# Fast Analytical-Scale Separations by Capillary Electrophoresis and Liquid Chromatography

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## I. Introduction

Separations are generally considered a slow step in any analytical methodology. HPLC and electrophoresis, the mainstay of separations of nonvolatile compounds in the modern laboratory, take anywhere from tens of minutes to hours to complete depending upon the complexity of the sample, the desired information, and selectivity available. This time requirement has limited the types of problems that separations can be asked to solve. For instance, separation methods are not typically used in high-throughput analyses or for monitoring applications. The time-consuming nature of separations has driven many researchers to investigate methods of improving the speed of separations techniques. In this review, we examine recent trends in improving the speed of liquid-phase analytical-scale separations based on chromatography and electrophoresis. While the present review is limited to liquid-phase separations, we note that recent advances have been made in the speed of gas chromatography separations as well, and this work has recently been reviewed elsewhere. Because many of the technical advances that have improved the speed of separations have occurred since 1990, our review emphasizes the past 9 years.

In developing a review on fast separations, we were faced with the dilemma of how to define a fast separation. Our approach was to consider articles in which the emphasis was on use of theory and technology which were developed specifically to improve the speed of separation over conventional practice. Conventional practice, while difficult to define, in HPLC includes separations performed in 1−4.6 mm bore by 10−25 cm long columns packed with 3−5 $\mu$m porous particles. Thus, innovations such as nonporous particles, particle diameters <2 $\mu$m, perfused particles, elevated temperature, or open tubular columns and their application are discussed. For electrophoresis, we considered only capillary electrophoresis (CE). CE, by its very nature, is "faster" than gel electrophoresis; however, the rapid development of CE has meant that it would be impractical to simply review CE relative to gel electrophoresis. (Furthermore, exhaustive reviews of CE are available.) Rather, we consider innovations in CE that led to speed over conventional practice. For CE, conventional practice was defined as separations in columns with 50−100 $\mu$m bore and 50−100 cm length and electric field ($E$) < 300 V/cm. Use of miniaturized systems, rapid and automated injection schemes, high E, and certain gels and additives are innovations which can dramatically improve the speed of the separation and are included here. Microfabricated systems have been extensively utilized in achieving fast separations, especially for CE. A separate review on microfabricated systems for analysis and separation has been commissioned for this special issue of *Chemical Reviews*; therefore, while we acknowledge such advances, we limit our discussion of these important techniques relative to other innovations. Further information on microfabricated systems can be found in this other review for a more complete picture of fast separation systems.

In addition to descriptions of the technical innovations, examples of the applications are included. We have emphasized applications which were made qualitatively possible by the improvement in speed. Such examples include on-line in vivo monitoring, using a fast separation as a second-dimension in two-dimensional separations, detection of short-lived spe-

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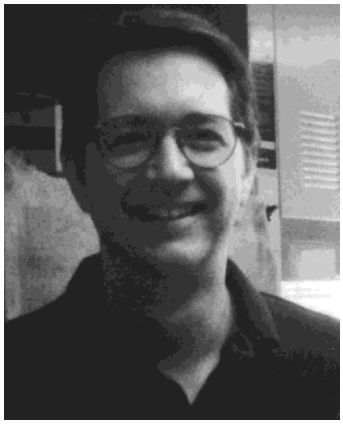

Robert T. Kennedy received his B.S. degree in Chemistry at the University of Florida, in 1984, where he performed undergraduate research in physical organic chemistry with Bill Dolbier. In 1984 he began his Ph.D. work at the University of North Carolina—Chapel Hill (UNC) in analytical chemistry under the direction of James W. Jorgenson. His work involved development of capillary LC columns and their application to single cell analysis. After graduation in 1988, he began an NSF post-doctoral fellowship with R. Mark Wightman, also at UNC, working in the study of neurotransmission using microelectrodes. In 1991 he joined the faculty at the University of Florida Chemistry Department where he is now Professor of Chemistry and a member of the UF Brain Institute. His research group is investigating capillary separations, confocal microscopy, and amperometry for the study of neutransmitters and hormones both in vivo and at the single cell level.

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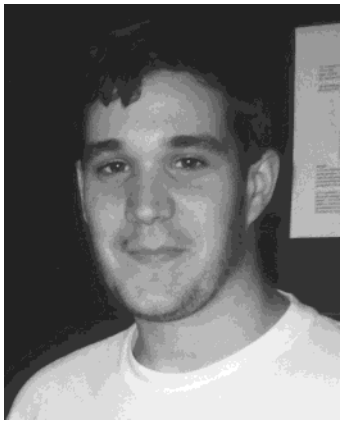
cies such as complexes or proteins in certain conformations, and process monitoring. These types of applications simply would not be practical with conventional separations conditions.

## *II. Capillary Electrophoresis*

### A. Introduction and Theory

In cases where diffusion is the only source of band broadening, simple theories for CE show that it is possible to simultaneously obtain increases in speed and efficiency by increasing the voltage. This property is illustrated by the following equations for migration time ($t_{mig}$) and theoretical plates ($N$)



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$$t_{mig} = l/v = L/V\mu \quad (1)$$

$$N = \mu V/2D \quad (2)$$

where $v$ is the analyte velocity, $l$ is the separation length, $L$ is the total capillary length, $V$ is the voltage dropped across $L$, $\mu$ is the electrophoretic mobility, and $D$ is the diffusion coefficient of the analyte. These equations show that $t_{mig}$ is inversely proportional to voltage while $N$ is proportional to voltage. The equations also show that separation efficiency is independent of column length. Thus, as long as the same voltage is dropped across the separation path, use of shorter columns will yield faster runs with the same plate counts. This fortuitous situation is quite different from chromatography where increases in speed by shortening the column or increasing the flow rate result in lower numbers of theoretical plates.

In most cases, electroosmotic flow (EOF) is present and this changes the above equations to the following

$$t_{mig} = L/(\mu + \mu_{EOF})V \quad (3)$$

$$N = (\mu + \mu_{EOF})V/2D \quad (4)$$

where $\mu_{EOF}$ is the electroosmotic mobility. These equations show that higher EOF improves speed and efficiency; however, the improvement in efficiency is misleading. The presence of EOF moves analytes off the column faster, allowing less time for diffusional band broadening and thus higher efficiency; however, since EOF is a nonselective mode of transport, it also moves analytes off the column with less time to be separated by electrophoresis (assuming that analytes are moving in the same direction as the EOF). It is more instructive to consider the expression for resolution ($R_s$) in the presence of EOF[1]

$$R_s = 0.177(\mu_1 - \mu_2)((V)/(D(\mu_{avg} + \mu_{EOF})))^{1/2} \quad (5)$$

where $\mu_1$ and $\mu_2$ refer to mobilities of two species and $\mu_{avg}$ is their average. This equation shows that increases in EOF will decrease resolution if the flow is in the same direction as the ion mobility and will increase resolution if flow is in the opposite direction of the ion mobility. Thus, while EOF decreases analysis time, its effect on resolution depends on the charge of the analyte.

The above equations are useful because they show what is possible in the limit where diffusion is the only source of band broadening. In reality, however, several other factors come into play to limit the efficiency as voltages are increased. To consider these other factors, we begin with the definition of efficiency

$$N = {t_{mig}}^2/{\sigma_{Tot}}^2 \quad (6)$$

where ${\sigma_{Tot}}^2$ is the zone variance due to all sources of band broadening. In CE, ${\sigma_{Tot}}^2$ can be expressed as

$${\sigma_{Tot}}^2 = {\sigma^2}_{Diff} + {\sigma^2}_{Inj} + {\sigma^2}_{Det} + {\sigma^2}_{Heat} + {\sigma^2}_{Ads} + {\sigma^2}_{ED} \quad (7)$$

where ${\sigma^2}_{Diff}$, ${\sigma^2}_{Inj}$, ${\sigma^2}_{Det}$, ${\sigma^2}_{Heat}$, ${\sigma^2}_{Ads}$, and ${\sigma^2}_{ED}$ are the variances due to longitudinal diffusion, sample injection, detection, Joule heating, adsorption, and electromigration dispersion (also known as "sample overloading"), respectively. When $t_{mig}$ is small, it is difficult to achieve diffusion-limited efficiency (${\sigma^2}_{Diff}$ dominates in the above equation) unless great care is taken to suppress these other band broadening effects. These effects and their suppression will be discussed below. As will be shown, the primary concern is minimizing Joule heat which is typically affected by using small bore capillaries (most fast separations use capillaries with inner diameter i.d. $<10$ $\mu$m) and low conductivity buffers.

### *1. Broadening Caused by Temperature*

For high-speed CE separations, we must consider the effects of Joule heat, which increases with increasing voltage, on efficiency. Although heat is generated uniformly throughout the capillary cross section, it is only dissipated through the capillary surface; consequently, a radial temperature gradient results inside the capillary. The temperature difference between the capillary wall and any radius within the capillary is given by

$$\Delta T = (G{r_c}^2/4\lambda)(1 - ({r_x}^2/{r_c}2)) \quad (8)$$

where $\Delta T$ is the temperature difference, $r_c$ is the radius of the capillary, $\lambda$ is the thermal conductivity of the solution, $r_x$ is the distance from the capillary wall, and $G$ is the heat generation rate (W/m$^3$). As $\Delta T$ is proportional to ${r_c}^2$, it is apparent that the use of small i.d. capillaries will reduce the temperature gradient. Additionally, the use of small i.d. capillaries increases the resistivity of the column, giving rise to less current and less heating.

The temperature gradients induced by Joule heat result in band broadening since many factors which control electromigration rates, such as viscosity, are temperature dependent. Electrophoretic mobility of a species can be altered due to regional viscosity changes in the solvent caused by the temperature differences. Uneven temperature also results in convection which additionally contributes to band broadening. Furthermore, Gas[2] has shown that the concentration of the background electrolyte may change at sites of jumps in the radial heat flux yielding a nonuniform separation media and nonuniform migration rates.

The effect of heating on band broadening has been quantified in the form of a plate height ($H$) equation for CE.[3] The term for heating was given as

$$H_{heating} = (\tau^2 S^2 \mu^2/\lambda^2 D))((K_1 E^5 {r_c}^6/\mu_{app}) + (K_2 E^6 {r_c}^8/LD)) \quad (9)$$

where $E$ is the electric field strength, $\mu_{app}$ is the apparent electrophoretic mobility ($\mu_{app} = \mu_{EOF} + \mu$), $S$ is the specific conductance, $\lambda$ is the thermal conductivity of the buffer, $\tau$ is the thermal coefficient of the solute mobility, $\mu$ is the electrophoretic mobility of the solute, $K_1$ and $K_2$ are unitless constants with $K_1 = 6.5 \times 10^{-4}$ and $K_2 = 4.34 \times 10^{-5}$, and the other terms have been described above. This equation allows prediction of the limits of voltage for a given set of parameters before heating begins to alter efficiency.

**Minimization of Joule Heat Effects.** Equations 7 and 8 show that decreasing the column radius should allow higher voltages and faster separations before heating degrades the separation. In many of the examples that will be given, the fastest separations are generally found to be performed in capillaries with i.d. of 10 $\mu$m or less with electric fields of 2−5 kV/cm. As will be discussed below, while decreasing the column radius is effective at reducing band broadening due to heating, reductions to capillary diameters below 50 $\mu$m place great demands on the injection and detection system.

Another approach to combat Joule heating is the use of low-conductivity electrophoresis buffers. Hjerten et al.[4] demonstrated this approach by utilizing high

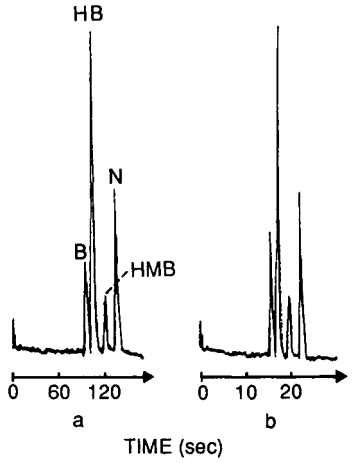


**Figure 1.** CE of low molecular weight compounds in a low conductivity buffer. Sample: benzoic acid (B), 4-hydroxybenzoic acid (HB), 4 hydroxy-3methoxybenzoic acid (HMB), and $\beta$-naphthylacetic acid (N), each compound 0.01 mg/mL. Buffer 0.3% w/v polyoxyethylene bis(3-amino-2-hydroxypropyl)/0.025% w/v polyoxyethylene bis(acetic acid), pH 8.6. Voltage (a) 5000 V ($E$ = 330 V/cm, 0.4 $\mu$A); (b) 30 000 V ($E$ = 2000 V/cm, 2.5 $\mu$A). A comparison between the electropherograms in a and b shows that the low conductivity buffer used permits analysis at high $E$, consequently minimizing analysis times (~5-fold) with no observable loss in resolution. (Reprinted with permission from ref 4. Copyright 1995.)

molecular weight buffers with small net charge or a narrow pH range of ampholytes for buffer. This approach is attractive because it requires no special instrumental design and therefore is applicable to commercial CE instruments. Using this approach, several carboxylic acids were separated within 25 s in 50 $\mu$m i.d. capillaries as shown in Figure 1. As shown, no loss in resolution is observed between the low-field case and the high-field case while analysis time is reduced ~5-fold.

### 2. Broadening Caused by Absorption

Adsorption to the capillary walls results in a contribution of chromatographic retention and band broadening to the electrophoretic separation. As is well-known from chromatography, increasing the speed of migration will increase band broadening due to such nonequilibrium effects. Novotny et al.[3] adapted equations derived by Wieme et al.[5] to describe this source of band broadening in CE as follows

$$H_{ads} = (R(1 - R)^2 \mu_{app} r_c E L n/Z\alpha)/L \qquad (10)$$

where $R$ is the fractional concentration of free solute, $n$ is the number of molecules per unit volume inside the tube, $\alpha$ is the fraction of molecules which stick on collision, $Z$ is the number of molecules striking a unit surface area/s, and the other terms are as defined above. This equation shows that, like the temperature effect, increasing voltage will increase this source of band broadening, thus lowering resolution at higher speeds. It is also interesting to consider that while utilizing small i.d. columns can suppress heating and associated band broadening, they may also increase broadening due to adsorptive interactions since the capillary surface area-to-volume ratio is increased in small bore capillaries resulting in greater chance for adsorption. Other authors,[6−10] have also contributed work in modeling solute adsorption to the capillary walls. These works have presented analytical solutions to equations describing electrophoretic migration in the presence of solute adsorption, and theoretical simulations based on these equations have been examined. The limit to speed in analysis when solute adsorption occurs is largely dependent upon the kinetics of the solute interaction with the capillary wall.

Most examples of high-speed separations to date involve small molecules or DNA, possibly because these compounds typically do not adsorb strongly to the capillary wall. Although adsorption of small molecules is usually considered inconsequential, comparisons of theory with experimental results under conditions of high $E$ ($E$ > 1000 V/cm) have shown that some band broadening is not accounted for by diffusion, injection, or heating, suggesting a contribution due to adsorption for small molecules under fast separation conditions.[11,12]

The adsorption problem is frequently important for peptides and proteins with pI lower than the migration buffer. A number of methods can be used to decrease adsorption to capillaries,[13−19] and implementation of these methods should allow fast separations of proteins as well, although this has yet to be demonstrated, except in a few isolated incidences related to fast immunoassays.[20−24]

### 3. Band Broadening Summary

The band broadening contributions from injection, diffusion, heating, and solute adsorption have been combined to form a complete plate height equation for CE[3]

$$H = \sigma_{Inj}^2/L + (2D/\mu_{app}E) + R(1 - R)^2 \mu_{app} r_c E n/Z\alpha + [(\tau^2\lambda^2\mu_o^2/k_1^2 D)] [(K_1 E^5 r_c^6/\mu_{app}) + (K_2 E^6 r_c^8/LD)] \quad (11)$$

Plots of $H$ versus electric field strength similar to van Deemter plots used in chromatography can be generated from this equation and used to show trends in efficiency with speed. This equation provided an acceptable fit when compared to experimentally observed plate heights as shown in Figure 2. At minimum plate height, it was shown that diffusion accounted for ~98% of zone broadening in 10 $\mu$m capillaries. Perhaps more importantly, the theory indicated that plate height would increase from ~3 $\mu$m to just 3.5 $\mu$m as $E$ was increased from 190 (the optimum) to 500 V/cm in a 10 $\mu$m capillary compared to a change from 4 $\mu$m to almost 7 $\mu$m for 50 $\mu$m capillaries. Again, the importance of using small i.d. capillaries is demonstrated.

