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In the Classroom

Tutorial: Capillary Electrophoresis*

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Capillary electrophoresis is the most efficient separation technique available for the analysis of both large and small molecules.

Capillary electrophoresis (CE) is electrophoresis performed in a capillary tube [1–3]. It is the most efficient separation technique available for the analysis of both large and small molecules. The transformation of conventional electrophoresis to modern CE was spurred by the production of inexpensive narrow-bore capillaries for gas chromatography (GC) and the development of highly sensitive on-line detection methods for high performance liquid chromatography (HPLC).

The basic instrumental set-up, which is illustrated in Figure 1, consists of a high voltage power supply (0 to 30 kV), a fused silica (SiO_2) capillary, two buffer reservoirs, two electrodes, and an on-column detector. Sample injection is accomplished by temporarily replacing one of the buffer reservoirs with a

*In the past 20 years, the number of instrumental techniques available to the chemist has grown exponentially. In order to help our readers keep up with this rapidly growing field, tutorial articles on chemical instrumentation will be a regular feature of *The Chemical Educator*. The articles are designed to serve as a brief introduction to emerging instrumental techniques, with an outline of the underlying principles and major applications.

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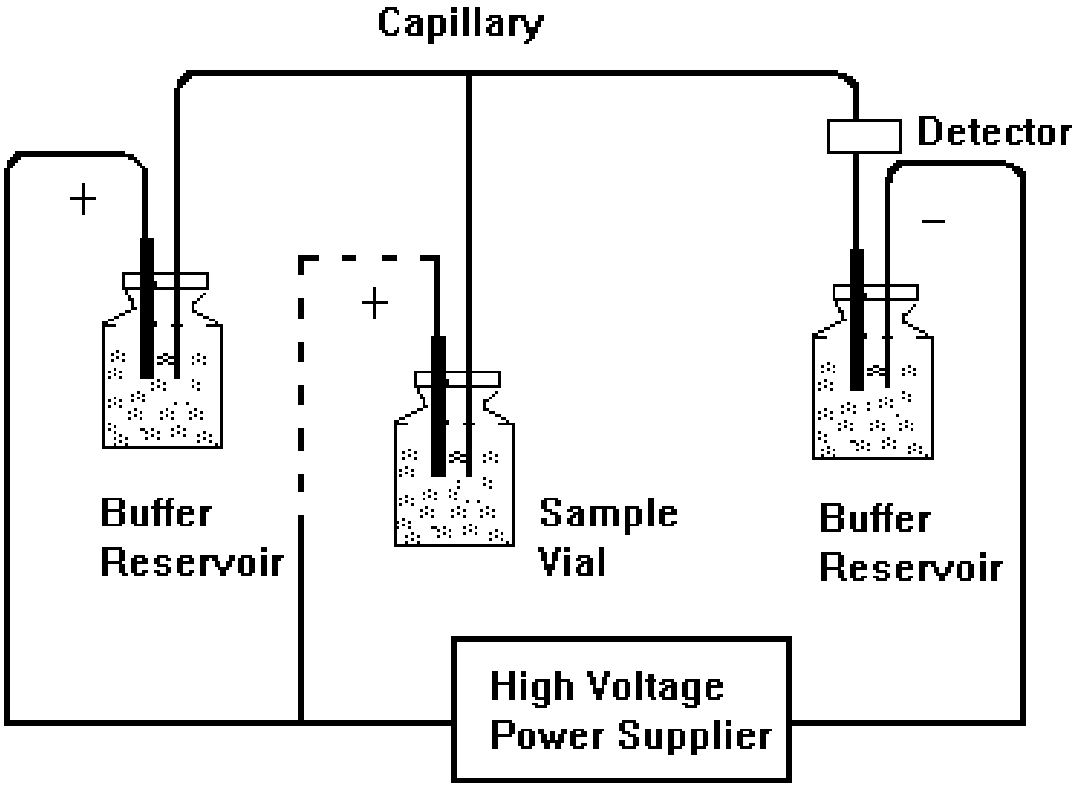

FIGURE 1. THE INSRUMENTAL SET-UP OF A CE SYSTEM.

sample vial. A specific amount of sample is introduced by controlling either the injection voltage or the injection pressure.

