported. Inorganic cations can also be detected by indirect means, but many of them are best determined via the formation of coloured complexes using non-discriminating ligands.

Fluorescence detection is also possible and is commercially available. However, few species display intrinsic fluorescence, so derivatization reactions have to be employed. Derivatization may be classified as pre-column, on-column or post-column according to the scheme employed. Fluorescence has the great advantage of much higher sensitivity than absorbance measurements. Detection limits approaching single molecule detection have been achieved. Lasers appear to be ideal light sources for fluorescence measurements, as the light is produced in a tightly focused beam well matched to capillaries, but inexpensive sources are not available for the UV range and available lasers are often plagued by insufficiently stable output intensities. This limits their use, especially for the more universal indirect detection scheme. Nevertheless, impressive results have been obtained for microbiological applications (e.g. in neuroscience) that include the analysis of single cells. Chemiluminescence detection is usually based on the influence of analytes on the efficiency of one of several available chemiluminescence reactions. The achievable sensitivities are very high, a feature this method has in common with fluorescence. Its implementation is similar to post-column fluorescence detection in that a pumped reagent stream has to be merged with the column effluent in a suitable small-scale mixing device prior to detection in a light-tight enclosure with a photomultiplier tube.

Electrochemical Methods

Electrochemical detection techniques in general are developing rather more slowly than optical techniques, even though some of the earliest examples of open tubular electrophoresis were based on electrochemical detection. It was considered that the electrical field applied for separation was a serious hindrance. Also, the exposure of the detector to the buffer solution (which is not the case for the optical methods) is a potential source of problems as the electrodes may corrode or be affected in other ways. While the common optical detection methods have reached maturity, the same cannot be said for the electrochemical methods. Of the three reported methods, namely conductimetry, amperometry and potentiometry, the former is the only one that is commercially available at this time. Nevertheless, these methods have attracted considerable attention and in general are much simpler than other methods.

In an early approach to conductivity detection, the cell was formed by drilling a hole perpendicularly through the capillary with a laser and then inserting two small wires that faced each other. In this way the two detector electrodes were not exposed to a voltage gradient. Another approach, which is still used by some workers for amperometric detection, is to

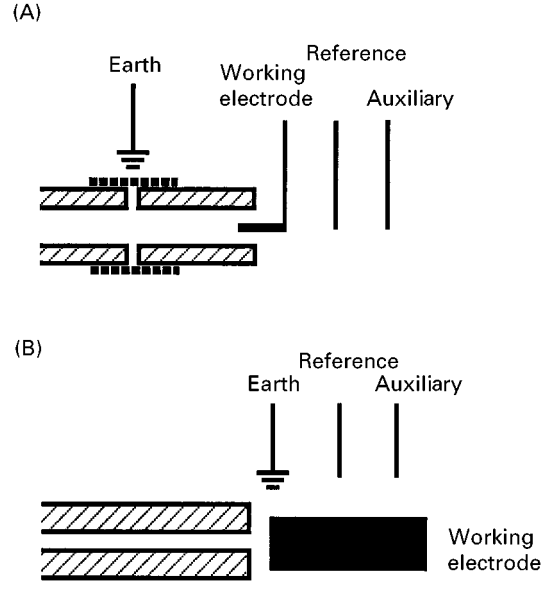

Figure 2 Common arrangements for electrochemical detection. (A) Decoupled configuration with on-column detection using a micro-electrode. (B) Wall-jet arrangement possible with capillaries with internal diameters of 50 μm or less, showing a relatively large electrode at a suitable distance from the capillary end.

decouple the detector from the electrical field. This is achieved by creating a small gap in the capillary and using a sleeve typically made of an ion exchange membrane to provide a contact to the electrical earth. This arrangement is illustrated in **Figure 2**A. The analytes are pushed forward to the detector electrode(s) by the pressure created by the electroosmotic flow. This arrangement is ideal in an electrical sense but cumbersome to implement. However, it was later realized that if electrodes are positioned immediately outside the end of capillaries that have internal diameters of 50 μm or less, then the electrical bias on the detector is minimal. This arises because the cross-section of the liquid volume outside the capillary is considerably larger than that inside, so that the remaining voltage drop between the end of the capillary and the electrophoretic earth electrode located a few millimetres away is a few hundred millivolts only. Inside the capillary voltage drops of 30 V mm^{-1} are typically encountered. Also, the electrical current through the capillaries is considerably lower for smaller internal diameters. This so-called wall-jet arrangement (**Figure 2**B) is the one used in commercial instruments for conductivity detection, and this same configuration is also frequently employed for the other two electrochemical detection methods. A further feature of the wall-jet configuration is the use of electrodes with diameters larger than the internal diameter of the capillary, which was found to be possible without significant loss of peak resolution. This allows the construction of relatively simple cells for the alignment of capillary ends and electrodes.