## B. Instrumentation

### 1. Detection

In addition to the above sources of band broadening, it also important to control band broadening

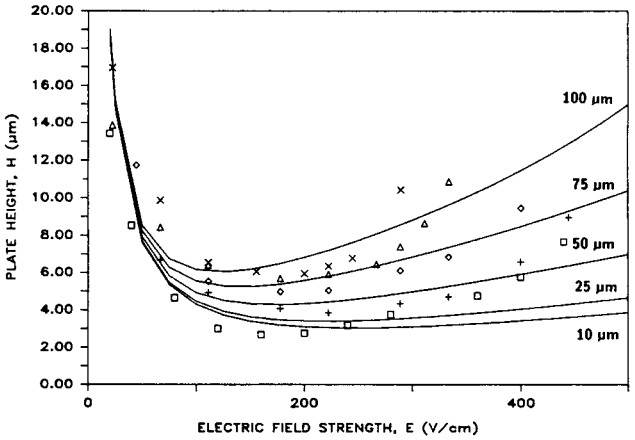


**Figure 2.** Dependence of efficiency on electric field strength in different i.d. capillaries. Experimental data: (□) 10 $\mu$m i.d.; (+) 25 $\mu$m i.d.; (◆) 50 $\mu$m i.d.; (△)75 $\mu$m i.d.; (×) 100 $\mu$m i.d. Lines: curves calculated from eq 11. (Reprinted with permission from ref 3. Copyright 1992 American Chemical Society.)

associated with instrumental effects such as injection and detection. We will first consider detection.

**Laser-Induced Fluorescence Detection.** Virtually all fast separations employ on-column detection in order to minimize extra-column band broadening. In addition, fast separations require extremely sensitive detectors as injected sample volumes are typically a few picoliters. For these reasons, laser-induced fluorescence (LIF) has become the method of choice for detection in fast CE. Lasers can easily be focused down to the micrometer sizes needed to minimize broadening associated with the detector. At the same time, as concentration sensitive detectors, they allow high sensitivity even in small capillaries. Detection limits on the level of yoctomoles ($10^{-24}$ mol)[25] have been achieved as has counting individual molecules during rapid separations.[26]

A limit of LIF detection is that most analytes are not fluorescent, therefore derivatization is required. Common derivatization methods for CE have recently been reviewed[27] and will not be discussed here. The requirement for derivatization can impact the strategy for utilizing high-speed separations. For example, if the goal of the high-speed separation is high throughput, then the samples should be derivatized in parallel and then analyzed either in parallel or sequentially by fast separation. On the other hand, if the goal is a rapid turnaround time, monitoring, or rapid feedback, then a rapid derivatization agent should be used. As will be seen in the applications sections, this may include rapid covalent derivatizations or use of affinity agents such as antibodies for labeling.

**Amperometric Detection.** Although LIF has been almost the exclusive mode of detection for rapid separations, in the long term it will be beneficial to utilize other modes of detection as well. Electrochemical detection has the appropriate sensitivity to be used with small columns and high sensitivity.[28,29] On-column and end-column designs have both been successfully used.[30−34] Any high-speed separation will require extreme care to avoid band broadening associated with the electrode size, adjoining capillary (off-column detection), or column−electrode spacing (end-column detection). The use of chip-based systems with integrated electrochemical detection may allow the control needed for rapid separation. As an example of this direction, Woolley et al.[35] separated three electroactive small molecules in under 100 s on-chip with an integrated Pt working electrode. Another potential limitation of using amperometry for fast separations is that fast separations will have the high electrophoresis currents which can contribute considerable noise in electrochemical detection.

**Absorbance Detection.** Although absorbance detection is one of the most frequently utilized detection techniques for CE, its application with fast CE is quite challenging. In particular, if small capillaries are used to prevent heating, then the path length is short, resulting in small absorbances. The short path lengths can be minimized by using *Z*-shaped flow cells,[36] multireflection cells,[37] rectangular capillaries,[38] bubble cell capillaries,[39,40] and signal averaging by use of a photodiode array;[41] however, most of these techniques are not likely to be compatible with fast separations. *Z*-shaped and bubble flow cells as well as multireflection flow cells can significantly increase $\sigma^2_{det}$, which is problematic in fast separations (see for example Xue[40]). Signal averaging offers an effective but time-consuming method for S/N enhancement and therefore does not appear to be a suitable solution for fast CE detection.

Rectangular capillaries may be a suitable approach to allowing absorbance detection with fast separations. It has been demonstrated that rectangular capillaries have better heat dissipation relative to cylindrical capillaries of the same surface area.[42−44] In this work, it was shown that the maximum temperature difference within the separation column in rectangular channels is 3 times less than that in circular capillaries of similar surface area. Thus, a gain in heat dissipation capability plus the opportunity for a longer detection axis[38] may prove a suitable combination for absorbance detection in fast separations. Such a combination has yet to be achieved however.

Another opportunity for using absorbance detection in high-speed CE is thermooptical absorbance detection because it offers both the spatial characteristics and sensitivity needed for detection in fast CE.[45−50] Thermooptical detection involves the use of two laser beams which intersect at the detection region of the separation capillary. The wavelength of the "pump" beam is selected to match the absorbance profile of the analyte. The "probe" beam is a longer wavelength laser which is used to monitor the changes in the refractive index of the solution in the capillary. As the analyte zone enters the detection area, it absorbs radiation from the pump beam. This absorption gives rise to thermal changes in the medium altering the refractive index, which is then detected by the probe beam. This form of detection contrasts with most other sensitive absorbance-based detectors because it maintains the spatial integrity of the zones. Yu and Dovichi[46] have reported a mass detection limit of 37 amol for DABSY L-methionine, and Bruno et al.[51] have detected 350 pg of sample in 25 $\mu$m i.d. capil-

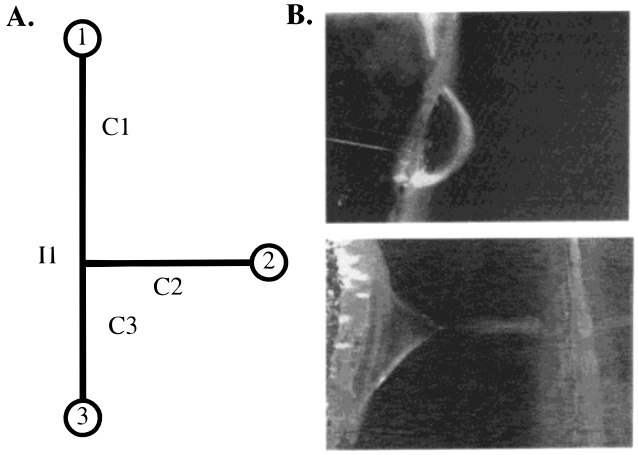


**Figure 3.** (A) Diagram of a structure, with intersection I1 and three channels, C1, C2, and C3, connecting three sample ports, P1, P2, and P3 using for interfacing a chip with ESI-MS. In this work, the walls of channel 2 (C2) were modified with linear polyacrylamide in order to reduce EOF. When a voltage is applied between ports 1 and 2, a strong EOF exists in channel 1 (C1), but not in channel 2. This difference in EOF's will generate a excess pressure at the intersection (I1) which allows pumping to port 3. (b) Photomicrographs of (top) a water droplet (~12 nL) forced through a channel by positive pressure and (bottom) the Taylor cone and electrospray generated at the opening of a channel that was electroosmotically pumped using a coated sidearm microchip. (Reprinted with permission from ref 59. Copyright 1997 American Chemical Society.)

laries by this method. These reports suggest thermooptical absorbance detection offers the potential of being applied to fast CE measurements utilizing small i.d. capillaries.

**Mass Spectrometry Detection.** Mass spectrometry also provides a possible alternative for detection in fast separations. While many of the fastest CE separations are applied to well characterized systems (i.e., chemical monitoring), there is a growing interest in high-throughput screening or high-throughput peptide/protein analysis for proteomics. The high separation efficiencies and rapid analysis of CE coupled with the structural information obtainable by various MS techniques provides an opportunity to obtain large amounts of analytical information in a short time.

Of all MS ionization/interface techniques electrospray ionization (ESI) interfaces have received the most attention and seem the most compatible with fast separations.[52−54] Providing electrical contact for the outlet of the CE capillary often is difficult when interfacing CE with ESI. An ideal interface must provide efficient mass transfer of the analytes while maintaining electrical contact for electrophoresis and limit band broadening resulting from pressure-driven flow or dead volume. Coaxial sheath−flow interfaces,[55] liquid junction interfaces,[56] microdialysis junctions,[57] and sheathless interfaces[58] have all been reported for CE-ESI-MS. Recently, another clever method for overcoming this difficulty has been demonstrated.[59] This work utilizes electroosmotic flow to "pump" solution to the exit of a microfabricated channel where a stable electrospray can be generated. Figure 3 illustrates both the function of the microchip device as well as the electrospray generated. The use of advanced fabrication processes allow for zero dead volume connections to be easily built into the instrument. To demonstrate this arrangement, the authors recorded mass spectra of a 10 $\mu$M solution of tetrabutylammonium iodide at an electric field strength of ~350 V/cm in a 60%/40% water/methanol solution. This work is significant because it integrates MS with the possibility of performing rapid separations as well as complex fluid handling/mixing on microfabricated chips.

Another ionization technique which has proven suitable for CE/MS analysis is chemical ionization.[60,61] In this mode, sample ions are ionized by corona discharge followed by ion/molecule reactions such as $M + SH^+ \rightarrow MH^+ + S$ (M, sample molecule; S, solvent molecule). Sample molecules which have a proton affinity greater than that of the solvent will be easily ionized by this method. This method is reported to have less sensitivity to salt content than ESI. For example, Takada et al.[60] have shown that at concentrations of sodium phosphate as low as 20 mM, ESI fails while chemical ionization is unaffected.

In terms of the mass spectrometer, certainly the time-of-flight instruments have the greatest compatibility with high-speed separations as spectra can be collected on the microsecond time scale. Recently, Lazar et al. examined TOFMS as a detector for CE[62] and found it suitable for routine analysis. Examples of fast CE with electrospray TOFMS are given in the applications section. A more comprehensive review of CE/MS analysis can be found in Smith et al.[63]

**Detection System Electronics.** In addition to the band broadening associated with the detector cell, the potential variance introduced by the detection system electronics must also be considered. This variance is introduced both by electronic filtering of the data as well as data acquisition rate. The variance introduced by the electronic filter is a function of the rise time of the filter and can be given as

$$\sigma_{EF}^2 = (T_R/2.2)^2 \qquad (12)$$

where $\sigma_{EF}^2$ is the variance contributed by the electronic filter and $T_R$ is the filter rise time.[11] Short rise times must be used with faster separations which increase the bandwidth of detection and the noise. Ultrafast analysis also requires high data collection frequencies to ensure adequate sampling of very sharp analyte peaks. The variance contributed by the data acquisition rate can be given as

$$\sigma_{DAQ}^2 = (1/2\pi f)^2 \qquad (13)$$

where $\sigma_{DAQ}^2$ is the variance contributed by data acquisition and $f$ is the sampling frequency. In the above equations, the data acquisition variance as well as the electronic filtering variance are given in units of $s^2$. Spatial variance of a zone can be converted to a temporal variance by dividing by the migration velocity squared of the compound of interest. With care, variance due to the detection system can be reduced to inconsequential levels except in extreme cases such as submillisecond separations.[64] For subsecond separations, sampling rates are typically on the order of 1000 Hz and low-pass filter cutoffs are 1

ms.[11,12] Examples of calculation of the effect of these various sources of band broadening can be found in several papers on fast CE.[11,12,64]

### 2. Sample Injection

Another contribution to band broadening in fast separations is that of sample injection. This is of particular interest for fast separations, which require very narrow plugs of sample injected onto the separation capillary. Most advances in separation speed have followed development of novel instrumentation for fast and reliable sample injection. In fact, most recent work has shown that for small molecules, which do not adsorb strongly to the capillary surface, the variance due to injection is often the largest contributor to band broadening.[11,12] Mathematically, the variance contributed by sample injection can be given as

$$\sigma_{\mathrm{Inj}}^2 = h^2/12 \qquad (14)$$

where $h$ is the initial width of a sample plug.[3] Thus, since narrow sample zones and small i.d. capillaries are a necessity, sample volumes on the order of hundreds of picoliters ($10^{-12}$ L) or less must be injected onto the column to achieve high-speed, high-efficiency separations. A frequent problem in high-speed CE separations is that to minimize the band broadening due to injection, small amounts must be injected, thus compounding the problem raised by using small i.d. capillaries.

In addition to broadening due to the width of the initial sample plug, broadening associated with sample overloading (or "electromigration dispersion") must also be considered. This type of broadening occurs as a result of the electrical discontinuity that occurs in the capillary from the sample to the buffer.[1] This type of broadening can be minimized by using low concentrations of analyte dissolved in a buffer identical to the electrophoresis buffer.

**Optically Gated CE.** One route to controlled, fast, small volume injections is the use of the optical gate. A block diagram of optically gated CE[65] is shown in Figure 4. In this system, the sample is fluorescently tagged and placed in one of the electrode vessels. When voltage is applied, the tagged sample is continuously drawn into the capillary according to electroosmotic flow and electrophoretic mobility. Near the entrance to the capillary, an intense laser beam is used to photodegrade the fluorescent tag, rendering it undetectable at the detector downstream. Injection is accomplished by blocking the gating beam for a brief period of time, which allows a narrow plug of labeled analyte onto the column, which then separates by electrophoresis and is detected by LIF. The ability to control the open time of the gate in the subsecond time range allows minimization of the band broadening due to injection. In one example, four fluorescein-labeled amino acids were separated in less than 140 ms[11] (see Figure 5). Optical-gating injection has also produced the highest efficiency separation per unit time at over 350 000 plates/s.[12] It has been shown[11,12] that efficiencies as high as 80% of the diffusion-limited value can be obtained by optical gating. As mentioned in the chromatography section, optical gating has also been used for capillary LC separation.[66]

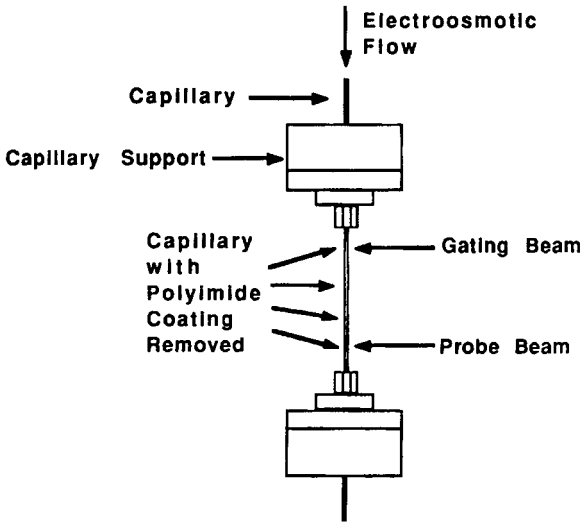


**Figure 4.** Schematic diagram of an optical-gating system. See text for description. (Reprinted with permission from ref 65. Copyright 1991 American Chemical Society.)

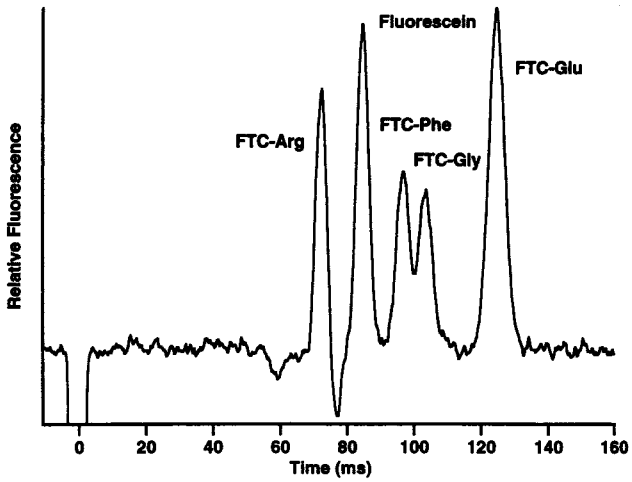


**Figure 5.** Separation of four fluorescein-labeled amino acids utilizing optically gated injection. Separation conditions: $l = 1$ mm, injection time = 5 ms, $E = 2500$ V/cm. (Reprinted with permission from ref 11. Copyright 1993 American Chemical Society.)