The unprecedented resolution of CE is a consequence of the technique's extremely high efficiency. The separation efficiency of CE and other high-resolution techniques such as chromatography and field-flow fractionation is modeled by the van Deemter equation, which relates the plate height, H, to the velocity, ν_x, of the carrier gas or liquid along the separation axis, x.

$$H = A + \frac{B}{\nu_x} + C\nu_x \tag{1}$$

Here, A, B and C are constants. A lower value of H corresponds to a higher separation efficiency — when the plate height is reduced, more theoretical plates (N) can be packed into a given length along the separation axis. The resolution of two components in the separations device is proportional to $N^{1/2}$. In CE, two of the three contributing plate

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height terms in equation 1 are eliminated: the multiple-path (eddy diffusion) term, *A*, and the mass-transfer term, Cv_x. These terms are eliminated because the separation is carried out in a single phase of uniformly flowing carrier liquid. As a result, the only fundamental source of band broadening under ideal conditions is longitudinal diffusion, B / v_x. Under typical conditions, a CE separation invokes 50,000 to 500,000 theoretical plates, which is an order-of-magnitude better than competing HPLC methods.

CE analyses are usually very fast, use little sample and reagents, and cost much less than chromatography or conventional electrophoresis. Although modern CE is still in its teenage years, it has demonstrated tremendous potential for a wide range of applications, from small molecules that include inorganic ions, organic acids, amino acids, peptides, drugs, nucleosides, nucleotides, vitamins, steroids, and carbohydrates, to larger molecules, such as hormones, proteins, nucleic acids, and even living cells.

Terminology

Electrophoresis. Electrophoresis is defined as the migration of ions under the influence of an electric field. The force ($F_E = qE$) imparted by the electrical field is proportional to its effective charge, q, and the electric field strength, E. The translational movement of the ion is opposed by a retarding frictional force ($F_f = fv_{ep}$), which is proportional to the velocity of the ion, v_{ep}, and the friction coefficient, f. The ion almost instantly reaches a steady state velocity where the accelerating force equals the frictional force.

$$qE = fv_{ep} \tag{2}$$

Rearranging equation 2 yields

$$v_{ep} = \frac{q}{f} E = \mu_{ep} E \tag{3}$$

Here μ_{ep} is the electrophoretic mobility of the ion, which is a constant of proportionality between the velocity of the ion and the electric field strength. The electrophoretic mobility is proportional to the charge of the ion and inversely proportional to the friction coefficient.

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The friction coefficient of the moving ion is related to the hydrodynamic radius, r of the ion and the viscosity, η, of the surrounding medium,

$$f = 6\pi\eta r \tag{4}$$

because $\mu_{ep} = q/f$, a larger hydrodynamic radius translates to a lower electrophoretic mobility.

Electroosmosis. Electroosmosis refers to the movement of the buffer in the capillary under the influence of the electric field. The inner surface of a fused silica capillary is covered with silanol groups (Si-OH), which are ionized to SiO^- at pH > 2. The negatively charged surface is counterbalanced by positive ions from the buffer, forming the so-called electric double layer. Under the influence of the electric field, the positive ions in the diffuse part of the double layer migrate towards the cathode; in doing so they entrain the waters of hydration, which results in electroosmotic flow. The equations of electroosmotic flow are identical to those developed for electrophoresis, as both phenomena are complementary. The electroosmotic velocity, υ_{eo} is defined by

$$\upsilon_{eo} = \mu_{eo} E \tag{5}$$

where μ_{eo} is the electroosmotic mobility, a constant of proportionality between the electroosmotic velocity and the electric field strength. Electroosmotic mobility is proportional to the dielectric constant, ε, of the medium and the zeta potential, ζ, at the capillary–buffer interface, and inversely proportional to the viscosity, η, of the medium.

$$\mu_{eo} = \frac{\varepsilon\zeta}{4\pi\eta} \tag{6}$$

The zeta potential is largely dependent on the electrostatic nature of the capillary surface, and to a small extent, on the ionic nature of the buffer. In fused silica capillaries, electroosmosis is diminished at low pH because protons convert the charged SiO^- surface to SiOH, causing a decrease in the zeta potential. Electroosmosis also decreases with increasing ionic strength, due to collapse of the double layer. Electroosmotic flow can be reduced by coating the capillary with a material that suppresses ionization of the silanol groups, such as polyacrylamide or methylcellulose.