In conductimetric detection it is essentially the same property which is responsible for the separation, namely the mobility in the electrical field giving rise to a detector signal. This means that in principle any species that can be separated by CE may be detected by conductimetry. However, the need for an electrolyte in the running buffer leads to the presence of a background signal against which the analyte signal has to be measured. As the analyte displaces ions of the same charge (the same feature exploited in indirect absorbance detection), it is the difference in conductivity (caused by a difference in mobility between background and analyte ions) that leads to a detectable signal. To optimize the sensitivity the conductivity of the background buffer should be low, a requirement that conflicts with the need for matching the mobility of the buffer to that of the analytes to prevent peak tailing or fronting. A compromise therefore has to be made. For analytes with low conductivity indirect detection may be employed using a background electrolyte with high conductivity.

Conductivity detection can also be carried out in a contactless configuration with two tubular electrodes placed over the capillary. These then form capacitors (albeit with small capacitance values) with the liquid, whose conductivity can be probed with an applied high frequency alternating current. Electrode degradation is prevented in this mode. The sensitivity of conductivity detection can be improved by the so-called suppressed detection technique, known from ion chromatography, in which the background conductivity is largely removed by using a weak acid or base that is rendered neutral by ion exchange before the detection cell. However, for CE an arrangement similar to that used for electrical decoupling is required for suppression. This is difficult to implement and the method has not found wide use.

Amperometric detection may be employed for ions that are electroactive, i.e. that can be reduced or oxidized at electrodes. Different classes of species show this property, including heavy metal ions, certain inorganic anions, and many different organic molecules that incorporate reactive groups such as phenols, aldehydes, amines, etc. Many applications of amperometric detection have been reported but these have certainly not been fully explored yet. As the detection limits of amperometry tend to be good this approach is useful when low concentrations are to be determined. Please note that the terms 'electrochemical detection' and 'EC detection' are often employed with the sole connotation of amperometric detection, a usage that has evolved in the context of HPLC detection methods. This may lead to confusion as

conductimetry and potentiometry clearly are electrochemical methods as well.

At this stage detector cells for amperometry have to be constructed in-house as (at least to our knowledge) no commercial units are available. However, all the other parts required to set up a CE instrument, including potentiostats to operate the detector, are available commercially in modular form. In the wall-jet arrangement, the voltage applied to the working electrode by the potentiostat circuitry is superimposed by a voltage bias that is not only dependent on the applied separation voltage but also on parameters such as buffer composition, capillary diameter and the exact position of the electrode. For this reason some workers continue to use the decoupled detector. Amperometric detection in CE in principle requires a total of four electrodes at the detector end of the column. Besides the detector electrode (the working electrode of the potentiostat circuitry) and the electrophoretic earth, a reference electrode and a counter (or auxiliary) electrode are required. It is possible to simplify this configuration by employing the electrophoretic earth as a pseudo-reference and as a counter electrode as well.

Different electrode materials may be used in amperometric detection including gold, platinum and glassy carbon to suit different applications. The use of copper wire electrodes has proved to be useful as several oxidation reactions are catalysed on this material. Pulsed amperometric detection (PAD) may be employed when reaction products lead to a fouling of the electrode. Voltammetric detection in which the applied electrode potential is swept rapidly and repeatedly over the range of interest to gain additional information on the peak identity via the redox potential is also possible. Somewhat higher detection limits may have to be accepted, however, for these pulsed methods.