In addition to allowing narrow, reproducible injections, optical gating enables repetitive injections to be made under constant voltage, allowing many separations to be performed rapidly in sequence. This capability has enabled several applications including as a second or third dimension in a multidimensional separation[67,68] and chemical monitoring[12] as discussed in the applications section.

Although optical gating allows fast separation, some shortcomings have been noted. Like other forms of electrokinetic injection, optical gating has bias in the injection process. Individual plug lengths are determined by each analytes' electrokinetic mobilities. Consequently, the injected plug composition is not representative of the actual sample composition. Another potential drawback of optically gated CE is the difficulty in photobleaching fluorescent tag. Jorgenson and Monnig report ~80% photobleaching

efficiency of fluorescein at 600 mW of the 488 nm line. Unbleached fluorophore molecules give rise to background fluorescence at the detector and result in poorer detection limits. More easily photobleached dyes, while poorer fluorophores, may be more amenable to optical gating applications. For example, Tao et al.[12] reported a photobleaching efficiency of over 98% for OPA/$\beta$−ME derivatives of amino acids at ~1 mW laser power. The low background possible with this reagent resulted in mass limits of detection between 10 and 35 zmol and concentration detection limits of ~15 nM. A final concern in optically gated CE is that the sample matrix is constantly being infused onto the capillary. If the sample matrix contains high salt content or adsorptive components which can alter the electroosmotic flow, then poor efficiency and reproducibility may be observed.

Because of the limitations of optical gating, it seems likely to be used primarily in certain niche applications where the above problems can be overcome. Such applications may include multidimensional separations or chemical monitoring. At the same time, however, opportunities exist for expanding the utility of this and related approaches. First, other fluorophores should be investigated for their applicability to optical gating. In addition, other destructive detectors could possibly be used with gating. One can envision, for example, using electrochemical gating in which an electrode is used to oxidize analytes preventing detection at a downstream electrode. Switching the potential on the upstream electrode could be used to inject a plug of intact molecules for separation and subsequent amperometric detection.

**Flow Gated CE.** Flow gating was introduced as a injection method in CE in 1993.[69] A diagram of a flow-gated interface is illustrated in Figure 6. In the system, a capillary containing sample is positioned across from the separation capillary with a gap of ~75 $\mu$m between the capillaries. Gating is accomplished by providing a rapid fluid flow of electrophoresis buffer between the inlet and separation capillaries (see lower portion of Figure 6). Typical volumetric flow rates for the gating flow are between 0.3 and 1.0 mL/min. While the gating flow is applied, sample that exits the inlet capillary is quickly swept away by the gating flow and is not injected onto the separation capillary. To perform an injection, the gating flow is stopped by a pneumatically actuated valve for a brief period of time, and small volumes of sample can then be drawn onto the separation capillary by electrokinetic effects. Refinements to this system have since been described[70] including altering the voltage during injection to achieve an additional level of control over the injection volume and using conically shaped capillary tips to improve the fluid dynamics around the column inlet. Photographs illustrating the injection process are shown in Figure 7. Using this improved interface, separations with over 480 000 plates in just 35 s have been achieved for several FITC-labeled amino acids.[70] Equally important, the precision of such injections result in relative standard deviations below 4% for all analytes. Finally, the method is well-suited for ultramicroscale analysis as flow rates through the sample capillary can be used from 79 nL/min to up over 1 $\mu$L/min.[71,72]

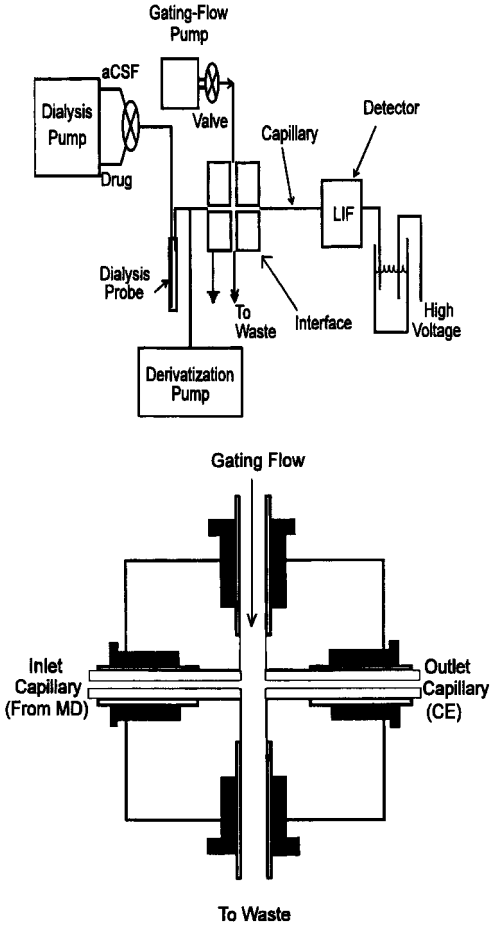


**Figure 6.** (Top) Block diagram of the flow-gating system coupled to a microdialysis (MD) probe for sampling. (bottom) Detailed diagram of the flow-gated interface. See text for details of the operation of the interface. The top figure also illustrates that dialysate sample is derivatized on-line by pumping derivatization reagent into the reaction capillary before the interface. (Reprinted with permission from ref 414. Copyright 1997 University of Florida.)

Flow-gated CE, like optically gated CE, has been used as a second dimension in multidimensional separations[69] and for in vivo monitoring.[71−74] Compared to the optical gate, the flow gate has not allowed as fast of a separation because of the difficulty of switching flows on a rapid time scale. At the same time, however, the flow gate has some advantages including ease of setup and it allows the separation buffer to be optimized independent of the sample. In addition, the voltage can be manipulated during the injection for optimal injection.

**Injection Valve Systems.** Another route to repeated injections for fairly rapid separations is based on a HPLC injection valve. In this system, the sample capillary and the separation capillary are aligned in a fashion similar to the flow-gated system. Sample is passed through an HPLC-type injection valve that is on the same line as the sample capillary. After sample is loaded into the valve, the valve is actuated to inject a slug of sample into the sample capillary. Flow through the capillary brings the slug of sample in the separation inlet for a brief period, allowing the

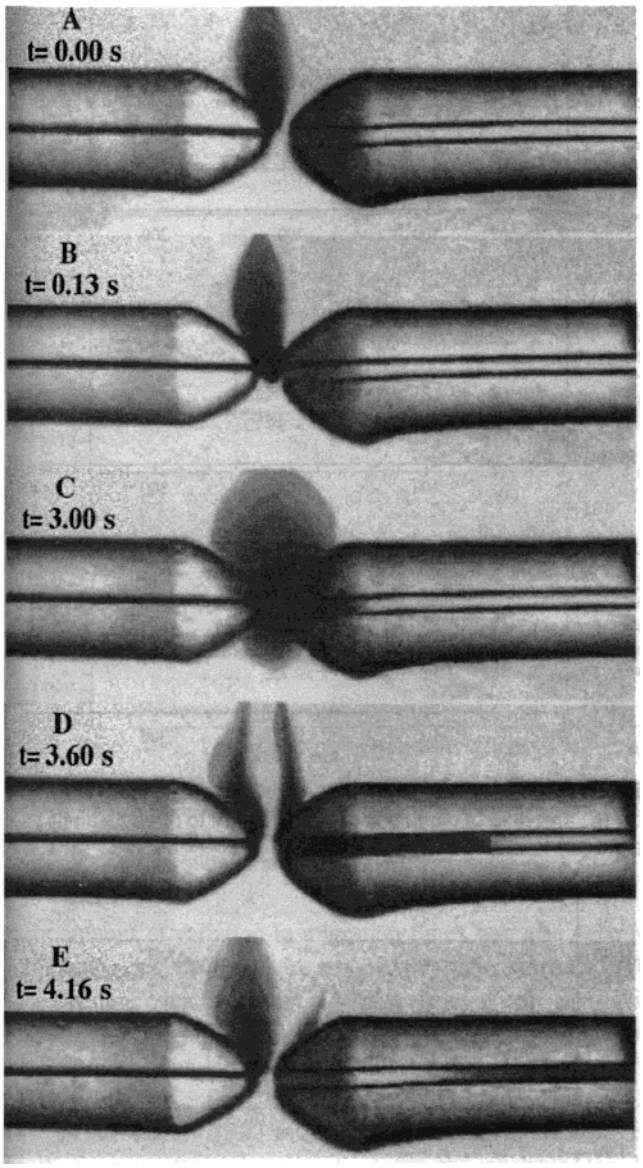


**Figure 7.** Video images of a flow-gated injection. (left) Solution of methylene blue dye is flowing out of a conically shaped capillary into the interface. The capillary on the right is the separation capillary. In frame A, the run voltage has been dropped to zero while the transverse flow remains on. In B, the transverse flow has been shut off allowing sample to flow across to the CE capillary. In C, the electrokinetic injection is underway and a plug of sample can be seen filling the inlet of the capillary. In D, the transverse flow has resumed and begun to wash away excess dye from the gap between capillaries. In E, the gap between capillaries is completely filled with buffer and the injected plug has begun to migrate down the capillary. (Reprinted with permission from ref 70. Copyright 1997 American Chemical Society.)

injection. The injection volume is controlled by the slug size (60 nL in most cases) and the flow rate. Between injections, electrophoresis buffer is brought through the injection valve. Although not as compatible as flow gating or optical gating for rapid separations, this system has proven reliable for coupling to microdialysis sampling for 2 min separation times.[75,76]

**Microchip-Based Devices.** High-speed CE separations have also been achieved in microfabricated devices or "chips". The use of lithographic techniques has enabled the formation of networks of channels on the micrometer scale which can be used as separations paths for CE as well as for sample manipulation. From the point-of-view of fast separations, an important advantage of chip-based devices is the capability of using electrokinetics to manipulate and inject samples on the picoliter scale. A separate review of chip-based systems for separations is available in this issue; therefore, our review will be limited to a few examples.

Various injection schemes have been reported in microchip-based CE analysis. Most commonly injection is accomplished by electroosmotically loading the sample into a fixed injection volume and then applying the separation voltage to pull the slug into the separation channel. Figure 8 illustrates this concept in better detail. During sample loading, a voltage is applied between the sample and injection waste reservoir. EOF pulls sample toward the injection waste reservoir, consequently filling up a region of the separation capillary. The loading voltage is then turned off and the separation voltage is applied, allowing the analyte zones to be detected down column. This approach injects a constant volume onto the separation channel as defined by the geometry of the injector region.

To prevent electrokinetic biasing, the loading voltage can be applied for a sufficiently long time to generate a representative sample in the injection plug region. A disadvantage of this approach is that the time required for injection ($t_{inj}$) can exceed the separation time. This effect was illustrated[77] in a demonstration of repetitive sample injection/electrophoretic migration of fluorescein on a chip. The separation step took 4 s ($t_{sepn}$), while the time required for injection was 3 s ($t_{inj}$) and a dead time ($t_{dead}$) of 2 s was necessary for washout of the region for a total cycle time of 9 s.

A concern with this type of injection is that sample may diffuse onto the separation channel during sample loading. The resultant "leaking" gives rise to injection plug broadening and also complicates the on-chip fluid mixing process as the solution being analyzed may often become contaminated by solution leaking from a side channel. This problem has been resolved by application of an opposing electric field which counteracts this leaking effect (see Figure 9).[78] Image b from the figure illustrates an example of this "pinched" sample loading.

Jacobson et al.[64] have pushed the limit of the chip-based injection/separation system by separating rhodamine B and dichlorofluorescein in <1 ms. For this separation a "T" type injector was used and the electric field was 53 kV/cm over a separation distance of 200 $\mu$m. This approach generated just over 100 theoretical plates for both species; however, ~230 000 plates/s were generated for dichlorofluorescein.

An important advantage of the chip-based approach is the capability of performing more complex sample manipulations prior to or after the actual separation. For example, various authors[79,80] have performed cell lysis, PCR, and electrophoretic sizing of the products on a chip. Additionally, Ramsey et al.[81] demonstrated electrokinetic focusing of an analyte stream by applying additional potentials per-

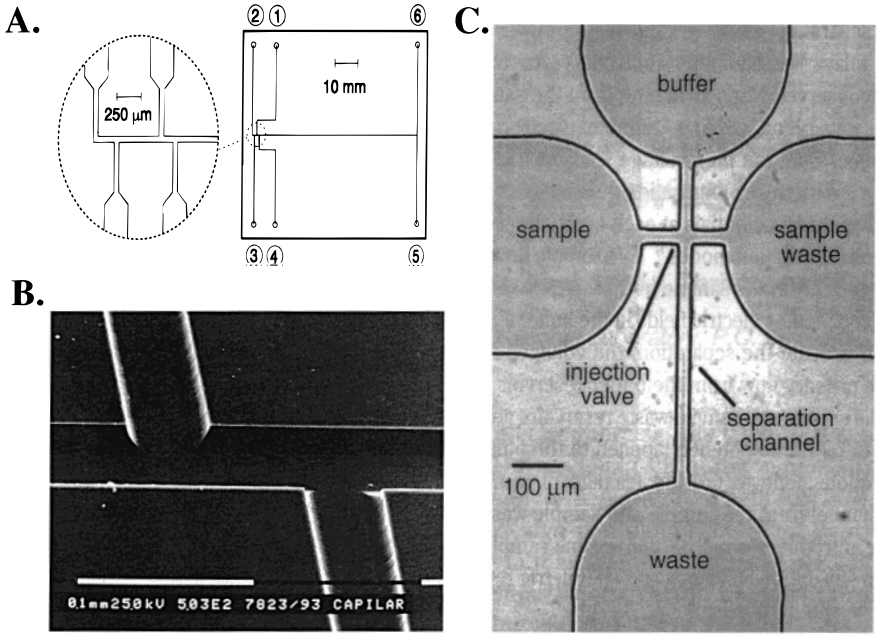


**Figure 8.** Diagrams of two injector types for microchip electrophoresis. (a) Layout of a glass chip with the "double T" type injector. The depth of the channels is 12 $\mu$m, and the width is 50 $\mu$m. When potential is applied between reservoir 1 and 4 EOF will draw sample into the main separation channel. After loading the sample, the separation voltage is applied and analytes begin to migrate down the channel. (b) Scanning electron micrograph of the sample injection region. (c) Drawing of an alternate injection interface for microchip electrophoresis. When a potential is applied between the sample and sample waste reservoirs, solution again is drawn into the region where the main separation channel intersects the injection channel. (Reprinted with permission from refs 77 and 64. Copyright 1993 and 1998 American Chemical Society and CRC Press.)