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Apparent mobility. The apparent mobility, μ_{app}, of a solute is a vector sum of the electrophoretic mobility, μ_{ep}, of the solute plus the electroosmotic mobility, μ_{eo}, of the solution.

$$\mu_{app} = \mu_{ep} + \mu_{eo} \tag{7}$$

The apparent velocity, υ_{app}, of a solute is directly proportional to μ_{app} and the electric field strength, E, across the capillary,

$$\upsilon_{app} = \mu_{app} E \tag{8}$$

Neutral solutes migrate in the same direction and velocity as the electroosmotic flow and are not separated. Cations and anions are separated based on differences in their apparent mobilities. For cations, which move in the same direction as the electroosmotic flow, μ_{ep} and μ_{eo} have the same sign, so $\mu_{app} > \mu_{ep}$. The electrophoresis of anions, on the other hand, is in the opposite direction of electroosmosis, so for anions μ_{ep} and μ_{eo} have opposite signs. At moderate pH values (pH > 3), electroosmotic flow is generally higher than electrophoretic flow causing anions to migrate towards the cathode, which is where the detector is typically located. At lower pH, electroosmosis is weak and anions may never reach the detector unless the polarity of the instrument is reversed in order to change the location of the detector from the cathode end to the anode end of the capillary.

General Aspects of Instrumentation

*Capillary column***.** The capillary column is a key element of the CE separation. Fused silica is by far the most frequently used material, although columns have been made of Teflon and borosilicate glass. The widespread use of fused silica is due to its intrinsic properties, which include transparency over a wide range of the electromagnetic spectrum and a high thermal conductance. Fused silica is also easy to manufacture into capillaries with diameters of a few micrometers. Many reports describe the covalent attachment of silanes with neutral or hydrophilic substituents to the inner wall of the capillary in order to reduce electroosmotic flow and prevent adsorption of the analyte; coatings also tend to stabilize the pH.

An uncoated fused silica capillary is prepared for its first use in electrophoresis by rinsing it with 10 to 15 column volumes of 0.1 M NaOH followed by 10 to 15 column

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volumes of water and 5 to 10 column volumes of the separation buffer. For a coated capillary, the preparation procedure is the same except that 0.1 M NaOH is replaced with methanol. In commercial instruments, the carrier fluid is forced through the capillary by either applying pressure to the inlet reservoir or reducing pressure at the outlet reservoir.

By adding a cationic surfactant, such as cetyltrimethylammonium bromide (CTAB), to the separation buffer, the direction of electroosmotic flow can be reversed. The positively charged group on one end of CTAB interacts with the negatively charged silanol groups on the capillary surface, while the hydrocarbon tail points away from the surface. A second layer of CTAB orients itself in the opposite direction so that the hydrophobic tails form a nonpolar layer. This surfactant bilayer adheres tightly to the wall of the capillary and effectively reverses the charge of the wall from negative to positive, resulting in reversed electroosmotic flow. This procedure is also known as dynamic coating.

*Sample injection***.** One of the main advantages of CE is its ability to inject extremely small volumes of sample. Typical injection volumes range from picoliters to nanoliters. There are two commonly used injection methods for CE: hydrodynamic and electrokinetic. Hydrodynamic injection is accomplished by the application of a pressure difference between the two ends of a capillary. The amount of sample injected can be calculated by the Poiseuille equation.

$$V_c = \frac{\Delta P \pi d^4 t}{128 \eta L_t} \tag{9}$$

Here V_c is the calculated injection volume, ΔP is the pressure difference between the ends of the capillary, d is the inner diameter of the capillary, t is the injection time, η is the sample viscosity, and L_t is the total length of the capillary.

Electrokinetic injection is performed by simply turning on the voltage for a certain period of time. The moles of each analyte injected, Q_i, are determined by the apparent velocity of each analyte, υ_{app}; the injection time, t; and the ratio of conductivities of the separation buffer and sample, k_b/k_s.

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$$Q_i = \upsilon_{app}(\frac{k_b}{k_a})t\pi r^2 C_i \quad (10)$$

Here, r is the capillary radius and C_i is the molar concentration of the ith analyte.

Because each analyte has a different mobility, electrokinetic injection is biased. For qualitative analysis, this is not usually a problem. For quantitative analysis, the concentration of the injected sample is different than that of the original sample. Despite this drawback, electrokinetic injection is useful for capillary gel electrophoresis (described later), in which the polymer inside the capillary is too viscous for hydrodynamic injection.