Potentiometric detection with ion selective electrodes is the least reported of the three electrochemical detection methods. The matching of a separation method with a sensor (rather than a detector which by definition is not selective) may appear to be a contradiction, but ion selective electrodes are in fact rarely highly selective and may be tailored to be responsive to a range of ions. So-called Hofmeister electrodes discriminate solely on the basis of the lipophilicity of the anions or cations, and are therefore at least in principle well suited for the determination of singly charged organic species. Early reports on this technique were based on micropipette ion selective electrodes known from physiological studies on single cells. These electrodes consisted of glass capillaries, with tip diameters of a few micrometres, that were filled with a viscous organic solvent incorporating an ionophore and acted as ion selective membranes. However, these electrodes were not very robust and have now been superseded by more reliable miniature coated-wire ion selective electrodes. These detectors have been used to detect a variety of inorganic and organic species that otherwise could not be detected with CE, or could only be detected with difficulty. It is possible to use a copper wire electrode as a simple potentiometric detector for amino acids in CE.

In summary, the three electrochemical methods may be considered to be complementary. Conductivity detection is a versatile general method that works best for small ions of high mobility. Amperometric detection is useful for electroactive ions and good detection limits can be expected. Potentiometric detection has currently been relatively poorly explored, but may prove to be a useful alternative for large, singly charged ions that cannot be detected amperometrically or by direct optical absorption measurements.

Other Methods

Detection methods other than optical or electrochemical methods have also been reported. One method that is fairly widely used is mass spectrometric detection, including detection by inductively-coupled plasma mass spectrometry, and commercial interfaces are available. Detection by nuclear magnetic resonance is also an established technique. These methods are covered elsewhere in this encyclopedia. Radioisotope detection has been reported and good detection limits have been achieved.

Future Developments

The acceptance of CE depends to a large extent on the availability of suitable robust detection methods with good detection limits. Shortcomings appear to exist for trace analysis of organic species that do not fluoresce, and for inorganic species. Thermooptic and chemiluminescence methods are promising in this regard as are the electrochemical methods of amperometry and conductivity. The last two methods appear to have reached some degree of maturity and it is hoped that these will become more readily available commercially.

See also: **II/Chromatography: Liquid:** Detectors: Ultraviolet and Visible Detection; Detectors: Mass Spectrometry; Detectors: Fluorescence Detection.

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Discontinuous Electrophoresis

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Introduction

Electrophoresis is one of the most powerful tools in the arsenal of separation scientists. It is commonly employed in the field of biochemistry, where separation of complex mixtures of proteins or nucleic acids is a continuing challenge. Numerous variants of electrophoresis have been described with the goal of optimizing the speed and effectiveness of the separations. One important electrophoretic variable is the separation matrix. It provides the retarding forces, or sieving qualities, that counter the electrophoretic transport. These forces can ultimately effect the separation and can be altered by the matrix type or concentration. Cross-linked or linear forms of polymers such as agarose or acrylamide are common choices. Different formats for the electrophoresis medium can also have dramatic effects on the resolution and separation time. This is exemplified by recent uses of microcapillary formats which greatly speed up separations. Another component that dictates the speed and resolution of electrophoretic separations is the charge-carrying buffer ion. The buffer is a universal component of electrophoresis, independent of gel constitution or format. This component is often overlooked, though attention to this aspect can be beneficial in developing electrophoresis-based separation techniques.

Proper buffer selection offers several practical advantages, including optimum separation times, increased band concentration and reduced effects of diffusion. The resolution of closely migrating species results from the proper choice of the pH, concentration, and type of buffer ion. These physical characteristics define the conductivity of the electrophoretic medium and affect the transport of the molecules to be separated. Inorganic ions, such as chloride anion, have high conductivities in comparison to the ionized form of weak acids and bases. Such high mobility ions offer little advantage when used for the electrophoretic separation of large, less mobile biomolecules but slower-moving ions, such as those of weak acids or weak bases, are more useful choices. These not only buffer the pH but, due to the slower mobility of these ions, lead to better separation of charged macromolecules.

Zonal electrophoresis utilizes a single buffer in the gel and reservoirs. An alternative to the continuous buffer, zonal separations is a discontinuous system where multiple ionic components are used. The presence of multiple ionic components in electrophoresis leads to discontinuities in the voltage gradient, pH and ionic strength due to the different physical mobilities of the ions involved. These different mobilities lead to the formation of discrete zones of ions that, under equilibrium conditions, travel at a constant rate in an applied electric field. Adjustment of these mobilities involves alteration of the ion concentration and potential gradient of the zone. Sharp boundaries can exist between these zones, with the ionic concentration being dictated by the Kohlrausch regulating function. The technique is similar or identical to a number of electrophoresis techniques that are known as discontinuous multiphasic, multizonal,