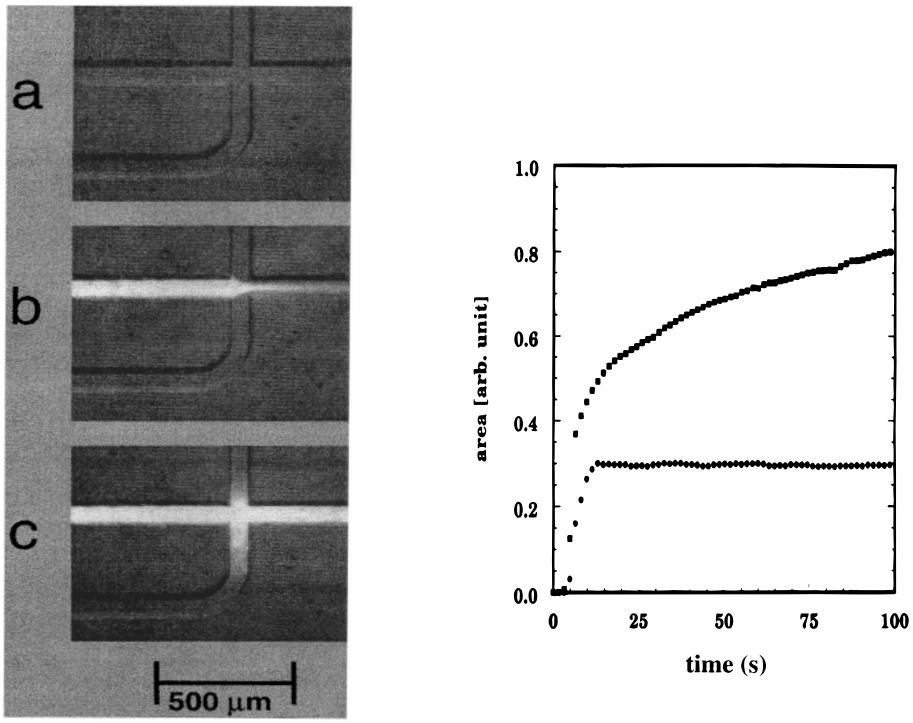


**Figure 9.** (left) CCD images of the injection cross on a microfabricated electrophoresis chip: (a) no fluorescent analyte present; (b) "pinched" sample loading (Notice the fluorescent analyte forms a narrow injection plug); (c) sample loading without applying a "pinching" voltage. In c, the sample can be seen "leaking" into the vertical channel. The fluorescent analyte for all images was rhodamine B. The figure on the right illustrates this effect graphically. It is observed that the peak area for the floating sample injection increases with time (■), while the pinched peak area from pinched injection remains relatively constant (●). (Reprinted with permission from ref 78. Copyright 1994 American Chemical Society.)

pendicular to the fluid flow on a microchip. Automated voltage switching on-chip provides the possibility of performing complex fluid handling and mixing operations which can be also used to facilitate sample derivatization. Indeed, both precolumn and postcolumn[82,83] reactor devices have been fabricated and characterized to illustrate the potential of mixing on-chip. Harrison[84] demonstrated between 22800−

83300 plates in a 20 s separation of four amino acids with postcolumn derivatization after optimization of the mixing chambers. These types of integrated systems are necessary for a rapid total analysis system.

### 3. Parallel Electrophoresis Separations

Optical-gating, flow-gating, and microchip-based methods all allow fast separations; however, many of the goals of rapid separations may also be achieved by using various forms of parallel separations. Some applications that may also be achieved by parallel separation include high-throughput analysis, high-temporal resolution monitoring, some types of kinetic analysis, and multidimensional separations. Interestingly, most of the fast work has involved only serial analysis while most of the parallel work has utilized fairly standard analysis times. Clearly, the ultimate in throughput could be achieved by fast, parallel separations, so these trends are likely to merge as instrumentation improves. Although the emphasis of the review is on fast separations, because of the applications overlap of parallel and fast separations, we will briefly discuss parallel separations as well. At the outset, we note that a recent article reviewed parallel separations, especially for genome analysis.[85]

Parallel CE separations have been performed in bundles of capillaries,[86−95] in multiple channels on chips,[96−100] or in open channels.[101−105] Parallel separation presents quite different problems from rapid separations. For example, the variance of zones is not so extremely small in parallel separations as it is in rapid separations; however, the associated instrumentation must be capable of injecting and detecting onto multiple capillaries or in a spatially resolved fashion.

**Capillary Bundles.** Several groups have demonstrated performing parallel separations in bundles of capillaries. This work has mainly been driven by the Human Genome project, and all of the published examples so far have been DNA separations. It may be expected, however, that this technology will now filter into other applications.

In these systems, arrays of capillaries are bundled together so that each capillary can simultaneously be inserted into a separate sample, samples loaded by electrokinetics, and then transferred to electrophoresis buffer for separation. Detection has been exclusively by fluorescence with spatially resolved detectors. Detection methods have included scanning detectors[106,107] and/or spatially resolved detectors such as CCD's.[93,108] As many as 100 capillaries have been bundled together to provide parallel analysis.[93] Recently,[85] an improved system, which will allow for more than 1000 capillaries to be imaged, has been described. Commercial availability of these types of instruments have led to a proposal to sequence the Human Genome by a "shot-gun" approach in just a few years.[109]

**Parallel Channels on Chip.** Application of microchip systems yields another approach to parallel analysis. While multicapillary chips and capillary bundles have been employed for similar analysis, some distinct differences are apparent. With the chip-based systems, each channel corresponds to a unique separation lane, thus eliminating the difficulty in preparing large capillary bundles and simplifying analyte detection. In addition, it has been shown that these devices can increase the speed of CE separations by an order of magnitude. Woolley et al.[96] applied this technology to DNA genotyping as 12 separations (<160 s each) were carried out simultaneously in a device that was monitored by a laser confocal scanner. Additionally, these devices offer the possibility of analysis of a diverse set of compounds quickly as each channel could be utilized to screen for a certain class of compound.

**Channel Electrophoresis.** Parallel analysis has also been performed using channel electrophoresis. An instrumental design for continuous channel electrophoresis is illustrated in Figure 10.[110] Separation is carried out in rectangular channels formed between two thin strips of uniform glass microspheres which define the outer edges of the channel. The channel thickness is defined by the diameter of the microspheres and is typically on the order of $<20\ \mu$m, while the width of the channel is typically ~1.5 cm. Sample is continuously introduced at one end of the separation channel via a fused silica sampling capillary. The sampling capillary constantly moves back and forth across the entrance to the separation channel, thus performing continuous sample "injection". Analytes continuously migrate through the channel and are detected either by spatially resolved LIF[101,111−116] or by an amperometric electrode array detector.[110,117] Electrode arrays used so far consist of 100 independent microelectrodes with 95 $\mu$m width spaced across the rectangular channel for site-specific amperometric detection. For LIF detection, the channel structure can be imaged by a CCD for sensitive detection of fluorescence.

In this method, the time required for separation does not determine the temporal resolution of the instrument; rather, the position of sample along the inlet axis encodes the time of introduction. Temporal resolution is limited by the broadening along the sample introduction axis. An example of the data obtained can be seen in Figure 11. The "zigzag" pattern results from electrokinetic migration in the vertical direction convoluted with the sampling capillary movement along the channel inlet. The separation can be performed in free solution or within a gel. The addition of gel helps to prevent lateral diffusional mixing of the sample as it is introduced and migrates down the channel. Channel electrophoresis has been used to monitor secretion from single cells,[118] in multidimensional separations,[116] for flow-injection analysis,[113] and for monitoring kinetics of formation of a fluorophore.[114,115]

This technique holds promise for temporally resolved monitoring using separations; however, some problems will need to be overcome. First of all, because the separation is performed in rectangular channels, lateral diffusional broadening cannot be overlooked. In recent work, it has been shown[119] that plugs of dopamine (2.3−5.5 mM) injected at the entrance to the separation channel laterally broaden

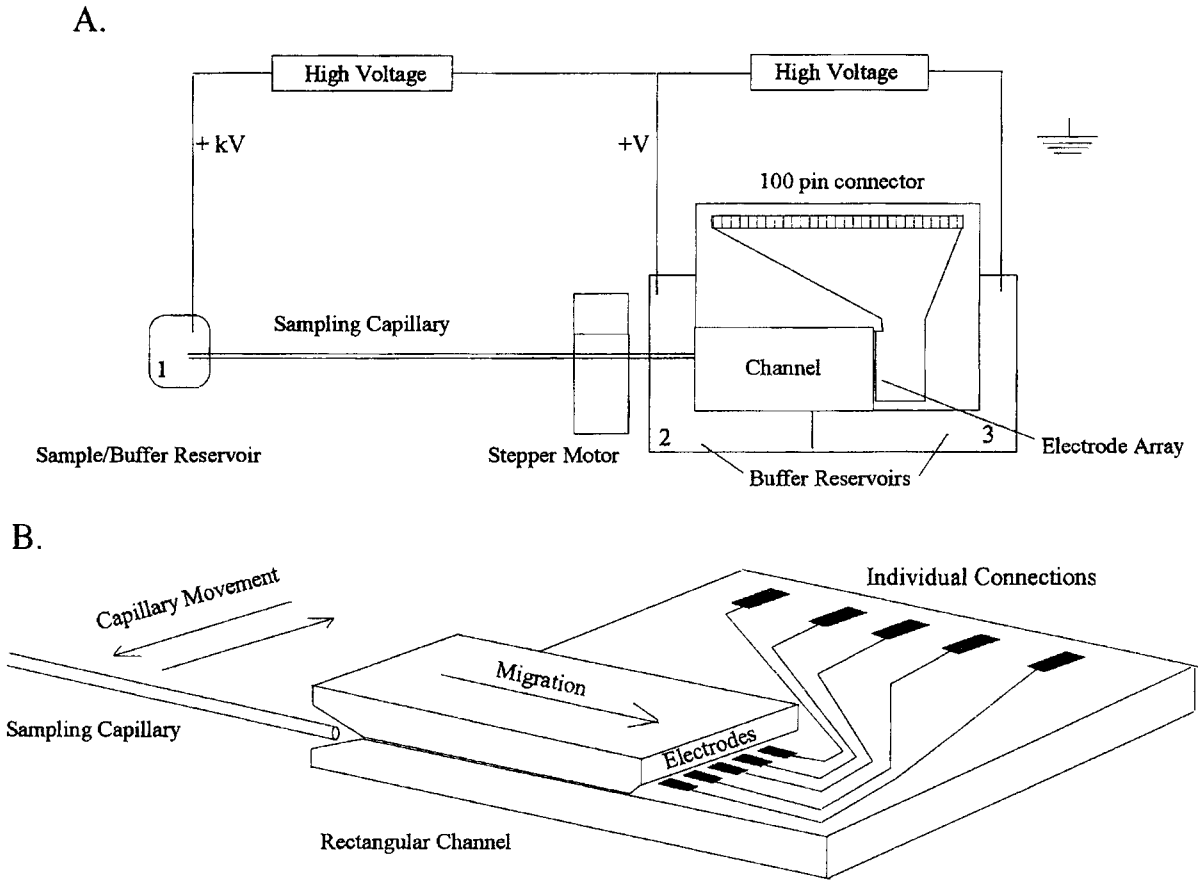


**Figure 10.** Depiction of the channel electrophoresis technique. (A) Sampling capillary is suspended across reservoirs 1 and 2, and the channel is suspended across buffer reservoirs 2 and 3. The stepper motor is used to move the capillary across the channel entrance. The counter and reference electrodes (not shown) are placed in reservoir 3. (B) Three-dimensional view of the channel electrophoresis scheme, shown with five electrodes, connections, and bond pads used for electrochemical detection. The actual electrode array has 100 electrodes, connections, and bond pads directly fabricated onto the bottom plate. (Reprinted with permission from ref 110. Copyright 1996 American Chemical Society.)

to between 1.2 and 1.8 mm at the time of detection. This lateral dispersion limits temporal resolution of the instrument to ~5 s as step changes in concentration will diffusionally "blur" in the channel before detection. Better results can be obtained with a high-density gel to limit broadening; however, this solution was shown to be incompatible with on-line derivatization, which was necessary for single cell monitoring.[118]

Broadening which affects separation resolution also cannot be overlooked. While all forms of broadening such as diffusion and adsorption still hold similar roles in channel electrophoresis, injection broadening is somewhat different. Injection broadening in channel electrophoresis is determined by several factors. The separation lane width, rate of sampling capillary movement, voltage applied to the channel, and analytes electrophoretic mobility all play a role in broadening the sample zones. The spatial broadening of analyte zones can be seen graphically in Figure 12 and can be given as

$$\sigma_{\mathrm{Inj}}{}^2 = ((\mu + \mu_{\mathrm{EOF}})E)(W/R_t))^2 \qquad (15)$$

where $W$ is the width of the individual separation lane, $R_t$ is the rate at which the sampling capillary is brought across the entrance to the channel, and other terms as discussed above. In the simplest case (neglecting diffusional effects), the width of each individual separation lane is determined by the detector element width (for electrochemical detection in the example given, this is the electrode width of 95 $\mu$m). As seen, this broadening is brought about by previously introduced molecules getting a "head start" on other molecules which will eventually be detected at the same electrode. This broadening can be minimized by utilizing high rates of sampling capillary movement across the channel entrance, but increasing the rate of movement also decreases the mass available for detection. A comprehensive review of channel electrophoresis can be found elsewhere.[120]

## C. Micellar Electrokinetic Chromatography (MEKC)

Since its introduction,[121] MEKC has become widely accepted as a valuable separation tool since it allows neutrals to be separated in an electrokinetic format and changes the selectivity for charged compounds. MEKC separation is based on differential migration induced by partitioning into a slow-moving micellar phase (pseudostationary phase) in addition to any electrophoretic separation that occurs for charged compounds. Thus, the band broadening and performance at high velocities of this separation is quite different from electrophoresis. Band broadening due to longitudinal diffusion, sorption−desorption kinet-

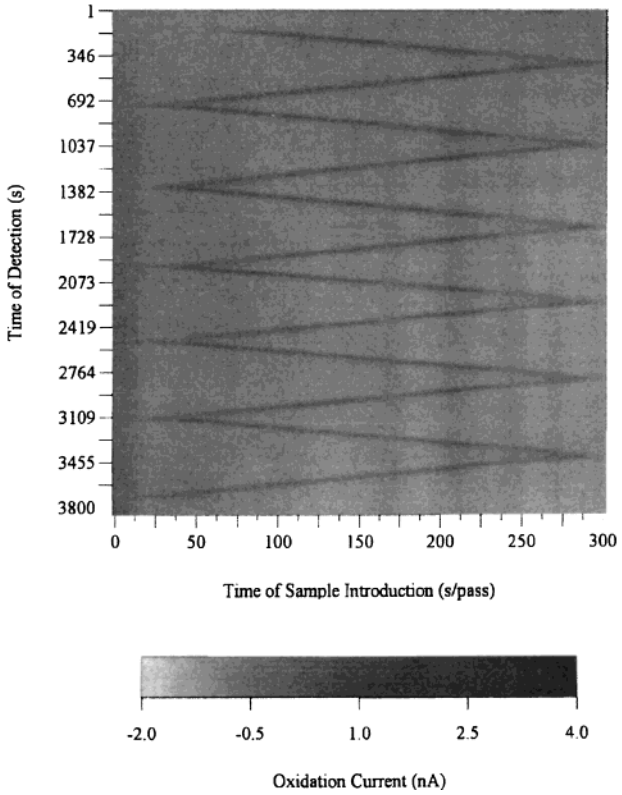


**Figure 11.** Continuous migration of a 7.2 mM solution of dopamine with electrochemical array detection as the sampling capillary is scanned across the inlet of the channel. Continuous scanning results in the zigzag pattern for dopamine detection. The channel was 4.8 cm long and had an internal height of 21 $\mu$m. The amount of material injected per 95 $\mu$m electrode was 1.4 pmol. (Reprinted with permission from ref 110. Copyright 1996 American Chemical Society.)

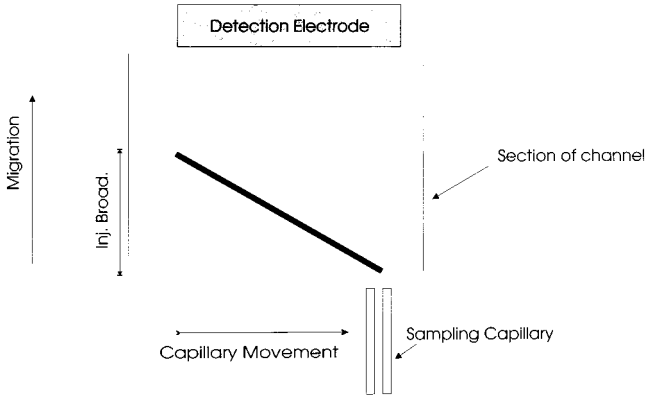


**Figure 12.** Depiction of injection broadening in continuous channel electrophoresis with electrode array detection. The detection electrode is a single 95 $\mu$m electrode in the array. As the sampling capillary is brought across the entrance to the channel, molecules introduced first (to the left) get a head start on molecules introduced at a later time. As these molecules will eventually be detected at the same electrode, broadening of zones results.

ics, intermicelle mass transfer, temperature gradient effects, and electrophoretic dispersion in MEKC have all been examined by Terabe et al.[122]

Terabe derived an equation for plate height in MEKC based on longitudinal diffusion alone

$$H_l = (2(D_{aq} + k'D_{mc}))/1 + (t_0/t_{mc})k'(v_{eo}) \quad (16)$$

where $D_{aq}$ and $D_{mc}$ are the diffusion coefficients in the aqueous and micelle phase respectively, $k'$ is the retention factor, $t_0$ and $t_{mc}$ are the retention times of an insolubilized solute and the micelle, respectively, and $v_{eo}$ is the electroosmotic velocity. Interestingly, longitudinal diffusion must take into account diffusion within the micelle as well as in free solution.