*Joule heating***.** Joule heating is a consequence of the resistance of the solution to the flow of current. The heat produced, H, is directly proportional to the applied voltage between the electrodes, V; the electric current, I; and the time, t.

$$H = VIt \quad (11)$$

If the heat is not sufficiently dissipated from the system the resulting temperature and density gradients can reduce the separation efficiency. The capillary walls used in CE can dissipate heat much more efficiently than the slab gels used in conventional electrophoresis due to the large ratio of surface area to volume. As a result, high potentials can be applied in CE; with current technology, up to 30 kV can be applied for extremely fast and efficient separations.

*Detection modes***.** With some modifications, most HPLC detection modes can be applied to CE. Table 1 contains a list of commonly used CE detectors and their representative limits of detection (LODs) [4].

Separation Modes and Principles

The main separation modes used in CE are capillary zone electrophoresis, micellar electrokinetic capillary chromatography, capillary isotachophoresis, capillary gel electrophoresis, and capillary isoelectric focusing. Each of these modes use a high voltage to achieve highly efficient separations.

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TABLE 1. CE detection modes and representative LODs.

Detection principle	LOD (mol)
Spectrophotometric	
Absorption	$10^{-15} – 10^{-}$
Fluorescence	
Precolumn derivatization	$10^{-20} – 10^{-17}$
On-column derivatization	8×10^{-16}
Postcolumn derivatization	2×10^{-17}
Indirect	
UV	$10^{-13} – 10^{-12}$
Fluorescence	5×10^{-17}
Thermal lens	4×10^{-17}
Raman	2×10^{-15}
Mass spectrometric	1×10^{-17}
Electrochemical	
Conductivity	1×10^{-16}
Amperometric	7×10^{-19}

Capillary zone electrophoresis (CZE) is the simplest form of CE. In this mode sample is applied as a narrow zone (band), which is surrounded by the separation buffer. As an electric field is applied, each component in the sample zone migrates according to its own apparent mobility. Ideally, all sample components will eventually separate from

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each other to form individual zones of pure material. However, neutral molecules cannot be separated because they migrate at the velocity of electroosmotic flow. The separation of charged molecules is accomplished most efficiently when differences among the apparent velocities of the components are maximized and random dispersion of the individual zones is minimized.

The apparent migration time of a sample component, t_{app}, is the time required for the component to move from the beginning of the capillary to the detection window; it can be calculated from the distance between the sample inlet and the detection window, L_d, and the apparent velocity:

$$t_{app} = \frac{L_d}{\upsilon_{app}} = \frac{L_d}{\mu_{app} E} \tag{12}$$

The development of *micellar electrokinetic capillary chromatography (MECC)* has extended CE applications to the separation of both neutral and charged molecules through the use of micelles in the separation buffer. Micelles are aggregates of amphiphilic monomers known as surfactants, which possess a hydrophilic head and a hydrophobic tail. The hydrophobic tail can be a straight or branched chain of hydrocarbon, or a steroidal skeleton; the hydrophilic head can be either cationic, anionic, zwitterionic or nonionic. When surfactant molecules exceed their critical micelle concentration (CMC), they are pushed together by the polar medium. In aqueous solutions, spherical micelles form with the hydrophobic tails pointing inward and the hydrophilic heads facing outward.

In MECC, micelles serve as a pseudo-stationary phase that resembles the stationary phase in reverse-phase HPLC. Various types of interaction can occur between solutes and micelles, including hydrophobic, electrostatic and hydrogen-bonding interactions. The partitioning of solutes between micelles and the aqueous buffer can be controlled by the concentration and chemical composition of the surfactant and other additives to the buffer, such as organic solvents, ionic salts, chiral selectors, ion-pairing and complexing agents. Partitioning can also be manipulated by changes in temperature, pH, and ionic strength. Separation in MECC is a result of the combined effect of the differential partitioning of molecules between the aqueous buffer and the micellar phase, as well as any differential migration of ionic species.