Sorption−desorption kinetics in and out of the micelle phase also play a role in band broadening in MEKC. Terabe identifies this contribution as

$$H_{mc} = ((2(1 - t_0/t_{mc})^2k')/((1 + (t_0/t_{mc})k')(1 + k')^2))(v_{eo}/k_d) \quad (17)$$

where $k_d$ is the desorption rate constant and all other terms as above. This equation predicts that plate height from kinetic broadening increases with an increase in velocity and with a decrease in the desorption rate constant. This band broadening is a limiting factor in fast MEKC as large $v_{eo}$ will be required.

Random walk theory allows for derivation of the plate height contribution of intermicelle diffusion as shown by Terabe

$$H_{aq} = (k'/1 + k')^2((1 - t_0/t_{mc})^2/1 + (t_0/t_{mc})k')(d^2v_{eo}/4D_{aq}) \quad (18)$$

where $d$ is one-half of the intermicelle distance and all other terms as above. Again, it is shown that large $v_{eo}$ values lead to larger plate heights. In most analysis, this term is considered negligible, but in ultrafast analysis it may be important.

As previously discussed, temperature gradients in capillaries give rise to band broadening. In MEKC, this holds true as well since

$$H_t = (k'r_c^2/24(D_{aq} + D_{mc}k'))(G_v/2 + G_v)^2 (v_{eo} - v_{mc}) \quad (19)$$

where $G_v$ indicates the extent of the differences in velocities between the axis and the inside wall of the capillary. The use of small i.d. capillaries helps reduce this effect.

Microheterogeneity of the micelles can also give rise to substantial broadening in MEKC. The polydispersity of the micelle size can lead to electrophoretic dispersion in the media as

$$H_{ep} = ((0.026(1 - t_0/t_{mc})^2k')/1 + (t_0/t_{mc})k')(v_{eo}/k_d) \quad (20)$$

Again, we see large $v_{eo}$ terms increase the broadening due to this effect.

Among these five band broadening terms, only longitudinal diffusion decreases as flow is increased. In addition to these terms, other aforementioned band broadening terms associated with injection and detection still apply.

MEKC has rarely been used for fast separations. In one example, Moore et al.[123] studied the separation of three coumarin dyes in a chip system that had a

separation capillary with ~70 $\mu$m width and 10 $\mu$m depth with either 16.5 or 1.3 cm length. They observed that with $E$ up to 1200 V/cm, the dominant source of band broadening was independent of velocity and was perhaps injection or detection broadening. More importantly from a theoretical standpoint, they observed that above 400 V/cm sorption−desorption kinetics began to contribute to plate height and above 700 V/cm Joule heating played a role. The fact that sorption−desorption kinetics began to contribute at 400 V/cm suggests that MEKC will not allow as fast a separation as CE. These results and theory show that the limit to speed in MEKC ultimately depends on the solutes retention factor as the mass-transfer band broadening terms become more significant as retention factor increases. Thus, applicability of MEKC to fast CE analysis is largely dependent on the analyte and sample matrix.

Despite this limitation, some good examples of rapid analysis by MEKC have been demonstrated. For instance, three neutral coumarin dyes were separated in <30 s with between 3200 and 8800 theoretical plates at 500 V/cm with a 1.3 cm separation distance in the Moore et al. work. In another work, MEKC separation was employed to separate 10 amino acids sampled by microdialysis from the rat caudate nucleus in under 160 s using a flow-gating system.[71] In this work, $E$ = 600 V/cm and the capillary dimensions were 10 $\mu$m i.d. and 10 cm separation length (Figure 13 illustrates electropherograms obtained from this work). This method allowed monitoring and quantification of these 10 amino acids with 3 min temporal resolution.

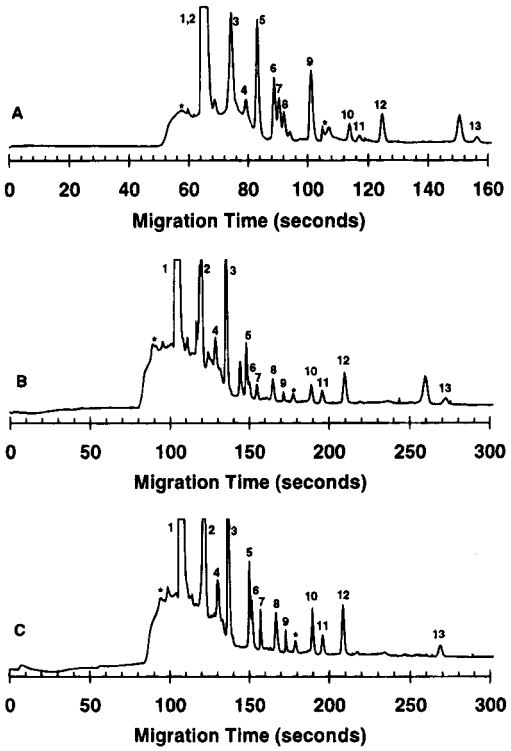


**Figure 13.** Fast MEKC of amino acids. The electropherograms were obtained utilizing the flow-gated interface. (A) Sample obtained by microdialysis from the rat caudate nucleus. $E$ = 600 V/cm, $l$ = 10 cm. (B) Sample obtained by microdialysis from the rat caudate nucleus. $E$ = 400 V/cm, $l$ = 15 cm. (C) Standard samples. $E$ = 400 V/cm, $l$ = 15 cm. Peak labels as follows: (1) glutamine, (2) serine/threonine, (3) alanine/glycine, (4) tyrosine, (5) taurine, (6) valine, (7) glutamate, (8) methionine, (9) aspartate, (10) leucine, (11) phenylalanine, (12) isoleucine, and (13) lysine. Impurities and reagent peaks are labeled with an asterisk, while unidentified peaks are unlabeled. (Reprinted with permission from ref 71. Copyright 1996 American Chemical Society.)

## D. Applications of Rapid CE

### *1. Rapid CE of Proteins and Peptides*

Separation of peptides and proteins by CE presents a special challenge to the analyst due to the large diversity of proteins and peptides in terms of size, shape, charge, and hydrophobicity. While a variety of CE modes have been successfully used for proteins and peptides, including isoelectric focusing[124] and capillary isotachophoresis,[125] the majority of fast protein and peptide CE has been performed using free solution CE or capillary SDS-gel electrophoresis. Rapid CE of proteins offers the opportunity for high-throughput assays and to observe short-lived intermediates or conformers. Specific examples of rapid CE separations of peptides and proteins and discussed in this section. A related topic, affinity probe assays, which often involves rapid separation of proteins, is discussed later in this review.

**Free Solution CE of Proteins and Peptides.** Separation of proteins in untreated capillaries frequently produce broad and irreproducible zones. Among the reasons for this phenomenon are hydrophobic protein−silica and ion-exchange interactions on the surface of the capillary,[126,127] which cause mass transfer band broadening as in chromatography. Such effects are especially troublesome for fast separations since this type of broadening is worsened with fast migration rates. As it is well-known, these effects can be ameliorated by use of capillaries coated with hydrophilic polymers or by use of buffer compositions, such as high salt or high pH, which prevent capillary wall−protein interactions. In an example using chemical modification of the silica surface to prevent adsorption during a fast separation of proteins, Hjerten and co-workers used allyl methyl cellulose-modified capillaries for the separation of four model proteins in less than 1 min.[128] A low-conductivity buffer (0.38% (w/v) polyoxyethylene bis-(3-amino-2-hydroxypropyl)−0.031% (w/v) polyoxyethylene bis(acetic acid), pH 8.6) was also used to allow application of a 2000 V/cm electric field over a 15 cm long capillary with a 50 $\mu$m i.d.

It is known that high-pH separation buffers can decrease the number of positively charged moieties on the surface of the protein and prevent electrostatic adsorption. The downside of this method is increasing separation time due to developing of more negative charge on the surface of proteins. Figure 14 illustrates separation of eight proteins in less than 200 s in an untreated fused silica capillary using 150 mM borate buffer at pH 10.5 in a 25 cm long by 25 $\mu$m i.d. capillary with 15 kV applied.[129] If the pH was increased to 11.5 better separation efficiency was achieved; however, the separation time was increased to about 350 s.

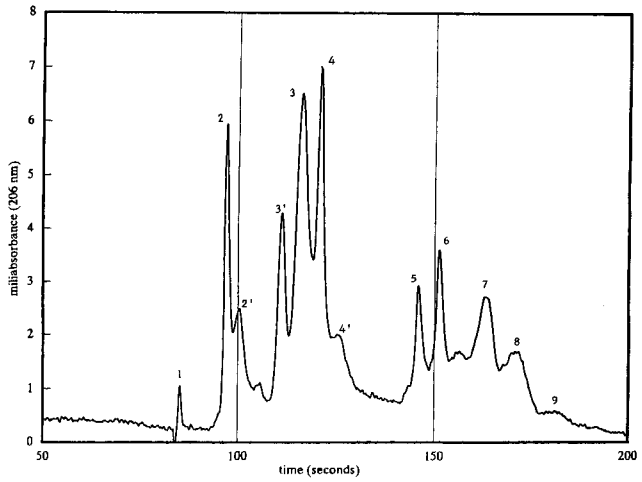


**Figure 14.** Electropherogram of model proteins at high pH with UV detection. Peaks: (1) DMF (neutral marker); (2) bovine ribonuclease A; (3) whale myoglobin; (4) horse myoglobin; (5) conalbumin; (6) $\beta$-lactoglobulin; (7) bovine serum albumin (BSA); (8) ferritin; (9) $\alpha$-amyloglycosidase. Peaks 2′, 3′, and 4′ are impurities from 2, 3, and 4, respectively. (Reprinted with permission from ref 129. Copyright 1991.)

The majority of CE separations of peptides performed to date took more than 5 min to accomplish. Among the faster examples were separation and characterization of isolated and synthetic peptides by Yildiz et al.[130] and separation of the hydrolysis products of glycine containing peptides by Vessillier et al.[131] Cifuentes and Poppe reported application of rectangular capillaries for separation of peptides.[43] Although separations were performed in about 5 min, advantages such as better heat dissipation, higher separation speed, and larger sample capacity compared to the cylindrical capillaries were demonstrated. Banks and Dresch[132] successfully interfaced CE with a TOF mass spectrometer using an electrospray interface for the separation and detection of peptides. One of the advantages of using a TOF mass spectrophotometer compared to other types is its inherently fast scan rate which gives the ability to detect narrow CE peaks. An electric field of 450 V/cm applied across an amine-coated 66 cm long 75 $\mu$m i.d. capillary was used to obtain a separation of five tripeptides in <100 s (total separation time 450 s) as shown in Figure 15. The peaks were 2 s wide or less, yet the fast scanning capability of the TOFMS allowed sufficient spectra to be collected to characterize the electrophoretic peaks. As shown in the figure, the use of a quadrupole MS greatly distorted the peaks because of the longer time to acquire individual spectra. Using the same experimental conditions, the authors also separated a mixture of three proteins, ubiquitin, myoglobin, and cytochrome *C*, in 125 s.

**Capillary SDS-Gel CE of Proteins.** Capillary SDS-gel electrophoresis can provide a high resolution size-based separation as a result of complexation between detergent and a protein. Initially, gels were covalently attached to the capillary walls and produced good results in separation of the mixtures of proteins. However, time-consuming preparation together with short lifetime of the columns have prompted the development of the techniques in which

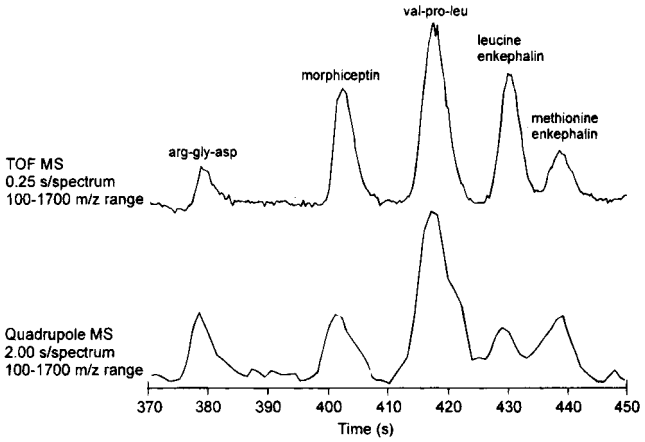


**Figure 15.** Comparison of total ion chromatograms from a peptide CE separation using TOF and quadrupole mass spectrometry detection. (Reprinted with permission from ref 132. Copyright 1996 American Chemical Society.)

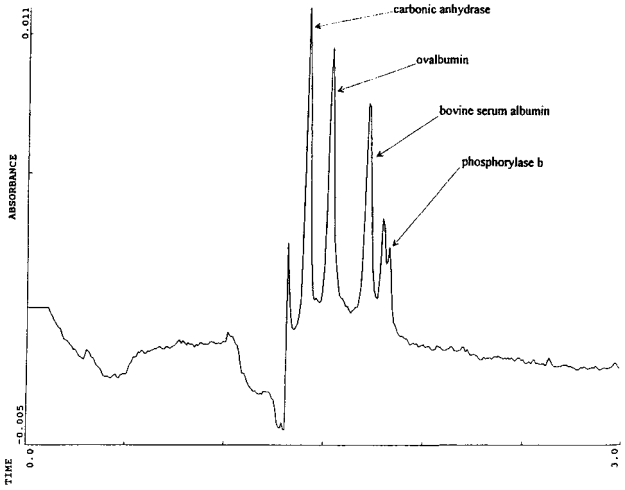


**Figure 16.** Rapid separation of four standard proteins according to their molecular mass by capillary SDS−PAGE. Electric field = 740 V/cm, time scale is in minutes. (Reprinted with permission from ref 133. Copyright 1993.)

the column could be refilled with the fresh gel solution before each separation. Lausch et al. have demonstrated the application of such refillable columns for rapid evaluation of the molecular weight of the light and heavy chain of the human immunoglobulin G.[133] Separations were performed with a 50 $\mu$m i.d. by 27 cm long (7 cm effective length) dextran-filled column previously coated with polyacrylamide to suppress EOF with 740 V/cm applied. Separation buffer was 0.1 M TRIS titrated to pH 8.6 with 0.1 M CHES buffer with a total of 0.1% SDS added. Separations were complete in 2 min and displayed a linear relationship between the migration time and the size of the protein as shown in Figure 16. The migration times were precise, with a 0.77% relative standard deviation ($n$ = 20), allowing the error in calculated molecular weight to be less than 1%. Thus, these separations demonstrated the potential for rapid determination of the molecular weight of proteins such as the complicated light and heavy chains of IgG.

Benedek and Guttman studied the effect of the amount of SDS present in sample and separation buffer on the efficiency of fast SDS-capillary gel

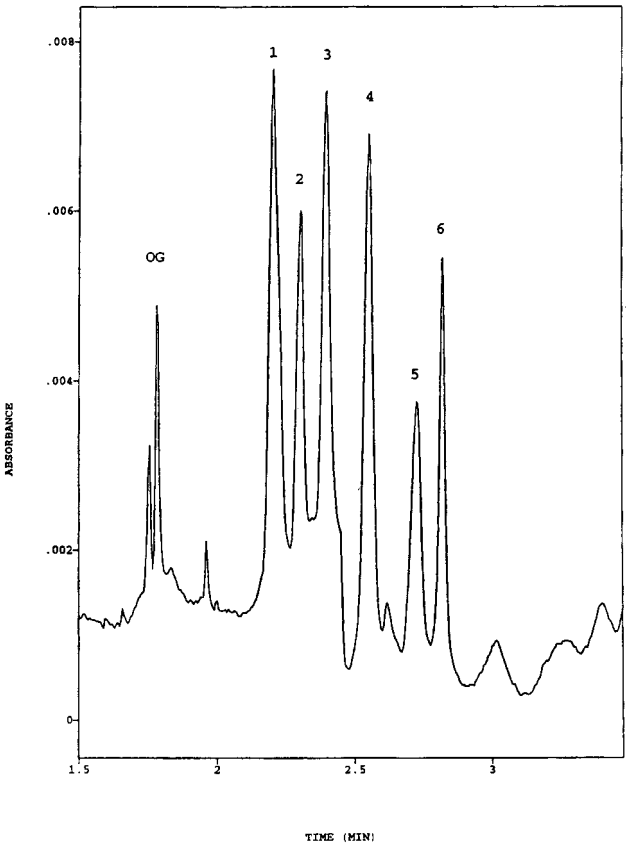


**Figure 17.** Separation of standard proteins at 888 V/cm by capillary SDS−PAGE. Peaks: (OG) orange G; (1) α-lactalbumin; (2) soybean trypsin inhibitor; (3) carbonic anhydrase; (4) ovalbumin; (5) BSA; (6) phosphorylase *b*. (Reprinted with permission from ref 134. Copyright 1994.)

electrophoresis separations.[134] This study utilized 50 and 100 $\mu$m i.d. columns with a 20 cm effective length (27 cm total) that were not coated with a hydrophilic material but treated with 1 M NaOH and 1 M HCl after every separation to remove adhered materials. The columns were filled with 3% PEO ($M_r$ 100 000) as the sieving media, and the separation buffer was 0.06 M Tris−HCl (pH 6.6) with various amounts of SDS. The authors demonstrated that increasing SDS concentration in the sample leads to the improvement in resolution; however, increasing SDS concentration in the separation buffer had an opposite effect. For optimized conditions, a separation of six proteins in less than 3 min at 888 V/cm electric field was possible as shown in Figure 17.

**Detection of Short-Lived Protein and Peptide Conformations.** As suggested above, the high-speed separation capability of CE allows short-lived species, such as various conformations of proteins or peptides, to be detected. Several examples have been reported which allow interesting observations on these molecules.

In one example,[135] a high-pH separation buffer (20 mM borate buffer at pH 9.6) was used as the media to monitor quaternary structure and structural changes induced by denaturants of nucleoside diphosphate (NDP) kinase, a protein shown to affect the metastatic potential of various cancers. Subunits and denaturants of the nucleoside diphosphate kinase were separated in less than 2 min using a 40 cm long by 50 $\mu$m i.d. capillary (20 kV applied voltage, 31.6 cm effective length). It was known that NDP kinase is a hexamer consisting of two subunits A and B; however, the exact ratio of the subunits was not known. The authors discovered using CE that recombinant protein is either $A_6$ or $B_6$. When two recombinant subunits were mixed at 37 °C, no interaction was observed. On the other hand, separation of NDP kinase obtained from human erythrocytes revealed five peaks indicating that the native protein has a heterogeneous population. Since calculated isoelectric points of both subunits were 6.13 and 8.64, the identity of the peaks could be determined from their migration time. The major peak was identified to have the structure of $A_3B_3$. The authors also studied effects of 6 M urea and heat on the structure and interactions of NDP kinase. The unfolding transition and possible formation of the intermediates are fast processes and could not be seen by conventional gel electrophoresis.

In another example, Kilar and Hjerten studied the unfolding of human serum transferrin in urea.[136] In this work, they demonstrate that monitoring the unfolding of proteins by denaturants can give information about conformational stability of the molecule. To perform the analysis, separations were performed with various concentrations of urea (0−8 M) added to the separation buffer (18 mM Tris−18 mM borate−0.03 mM EDTA, pH 8.4). Since transferrin is a iron-binding molecule, the authors also investigated the effect of iron on the transitions. Using 20 cm long by 100 $\mu$m i.d. capillaries, five isoforms of transferrin were separated in under 5 min (applied voltage was 8 kV). After performing the separations in various concentrations of urea, it was determined that the unfolding of the iron-free protein is initiated at 3 M urea and intermediates could be seen up to 5 M urea. Above 5 M urea, the unfolding is faster than the separation and intermediates could not be detected. In the presence of iron, the protein adopts a more rigid conformation and cannot be denatured by 8 M urea. In this study, it was clear that the speed of separation was critical in allowing the intermediates to be observed. Such observations would not have been possible with conventional slab gel electrophoresis.

Peptides containing proline residues, except at the *N*-terminus, exist in cis and trans forms due to the rigidity of the peptidyl−proline bond. At room temperature, the cis and trans forms interconvert (isomerize) with rates on the order of seconds to minutes.[137] Although extensive studies to characterize isomerization have been done using NMR, CE separations that can be performed on a time scale faster than the isomerization rate can be used to complement these data. Rathore and Horvath obtained kinetic data for isomerization of phenylalanine−proline dipeptide using CE and compared it to the data obtained by NMR.[138] To perform separations, the authors applied 810 V/cm across a 30 cm effective length of 50 $\mu$m i.d. capillary filled with 100 mM borate buffer (pH 8.4). Although peaks migrated past the detector in less than 3 min, the complete separation could be obtained only when the entire system was cooled to

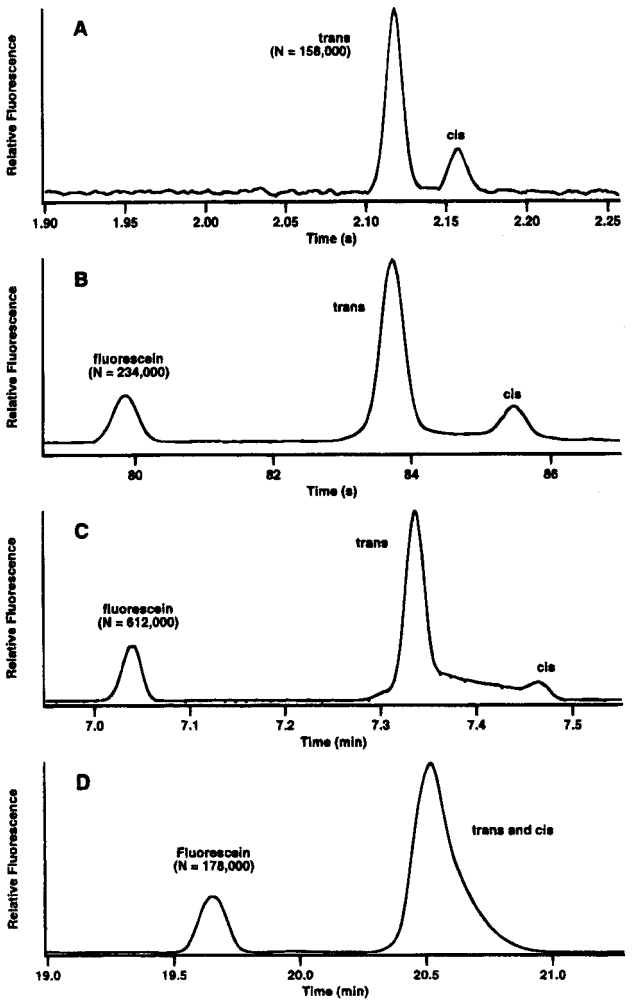


**Figure 18.** Effect of varying analysis time on resolution of cis and trans isomers of Ala−Pro by CE-LIF. (A) $L = 8$ cm, $l = 2$ cm, 20 kV. (B) $L = 29.5$ cm, $l = 13.7$ cm, 15 kV. (C) $L = 70$ cm, $l = 65.2$ cm, 28 kV. (D) $L = 70$ cm, $l = 65.2$ cm, 10 kV. Separation efficiency is less than optimal in D due to overinjection of the sample. (Reprinted with permission from ref 139. Copyright 1995 American Chemical Society.)

1.5 °C to slow the isomerization rate. At higher temperatures, a bridge forms between the two peaks, indicating interconversion during the separation. The authors used simulation techniques to determine kinetic parameters from peak shapes and obtained results that were in good agreement with NMR data (average error less than 5%).

Moore and Jorgenson also analyzed prolyl−peptide isomerization by CE. In their study, they used short capillaries with optically gated injections to obtain faster separations and, thus, better resolution between peaks.[139] To obtain a sufficiently fast separation to completely resolve the isomers at room temperature, a 2.5 kV/cm electric field strength was across an effective length of 2 cm capillary (6 $\mu$m i.d.). With these conditions they were able to obtain complete separation between cis and trans isomers of alanine−proline dipeptide with separation times less than 2.3 s as depicted in Figure 18.

**Monitoring Peptide Secretion.** In an unusual combination of CE and intra-capillary manipulation, Tong and Yeung have utilized CE with a laser-induced native fluorescence (LINF) system for monitoring secretion from single cells.[140] To perform the monitoring, a single $\beta$-cell was hydrodynamically injected onto a 63 cm long (46 cm effective) by 22 $\mu$m i.d. capillary and allowed to adhere. To induce secretion, a 3.2 mm long plug of digitonin, a reagent that permeabilizes cells, was injected so that it covered the cell. After 15 min of incubation, the released insulin was separated from the cell by either applying 3 kV for 5 min or by hydrodynamically flowing separation buffer (20 mM Tricine, pH 8.5) through to the column. Finally, a separation voltage of 30 kV was applied to cause the cell to lyse and release its remaining insulin. Both insulin bands migrate to the detector and are detected in less than 3 min. This work demonstrates the ability of CE to perform simple and rapid determinations of peptides in a single cell and the effect of the various pharmacological and toxicological compounds on the cell. The authors used the same experimental setup to monitor secretion of epinephrine and norepinephrine by bovine adrenal chromaffin cells.[141] In this case, however, the separation took about 9 min to perform.

**Multidimensional Separations.** One of the most effective uses of fast separations has been as a part of comprehensive multidimensional separations.[142] Multidimensional separations are an effective means of analyzing complex mixtures since the peak capacity of the combined techniques is approximately the product of the independent methods. In a comprehensive multidimensional separation, all components are separated by both techniques, in contrast to heart-cutting approaches where only certain bands from the first dimension are separated by subsequent dimensions. A comprehensive multidimensional separation can be performed in space, such as on TLC plates or in two-dimensional electrophoresis, or in time in which two or more serial columns are coupled together. In the latter approach, effluent from the first dimension is sampled and separated by the second dimension either off-line or on-line. In the off-line case, a fast second dimension is desirable to prevent long analysis times caused by the many fractions collected from the first dimension. In the on-line case, the second dimension must be fast enough to effectively sample the peaks eluting from the first dimension to preserve resolution.[143] As a result of these considerations, it is apparent that the separation time of a multidimensional separation is primarily dependent upon the time of the separation in the second and higher dimensions. Rapid CE has been effectively used to sample chromatographic effluents for multidimensional separations in several formats.[144,145] In two-dimensional separations, the resulting separation is represented as a pseudo three-dimensional or contour plot with the $x$ and $y$ axes being migration times of the first and second separations and the $z$ axis representing intensity at the detector.

A good example of an off-line multidimensional separation was presented by Hynek et al.[146,147] The authors developed a system to perform analysis of phosphorylation of pepsin isoenzymes and pepsinogens using peptide mapping. Fractions from RPLC separation of α-chymotryptic digests were collected

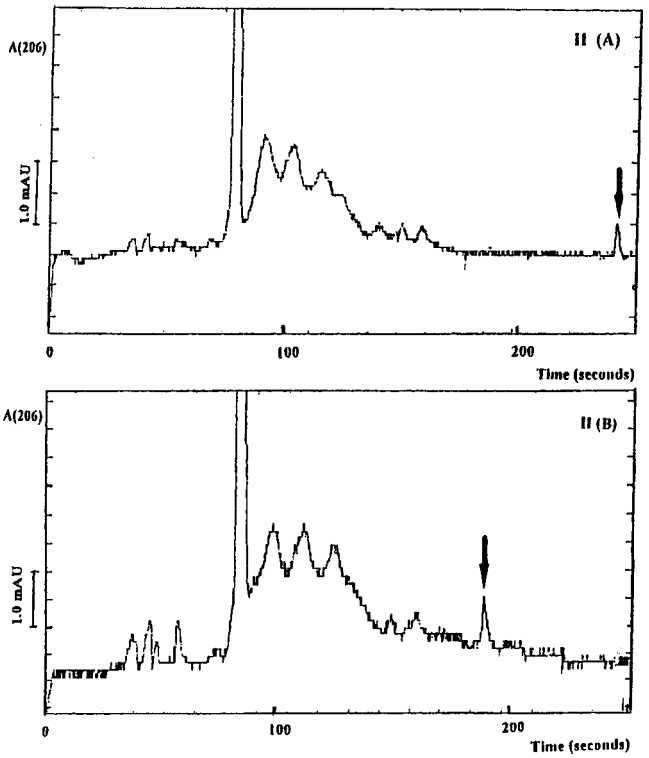


**Figure 19.** Comparison of CE separation of a chromatographic fraction of chymotryptic digest of human pepsinogen *C* that was phoshporylated (A) and dephosphorylated (B). Arrows indicate the peaks which exhibit significantly different migration times in the peptide maps of two pepsinogen forms due to differences in phosphorylation. (Reprinted with permission from ref 147. Copyright 1997.)

and frozen for future analysis. CE separations of the collected fractions were performed using 20 cm long, 50 $\mu$m i.d. untreated fused silica capillaries with an applied voltage of 12 kV. The resulting separations were less than 200 s long and contained many peptide peaks which had not been resolved by the reversed-phase separation. Figure 19 indicates the shift in the electrophoretic mobility for a phosphorylated peptide fragment in the expected direction. This work demonstrates high potential for the rapid identification of modifications in the proteins by CE.

Even faster peptide CE separations have been used in on-line two- and three-dimensional separations developed by Jorgenson.[145] In one experimental setup, an RPLC column was used as the first dimension and was coupled to the CE column, by means of an 8-port valve and a stainless steel splitter tee. The valve normally allowed flow of electrophoresis buffer past the inlet of the CE column, and injections were performed by actuating the valve so that a RPLC fraction collected in the injection loop would flow past the inlet allowing for injection. In this system, most of the fraction went to waste and only a tiny fraction was actually loaded onto the CE column. The 8-port valve allowed two injection loops to be placed on-line so that one could collect while the other was injecting. CE separations were performed in about 10 s on this system.

In the above case, the separation time was limited by the use of the valving system for injection. To increase the CE separation and rate of sampling of

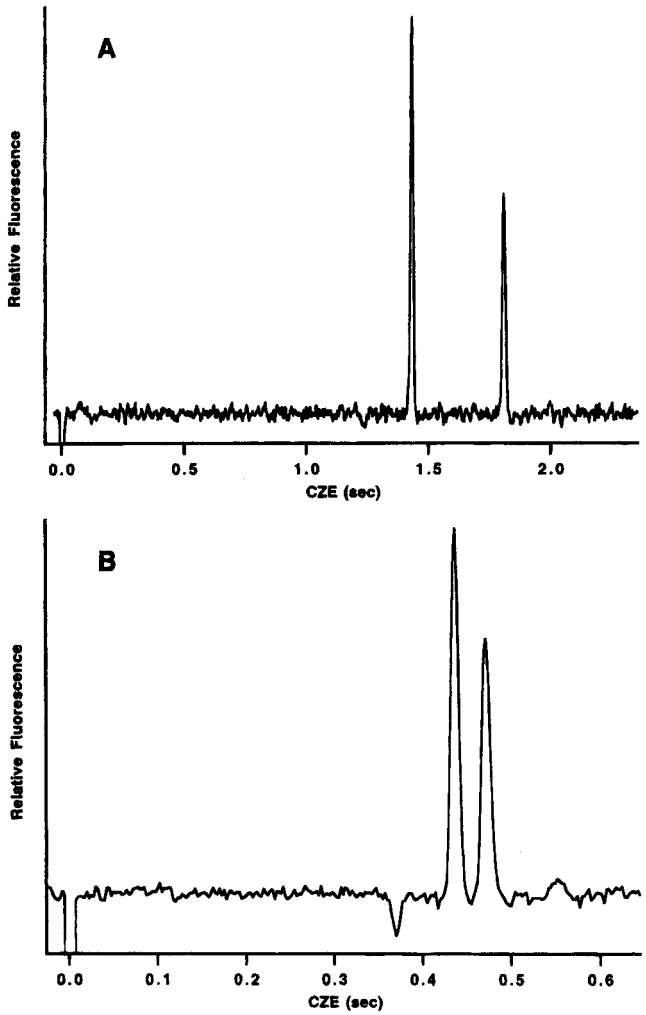


**Figure 20.** Individual electropherograms from a comprehensive two-dimensional separation of a tryptic digest where chromatographic effluent was continuously sampled by fast CE. (A) Electropherogram taken from the 6.3 min point of the LC time with 2 cm between the gating and probe beams and a total analysis time of 2.5 s. (B) Separation distance was 0.45 cm, and a total analysis time was 0.7 s. (Reprinted with permission from ref 67. Copyright 1995 American Chemical Society.)