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Isotachophoresis comes from the terms “iso” (same) + “tacho” (velocity) + “phoresis” (electrophoresis). It is also known as displacement electrophoresis and multizonal electrophoresis. *Capillary isotachophoresis (CITP)* is isotachophoresis performed in a capillary. In CITP, a sample is inserted between a leading electrolyte and a trailing (or terminating) electrolyte without electroosmotic flow. The leading electrolyte has a higher mobility and the trailing electrolyte has a lower mobility than ions in the sample zone. Separation in CITP relies on differences in the velocities, υ_i, of analyte ions within the sample zone.

$$\upsilon_i = \mu_{ep,i} E_{sz} \tag{13}$$

Here $\mu_{ep,i}$ is the electrophoretic mobility of species i and E_{sz} is the electric field strength of the sample zone before separation. During the transient separation process, all analyte ions are separated into consecutive bands under constant electric current and temperature. According to Ohm's law, the electric field strength increases as the mobility of ion band decreases (an ion band is defined as a homogeneous solution separated by moving or stationary boundaries). After separation, each of the analyte ions in different bands migrate at the same velocity, υ; therefore, a steady-state stacking of bands is achieved.

$$\upsilon = \mu_{ep,i} E_i = \mu_{ep,j} E_j \tag{14}$$

Here the subscripts i and j represent different analyte ions. The analyte concentrations in each band are adjusted to the concentration of the leading electrolyte ion according to the Kohlraugh regulating function

$$C_i = C_l \frac{\mu_i(\mu_l + \mu_c)}{\mu_l(\mu_i + \mu_c)} \tag{15}$$

where C_i is the concentration of species i in ion band i, C_l is the concentration of the leading electrolyte, and μ_i, μ_l and μ_c are the mobilities of species i, the leading electrolyte ion, and the counter ion in the steady state, respectively. CITP will concentrate those analyte ions whose concentration in the sample is lower than their steady-state concentration defined by equation 15.

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CITP cannot be used to separate cations and anions at the same time. Detection methods are based on conductivity, differential conductivity, or direct UV adsorption. In the latter case, spacers are placed between analyte bands. The spacers contain solutes that do not absorb in the UV and have mobilities between those of the two neighboring bands.

Capillary gel electrophoresis (CGE) is an adaptation of traditional slab gel electrophoresis to the capillary format; it is CZE performed in a polymeric gel medium. CGE is potentially useful for the separation of large biological molecules such as proteins and DNAs, which have similar electrophoretic migration rates in free solution due to their similar charge-to-mass ratios. CGE separates molecules according to their size in an nonconvective medium. Separation media include non-crosslinked polymers such as linear polyacrylamide, polyethylene glycol and cellulose derivatives, as well as crosslinked polymers or gels, such as polyacrylamide and agarose.

The entangled polymer network inside the capillary serves as a molecular sieve in which smaller molecules migrate faster than large. The polymer network reduces the solute diffusion rate and the adsorption of solute to the capillary wall, while suppressing electroosmotic flow. These features increase efficiency, which permits the use of a shorter column. With crosslinked polymers, the resolution of the capillary can be easily optimized for a given range of molecular weights by varying the total monomer concentration and degree of cross-linking. However, non-crosslinked polymers can be easily flushed out of the capillary when a problem develops (such as a trapped air bubble inside the capillary) and can be reloaded to generate a fresh capillary for each separation.

Capillary isoelectric focusing (CIEF) is used for the separation of amphoteric substances such as proteins, peptides, amino acids, and pharmaceuticals in polymer matrices as well as free solutions. Separation in CIEF is based on differences in the isoelectric point (p*I*) of sample components rather than differences in apparent velocity. A series of zwitterions (called ampholytes) are used to generate a pH gradient inside the focusing capillary. Ampholytes that are positively charged migrate towards the cathode while those that are negatively charged migrate towards the anode. Therefore, the pH increases at the cathode side of the capillary and decreases at the anode side. When an ampholyte reaches its own p*I* and is no longer charged, its migration ceases. As a result, a stable pH gradient is formed. If the analyte has a net positive charge, it migrates towards the cathode. During its migration, it eventually encounters a pH at which it has a

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zero net charge and ceases to migrate. The pH gradient is smoother when a larger number of ampholytes are used. In order to prevent the migrations of buffers from the buffer reservoirs into the capillary, the pH of the electrolyte in the cathode must be higher than the p*I*s of all the basic ampholytes and the pH of the buffer in the anode must be lower than the p*I*s of all the acidic ampholytes.

CIEF is most effective when electroosmotic flow and other convective forces are eliminated or greatly suppressed. However, it is still possible to perform CIEF in the presence of electroosmosis, as long as the electroosmotic velocity of the solution inside the capillary does not exceed the electrophoretic velocities of the analytes.