LC separation, an optical-gating coupling was used.[67] In this case, the outlet of the LC column was connected directly to the CE column via a splitter and the sample was continuously loaded onto the CE capillary and optically gated to control the separation. Figure 20 shows complete separation of two peaks injected from the LC fraction collected at 6.3 min in just 700 ms. CE separation conditions were 0.45 cm long effective length (10 $\mu$m i.d.), 2500 V/cm electric field, and 10 mM phosphate buffer + 20 mM triethylamine (TEA) pH 11 as the separation buffer. Since the entire separation space was not used, the authors performed overlapping injections to further increase the rate of sampling in the second dimension. Using such a system, the authors successfully analyzed an FITC-labeled tryptic digest of the horse heart cytochrome *c*. The total analysis time was 20 min with LC peaks typically 20 s wide at the base and CE peaks about 45 ms wide at the base.

This system was further expanded to include a SEC column in the first dimension, RPLC column in

the second dimension, and CE column in the third dimension.[68] This 3D system was reported to have a peak capacity of ~2800 compared to ~550 of a 2D system and ~25 of the individual CE separation. The authors successfully used the system to analyze FITC labeled tryptic digest of the ovalbumin. The demonstrated 3D system utilizes separation by size, hydrophobicity, and charge and can potentially be used for identification purposes. Among the drawbacks of the system are the requirement of stringent temperature control of all three separations since temperature fluctuations could affect the migration times of one or more separations and the necessity of having high concentration of sample since dilution after each separation may be significant.

Multidimensional separation of proteins was also performed using the above setup.[68,145] A 105 cm long, 250 $\mu$m i.d. fused silica column packed with 6 $\mu$m spherical Zorbax GF450 particles was used in the first dimension of the separation. This column was connected to the electrically actuated injection valve with an internal loop of 500 $\mu$L. A 50 $\mu$m i.d. CE capillary was connected to the injection valve using a Delrin tee. A sample was flushed from the loop through the tee by the pump. During the time the sample has reached the tee, the injection voltage is applied to make a CE injection. After separation, the analytes are detected with a LIF detector. Although the CE separation was performed in about 500 s, it is important to note that no peaks appear on the electropherograms before 320 s. This indicates that if necessary, it would be possible to inject twice as often to fully utilize separation space. To demonstrate practical application of the described multidimensional system, the authors analyzed human serum. As in the case of the standard protein mixture, only part of the separation space was used (all peaks are between 150 and 230 s on the electropherograms), indicating a potential for further increase in the rate of injection. The CZE conditions were a 12 cm long $\times$ 10 $\mu$m i.d. capillary, an electric field of 1.7 kV/cm, and 0.01 M phosphate buffer (pH 6.8) as background electrolyte.

A flow-gated interface has also been used to couple LC and CE in two-dimensional separations.[148] The flow-gated interface possesses several advantages over an optically gated interface including a significantly less expensive system (high-power laser is not required) and lower background levels. However, the injection time in the flow-gated interface is limited by the speed of the cross-flow switching and is usually longer than that of the optically gated interface. Hooker and Jorgenson used an improved flow-gated interface to couple LC and CE.[70] Using their system, they were able to obtain a complete separation of FITC-tagged human urine. The outlet of the 76 cm long, 50 $\mu$m i.d. microcolumn was connected to the flow-gated interface, and the eluting solution was injected on to a 25 cm long (15 cm effective length), 17 $\mu$m i.d. CE column every 29 s. Such frequent injections were possible since during a 58 s long CE separation, no peaks migrated past the detector in the first 30 s. Other experimental conditions were CE potential of 30 kV, separation buffer 10 mM phosphate, 0.22% TEA, 15% acetonitrile, pH 10.5.

### *2. Rapid CE Separations of Small Molecules and Ions*

Although CE was originally seen as being primarily advantageous for large molecules, a significant speed advantage can be gained relative to HPLC for small molecules as well. The need for high-throughput screening, monitoring chemical processes, following chemical dynamics in biological systems, and detecting rapidly decaying substances generates a strong drive for developing high-speed separations of small molecules. In this section, we will present examples of the fast separations published for analysis of small organic acids, sugars, drugs, amino acids, catecholamines, and inorganic ions as well as some applications of these separations.

**Organic Acids.** Chen and co-workers have been involved in developing a "universal" CE method for the analysis of organic acids in a wide range of industrial samples with minimal sample preparation and analysis time.[149] To develop a rugged method, the authors have experimentally optimized buffer composition, pH, type and concentration of the EOF modifier, capillary parameters, and detection methods. When separation of the negatively charged species is required, cationic surfactants are often added to reverse the EOF and increase the speed of analysis. In this work, the authors used the surfactant CTAB to accomplish this task. Figure 21 shows a separation of an 18 component mixture in about 3 min performed using 412 V/cm across a 45 cm effective separation length (50 $\mu$m i.d. capillary). The buffer was 5 mM phthalate with 0.25 mM CTAB (pH 7.0) with indirect detection at 340 nm. Ion chromatography of a similar mixture with similar resolution shown in the report required an ~32 min analysis time. This system was successfully applied to determine low ppm levels of organic acids in process monitoring. It is important to indicate that all of the peaks migrate past the detector between 1.6 and 3.2 min, which means that temporal resolution of the monitoring can be doubled with overlapping injections if desired.

**Sugars.** Zemann et al. have utilized CE with reversed electroosmotic flow for separation of underivatized carbohydrates.[150] A polycationic surfactant hexadimethrine bromide (HDB) was used for reversal of EOF since it exhibits no disadvantageous interaction with the analytes nor with the separation buffer. Separation of 11 carbohydrates was achieved in 3.5 min using 687 V/cm across a 24.5 cm effective separation length (50 $\mu$m i.d.) with 6 mM sorbate with 0.001% (w/v) HDB at pH 12.1 as the electrophoresis buffer. It was found that addition of 5% (v/v) acetone improves resolution of several carbohydrates; however, total analysis time is increased to about 6 min. The authors applied their methods to analyze various soft drinks for fructose, glucose, and sucrose content. With the simpler mixture, separation time was reduced to 75 s. Detection limits were 100 $\mu$M, and the linear dynamic range was up to 4 mM.

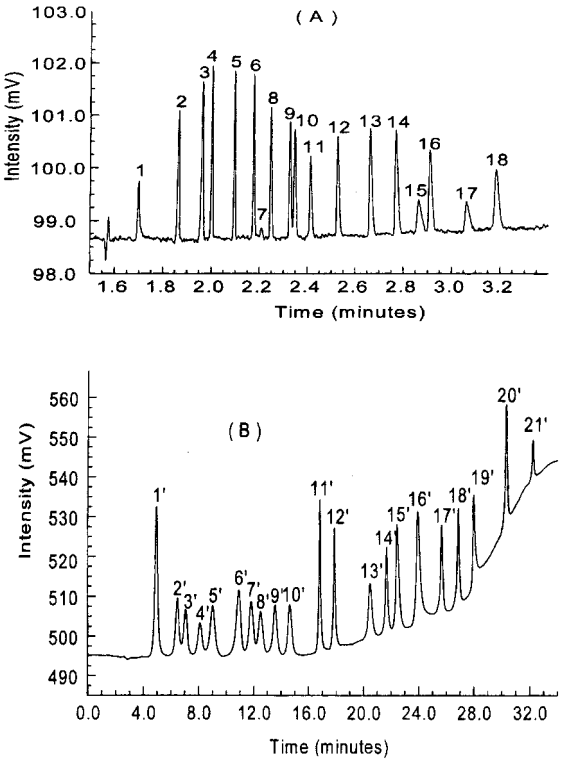


**Figure 21.** Comparison of CE (A) and ion-chromatography (B) separations of organic acids. Peaks (A): (1) oxalate; (2) malonate; (3) formate; (4) succinate; (5) glutarate; (6) adipate; (7) carbonate; (8) pemilate; (9) suberate; (10) acetate; (11) acrylate; (12) propionate; (13) butyrate; (14) valerate; (15) benzoate; (16) hexanoate; (17) hydrocinnamate; (18) 2-ethyl henanoate. Peaks (B): (1′) fluoride; (2′) acetate; (3′) propionate; (4′) butyrate; (5′) iso-valerate; (6′) formate; (7′) valerate; (8′) isocaproate; (9′) pyruvate; (10′) sec.-caproate; (11′) chloride; (12′) nitrate; (13′) benzoate; (14′) bromide; (15′) nitrate; (16′) carbonate; (17′) malonate; (18′) sulfate; (19′) oxalate; (20′) phthalate; (21′) phosphate. (Reprinted with permission from ref 149. Copyright 1997.)

Extensive research on CE separations of oligosaccharides was done by Novotny and co-workers.[151,152] Although these separations were frequently several minutes long, the high peak capacity resulted in a high rate of resolution. One of the faster separations was shown by Stefansson et al., where the authors developed a rapid and efficient separation of oligosaccharide mixtures in polyacrylamide-coated capillaries.[153] To improve detection limits, the analyte mixture was derivatized with the 8-aminonaphathalene-1,3,6-trisulfonate (ANTS). Although application of other fluorescent tags was investigated, ANTS derivatization exhibited the advantages of narrow peaks, greater resolution, shorter analysis time, and improved detection. Using this approach, at least 40 peaks were resolved in ~3 min from a corn amylose sample using a 15 cm long, 50 $\mu$m i.d. capillary with 0.1 M borate−TRIS at pH 8.65 as the separation buffer (see Figure 22). This report also described the effect of buffer composition and concentration on the separation.

**Rapid CE of Drugs.** Hyotylainen et al. developed a methods for fast screening of caffeine, morphine, heroin, codeine, amphetamine, and cannabis compounds in biological fluids.[154] The authors evaluated

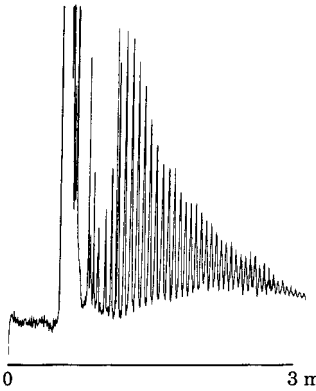


**Figure 22.** High-speed CE separation of corn amylose derivatized with ANTS (see text for details of the separation conditions). (Reprinted with permission from ref 153. Copyright 1994 American Chemical Society.)

both CZE and MEKC for the analysis and found that MEKC was superior to CZE with greater flexibility and selectivity for compounds of similar electrophoretic mobility. For fast screening, 1086 V/cm over short capillaries (23 cm long, 50 $\mu$m i.d.) was used with 0.05 M glycine + 0.05 M SDS as the electrolyte. These conditions allowed separation of six compounds of interest in less than 2 min. One of the drawbacks of the CE methods is poor reproducibility of the migration times between runs. Therefore, two marker compounds, *meso*-2,3-succinic acid and phthalic acid, were used to simplify peak identification. According to the authors, the RSD of the migration times corrected using marker compounds were significantly improved, aiding in confirmation of peak identity.

Perrett and Ross studied the determination of antipyrine, a drug that possesses physicochemical, biochemical, and pharmacokinetic characteristics ideal for probing liver function and hepatic oxidative metabolism.[155] Initially, a 37 cm separation distance (75 $\mu$m i.d.) and electrolyte consisting of 25 mM phosphate with 50 mM SDS at pH 9.6 was used. Such conditions produced a well-resolved peak for the antipyrine at about 2.7 min. To reduce the analysis time, the separation distance was shortened to 23 cm and the capillary cooled to 15 °C to decrease Joule heating. As a result of these improvements, the analysis could be completed in less than 1 min. The authors indicated that the speed of separation made the analysis suitable for pharmacokinetic studies. The results of the assay had good correlation with the HPLC analysis.

**Rapid CE of Inorganic Ions.** Since inorganic ions possess little absorbance in the UV−Vis range, their detection during a CE separation generally requires indirect methods. Additionally, there are subtle differences in separation of inorganic cations and anions. Inorganic cations of the same charge usually have little difference in their electrophoretic mobility and may require complexation with a ligand for effective separation. Foret et al. demonstrated this by separating 18 cations with and without the ligand hydroxyisobutyric acid (HIBA).[156] Figure 23 demonstrates the result of these experiments. In the absence of HIBA, most of the lanthanide ions migrate unresolved due to a small difference in their effective mobilities. Following addition of a low concentration

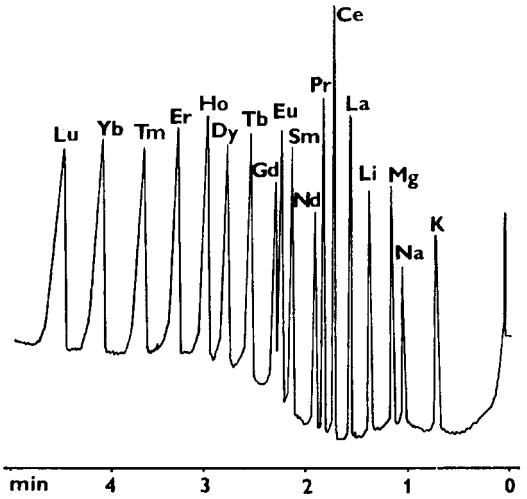


**Figure 23.** CE separation of a model mixture of cations using creatine-acetate buffer at pH 4.8 with 0.004 M HIBA. (Reprinted with permission from ref 156. Copyright 1990.)

of HIBA to the separation buffer, the separation was markedly improved. Using 0.03 M creatinine−acetate buffer, pH 4.8, containing 0.004 M HIBA with a 20 cm long, 25 $\mu$m i.d. capillary, the authors were able to separate all 18 ions in under 5 min. Shi and Fritz have used lactate as a complexing agent.[157] Using 60 cm long (57 cm effective length), 75 $\mu$m i.d. capillary filled with 15 mM lactic acid, 8 mM 4-methylbenzylamine, 5% methanol at pH 4.25, and a separation voltage of 30 kV, they were able to separate 27 cations in a 4 min interval (peak widths about 3−6 s, overall separation time was 6 min).

In the case of inorganic anions, their electrophoretic mobility toward the cathode is usually strong, making it beneficial to reverse the EOF. Zemann has added a polycationic surfactant, hexadimethrine bromide (HDB), to the background electrolyte to dynamically reverse the EOF.[158] Separation of a solution containing thiosulfate, chloride, sulfate, oxalate, sulfite, and carbonate with $\sim 10^6$ theoretical plates was performed in less than 21 s using $E = 625$ V/cm and a 20 cm separation distance. (In addition to inorganic ions, this paper also discusses separation of various organic ions and sugars discussed above.) The authors discuss fast separations for process control and rapid analysis of large sample numbers. Jones and Jandik also reversed the EOF so that it augments the direction of movement of analytes.[159] For their analysis, the authors used 5 mM chromate with 0.5 mM NICE-Pak OFM Anion-BT EOF modifier (Waters) at pH 8. Using a 50 $\mu$m i.d. capillary and 30 kV, 30 anions were separated in a 89 s window (total separation time is 3.1 min). The authors investigated several separation parameters and determined that pH and EOF modifier concentration provided significant selectivity control over the analytes while other electrolyte parameters permitted only subtle changes in selectivity.