CIEF is a true focusing technique. If a solute molecule from a focused band happens to diffuse away from the zone center, it immediately loses or gains protons, and thus acquires charge. In its charged state, the solute migrates back toward the zone center. Eventually, a steady state is reached where the zones are stationary and sharply focused. The width of solute zone can be characterized by the variance of Gaussian distribution

$$\sigma^2 = \frac{D}{E} \cdot \frac{d(pH)}{d\mu_{app}} \cdot \frac{dx}{pH} \tag{16}$$

Smaller variance, which results in a sharper zone, is favored by a high field strength (E), a low diffusion coefficient (D), and high values of $d\mu_{app}/d(\text{pH})$ (the rate of change of mobility with pH) and $d(\text{pH})/dx$ (the slope of the pH gradient). For a complete separation of two solutes, a difference between the two p*I*s must be greater than 4σ.

Analytical Considerations

Qualitative analysis. Qualitative analysis in CE provides information about the identity of a peak in an electropherogram. The simplest way to identify a CE peak is to compare its migration time with that of a known compound. As with other separation techniques, however, the migration time alone is not always reliable for confirming peak identity and purity; final confirmation requires additional information. One method of confirmation entails comparing the ratio of absorbances at different wavelengths in the unknown with that ratio in the suspected compound using spectrophotometric detection. Another method is to compare the ratio of currents obtained from two different electrical potentials using amperometric detection.

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Quantitative analysis. Quantitative analysis provides information about the amount or concentration of a substance in a given sample. Although quantitative analysis by CE is still in its early stages of development, several aspects deserve special attention.

(a) *External (direct) calibration.* Solute concentration is directly related to peak height, while residence time in the detector is directly related to peak width. Therefore, the amount of solute is directly related to peak area and quantitative information can be obtained in CE by directly comparing an analyte's peak area or height with those of calibration standards.

(b) *Internal calibration.* Internal calibration usually results in better precision compared to methods that rely on direct calibration because neither the quantity injected nor the detector response needs to remain constant. In internal calibration, a known amount of internal standard is added to each sample prior to the sample pretreatment procedure, be it the calibration standard or unknown. After pretreatment, the solution of sample and internal standard is subjected to electrophoresis. The calibration graph consists of ratios of peak area (or height) of the calibration standards to that of the internal standard plotted against the concentration of the standard. The unknown concentration is determined from the peak area (or height) ratio of the unknown mixture.

Estimation of impurity. In CE, the time required for injected solutes to reach the detector is governed by their apparent mobilities. Even the time a solute spends in the detector is governed by its apparent mobility. This is unlike HPLC, where the residence time in the detector is equal for all solutes, and governed by the flow rate of the carrier liquid. Because the detector signal is proportional to both concentration and residence time, the presence of impurities should not be made by directly comparing the peak areas among components. Instead, such comparisons should be made using normalized peak areas, which is the ratio of peak area to migration time.

Sample matrix effect. The sample matrix strongly influences quantitative precision and accuracy in CE, especially when electrokinetic injection is used. The matrix effect can be accounted for by using matrix-corrected peak areas combined with internal calibration [5].

Sample preparation. Many CE applications do not require sample pretreatment other than a possible dilution. Other applications require the sample to be treated before

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injection, especially those dealing with the analysis of biological samples. Pretreatments include liquid-liquid extraction, solid-liquid extraction, ultrafiltration, and microdialysis.

Limit of detection. Spectrophotometric detectors used in CE may be an order of magnitude less sensitive compared to those available for HPLC because the former detectors use shorter pathlengths. The reduced sensitivity is partially compensated by the high separation efficiency of CE, which allows for more precise integration of peak areas. Other methods for improving detection limits include the use of a low detection wavelength (down to 185 nm), where many solutes have greater absorptivities, and by using sample stacking or a packed-inlet capillary for on-column concentration of samples. Without sample concentration, limits of detection (LODs) in the low μM (or μg/mL) range can be obtained with UV detection; with sample preconcentration, LODs as low as a few nM (or ng/mL) in the original sample can be achieved. LODs at sub-μM and sub-pM levels are typical with amperometric detection and laser-induced fluorescence detection, respectively.

Range of linearity and reproducibility. The linear dynamic range of CE applications has been as narrow as one order of magnitude or as wide as six orders of magnitude, depending on the analyte. Reproducibility is typically in the range of 1-2% for peak area and 3-7% for peak height.

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