**Rapid CE of Amino Acids and Catecholamines.** Amino acids and catecholamines have also been popular targets for generating fast separations. Most of this work has been directed toward monitoring these compounds in vivo as described in a separate section below; however, a unique route to rapid separation of amino acids was described by Pawliszyn and Wu who utilized frontal analysis, or moving boundary CE, with a refractive index detector.[160] In moving boundary CE, buffer is removed from the anodic reservoir and replaced with the sample using a syringe. Continuous injection of analytes that move through the capillary at different velocities related to their electrophoretic mobility results in formation of boundaries that are monitored at the detector. As a demonstration of this method, the authors separated a standard solution of three amino acids, alanine, histidine, and tryptophan, in 13 s using an applied field >1000 V/cm over a 11 cm separation distance (20 $\mu$m i.d. capillary). Similar conditions allowed separation and detection of three sugars in corn syrup in <30 s. The detection system was universal and therefore required no derivatization for the detection of analytes; however, the detection limit appeared to be high. Although no detection limits were reported in the manuscript, a signal-to-noise ratio for 1 mM tryptophan calculated from one of the figures was approximately 5.

Amino acids have been also separated using microchip devices.[161,162] In a representative example, five amino acids were separated within 14 s using 1060 V/cm applied over a 24 mm channel generating 8300 plates/s.[163]

**Fast Chiral Separations.** Rapid resolution of enantiomers is of importance to many fields. In particular, the pharmaceutical industry may benefit from the rapid determination of enantiomeric purity of batch synthesis products or with an on-line analysis for process monitoring. As will be shown, CE has potential for rapid CE separations that is just beginning to be tapped.

Chiral resolution by CE may be achieved by an indirect method, where chiral analytes are derivatized with a chiral reagent to form diastereomers which can be separated in achiral media, or a direct method, where the electrophoresis buffer is supplemented with chiral selectors to achieve separation. Controlling the stereoselectivity of enantiomer−selector complex formation is of paramount importance in fast chiral analysis. Background electrolyte, capillary temperature as well as type and concentration of complexing agent play key roles in determining stereospecificity.[164−169] Wren[164] has shown that the difference of the apparent mobility of two enantiomers ($\Delta\mu$) in an complexation system is given by

$$\Delta\mu = ((\mu_f - \mu_{com})(K_2 - K_1)C)/(1 + C(K_1 + K_2) + K_1K_2C^2) \quad (21)$$

where $\mu_f$ and $\mu_{com}$ are the electrophoretic mobility of the free and complexed analytes, respectively, $K_1$ and $K_2$ are the association constants for each enantiomer with the buffer additive, and $C$ is the concentration of the selector. From this equation it is seen that a maximum difference in mobilities is obtained at $C$ equal to the inverse square root of the product of the association constants $K_1$, $K_2$. Also, large differences in association constants will cause large mobility differences. Chelate−metal complexes,[170] cyclodextrins[171] (CD), crown ethers,[172] and macrocyclic anti-

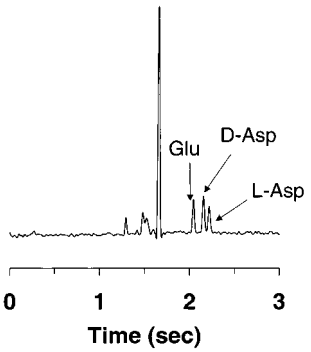


**Figure 24.** Fast separation of aspartate enantiomers by optically gated CE. Separation distance = 15 mm, injection time = 10 ms, $E$ = 2500 V/cm, electrophoresis buffer consisted of 50 mM borate at pH 10 with 10 mM $\beta$-CD. Amino acid concentrations were 5 $\mu$M. (Reprinted with permission from ref 175. Copyright 1999 American Chemical Society.)

biotics[173] have all been utilized as chiral selectors and can yield high selectivity. Given the capabilities of modern instrumentation for achieving high voltages (to maximize differences in mobilities) and minimize band broadening, it is apparent that fast chiral separations are possible if $C$ is optimized and large differences in association constants can be realized.

In one example, Aumatell and Guttman reported a CD-based CE chiral separation of isoproterenol in as little as 45 s on a commercial instrument.[174] In more recent work designed for speed in separation, it was shown[175] that enantiomers of isoindole aspartate derivatives could be resolved in as little as 3 s utilizing optical gating injection and $\beta$-CD as the chiral selector. This separation was coupled with on-line sampling and derivatization to allow high-throughput assays of D,L-aspartate in tissue homogenates (see Figure 24).

### 3. Rapid CE of DNA

The Human Genome project generated considerable interest in increasing the speed of determination of DNA. Additionally, analysis of polymerase chain reaction (PCR) products, identification of a single-point mutation, and separation of a large supercoiled DNA is important for the variety of clinical assays, suggesting a need for rapid separations. We will not review fast DNA separations, especially for sequencing, extensively as many other reviews exist on this topic.[176,177]

The speed at which DNA can be sequenced is determined in large part by the rate of separation of DNA fragments. Among the methods capable of separating DNA fragments, such as ion-exchange chromatography and gel electrophoresis, CE-based separation with replaceable polymer solutions has shown the greatest potential in increasing the rate of sequencing. In sequencing operations, the separations usually take several minutes to complete; however, since a large number of compounds are separated, they can be considered "fast" separations. The majority of fast DNA separations are performed in capillaries filled with dilute solution of various polymers such as poly(ethyleneoxide),[178] polyacrylamide,[179] and methyl cellulose.[180] As an example of the high-throughput possible with these gels, one DNA sequencing run demonstrated separation of 350 oligonucleotides in about 30 min, which corresponds to about 11 compounds per minute.[181] Several groups have also demonstrated similar fast sequencing separations.[182−184]

Carrilho et al. have designed a system capable of sequencing more than 1000 bases in 80 min.[185] The effect of size and concentration of polyacrylamide gel, temperature, and electric field on separation was studied in this work. The authors determined that the optimal parameters for the separations were 2% (v/w) linear polyacrylamide ($>5.5 \times 10^6$ Da) solution, column operation at 50 °C, and an electric field of 150 V/cm. (Note that lower electric fields are used for these separations.) The authors also indicated that commercially available polyacrylamide has several deficiencies, making it unfit for high-resolution separations. These include significant amount of impurities, poor solubility, and decreasing lifetime of the capillary coatings. Fung and Yeung also indicated difficulties with cross-linked polymers and capillary coatings.[186] The authors used bare fused silica capillaries pretreated with HCl and PEO to separate 80 fragments between 9.5 and 13 min of the run.

Extensive development of CE separation of large DNA molecules has been carried out by Morris's group. Oana et al. developed a system for rapid separation of double-stranded DNA (ds-DNA) and plasmid DNA using dilute and semidilute hydroxyethyl cellulose (HEC)-filled capillaries.[187] The inside walls of the capillary were coated with polyacrylamide to reduce electroosmosis. Separation in the order of increasing molecular mass of 1 kbp ladders of 12 different sizes of ds-DNA in about 2.2 min was demonstrated. Additionally, the authors performed fast separations of supercoiled DNA and plasmids. Separation conditions were 22.5 cm separation length (75 $\mu$m i.d. capillary), 0.045 M TRIS/boric acid, 0.001 M EDTA, and an electric field of 600 V/cm. Prior to the experiment, 3 ng/mL ethidium bromide was added to the buffer to aid in detection of DNA. The column was filled with 0.063 wt % HEC ($M_n$ = 90 000−105 000). Upon investigation of effects of separation parameters, it was concluded that electric field strength and buffer composition have a lesser effect on electrophoretic behavior of plasmids than that of the ds-DNA. Such a difference can possibly be used to adjust the relative mobilities of ds-DNA and plasmids. Similar results were obtained when uncoated capillaries were used; however, in this case the order of migration was reversed with larger sizes of DNA migrating faster.[188]

Separation of 15 DNA fragments ranging from 200 kbp to 1.9 Mbp long in under 3.8 min was reported by Kim et al.[189,190] using pulsed field electrophoresis in polyacrylamide-coated 8−12 cm long, 75 $\mu$m i.d. capillaries filled with 0.0033% HEC/0.0040% PEO. Pulsed field electrophoresis is a technique in which the electric field is periodically reversed in order to take advantage of reorientation effects for large DNA. Migration of molecules is achieved by application of higher potential in the forward direction of the

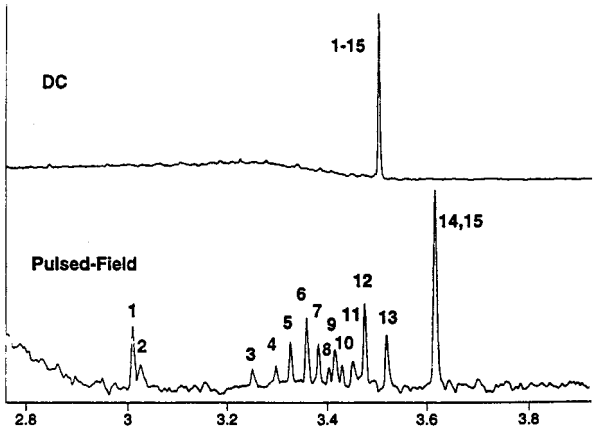


**Figure 25.** Comparison of DC and pulsed field electropherograms of DNA. DC = 200 V/cm. Pulsed field = 35 Hz square wave, 300% modulation. The fragment lengths are (1) 0.21 Mbp, (2) 0.28 Mbp, (3) 0.35 Mbp, (4) 0.44 Mbo, (5) 0.55 Mbp, (6) 0.60 Mbp, (7) 0.68 Mbp, (8) 0.75 Mbp, (9) 0.79 Mbp, (10) 0.83 Mbp, (11) 0.94 Mbp, (12) 0.97 Mbp, (13) 0.97 Mbp, (14) 1.6 Mbp, (15) 1.9 Mbp. (Reprinted with permission from ref 189. Copyright 1996.)

electric field. Figure 25 compares DNA separation in DC electric field and 35 Hz square wave, 300% modulation. The authors believe that improved separation is due to a change in the shape of the DNA molecules as a result of a reverse in the electric field.[191] It is important to note that the actual separation is only between about 3 and 3.8 min, offering potential for overlapping injections to increase throughput.

Chan, Issaq and others developed a system for rapid separation of smaller sizes of DNA.[192−194] Effects of various buffers and gels, applied electric field, buffer concentration, and capillary length on separation of a 20 bp ladder up to 1000 bp were studied. A 50 $\mu$m i.d. commercially available DB-17 (J&W, Folsom, CA) column was used in all experiments. In the report, increasing $E$ was demonstrated to shorten analysis time; however, the resolution of fragments greater than 500 bp was lost. Three different gels were compared: Sigma DNA separation buffer, 0.4% HEC, and 0.75% polyacrylamide with Sigma buffer and HEC producing the best results. Increasing the buffer concentration resulted in improved resolution but increased analysis time. Shortening the column length from 7 to 2 cm had decreased analysis time from 4.5 to ~1.1 min without significant loss of resolution. Further decrease of the column length to 1 cm shortened the analysis time to about 0.6 min with some loss of resolution resulting from poorly optimized injection. This system was used to analyze wild-type and mutant PCR products of the TGF-$\beta_1$ gene. Figure 26 shows the separation of PCR products fortified with the 20−1000 bp ladder performed on 2 cm long capillary using an electric field of 260 V/cm. This less than a minute separation demonstrates clear distinction between mutant and wild-type PCR products and also allows determination of their size. This rapid separation system can potentially be used for high-throughput detection of disease-causing mutations.

High-throughput detection of single-point mutations has significant importance in medical diagnosis.

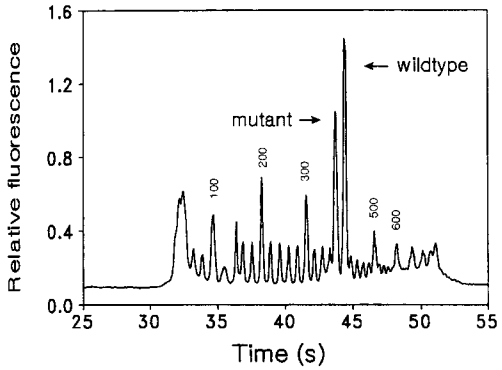


**Figure 26.** Separation of the wild-type and mutant TGF-$\beta_1$ PCR products fortified with the 20 1000 bp ladder. A 2-cm effective length of capillary and 260 V/cm were used. (Reprinted with permission from ref 193. Copyright 1997.)

Barta et al. developed a system for detection of single-point mutations based on a rapid separation.[195] To detect single-point mutations, the authors used a PCR-based multiallele specific amplification technique in conjunction with capillary gel electrophoresis-LIF for detection of the PCR products. The main advantage of this method is the ability to simultaneous detect several single-point mutations. To develop the separation part of the method, a test mixture of 11 DNA fragments ranging from 72 to 1353 base pairs in size was separated in less than 2.5 min using 50 mM Tris/TAPS buffer containing 2 mM EDTA (pH 8.3) and 4% polyacrylamide with 400 V/cm over an 8 cm effective length. Using this method, the authors were able to simultaneously identify several mutations on the 21-hydroxylase gene of a patient with congenital adrenal hyperplasea.

Muller and co-workers have used a constant denaturant capillary electrophoresis (CDCE) for the analysis of the single-base mutation.[196] In CDCE, a migration time shift depending on the degree of thermal denaturation (melting) is induced for different DNA species. Strands containing mismatches melt at lower temperatures than wild-type strands. A fast separation was developed in order to shorten CDCE analysis time. The authors performed extensive theoretical analysis to determine the best electric field strength and capillary length. Using a 3 cm effective length of the capillary with the electric field of 800 V/cm, 11 fragments of the ΦX174/*Hae*III digest were separated in 30 s. Using similar experimental parameters, the authors were able to detect a mutation in a mixture of a wild-type/mutant mitochondrial DNA. To further develop the capability of the separation, the authors also examined the analysis of ss-DNA. As a result of this experiment, single-base resolution was observed up to 300 bases in 3 min of analysis time.

Microchip devices had been used extensively to perform DNA separation.[96,197] A more complete review of microchip DNA separations will be presented in a separate article of *Chemical Reviews*. As in the capillary DNA separations, microchip separations involve sizing media. Effenhouser et al. described one of the first successful uses of microchannels filled with non-cross-linked polyacrylamide.[198] Using an

electric field of 2300 V/cm, the authors were able to highly efficiently separate 10−25 bases in less than 45 s. The experiments were performed in am unmodified channel filled with 10%T solution of polyacrylamide.

### 4. Rapid Affinity Probe CE Assays

Affinity probe CE is an analytical technique in which an affinity probe (AP) a compound which has a specific interaction with the analyte or target (T), is allowed to bind to the analyte and the resulting complexes are separated from free reagent by CE. Two types of AP assays, competitive and noncompetitive, are possible. In a competitive assay, a fluorescently labeled target (T*) (all APCE to date has used fluorescent labels, although other labels could be used) competes with T for binding to a limiting amount of AP. CE-LIF separation of the mixture produces two distinct peaks corresponding to T* and T*−AP complex, the intensities of which can be related to the original concentration of T. In a noncompetitive assay, excess of fluorescently labeled affinity probe (AP*) is added to the solution containing T. A CE separation of AP* and T−AP* allows for determination of the concentration of the T. Most examples of affinity probe CE are immunoassays since the AP is an antibody; however, other ligands may be used such as phage display peptides,[199] aptamers,[200,201] combinatorially selected ligands,[202] or naturally occurring ligands such as receptors[203] or transcription factors.[204,205]

CE-based immunoassays have been reviewed several times in the recent literature,[206,207] and the principles, are outlined in the Immunoassays and Enzyme Assays chapter of the *Handbook of Capillary Electrophoresis*;[208] therefore we will emphasize the speed of separation and how it is utilized with APCE. In any affinity probe assay, the binding reaction between AP and T is governed by the following equilibrium

$$\mathrm{AP} + \mathrm{T} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{AP\text{-}T} \qquad (22)$$

Importantly, the noncovalent AP−T complexes have a limited half-life ($t_{1/2}$) which can be expressed by the following equation

$$t_{1/2} = \ln 2/k_{-1} \qquad (23)$$

To detect complex quantitatively, the separation must be considerably faster than the $t_{1/2}$ of the complex; therefore, speed of separation becomes important, especially in cases where the dissociation rate is fast. If separation is significantly fast, the size of both free and complex peaks should represent the equilibrium concentration of the respective species, thus allowing determination of dissociation constants. As will be seen in some of the examples given below, the speed of separation is also important since immunoassays are an important component of clinical assays and drug monitoring where high throughput or rapid feedback are necessary.

Compared to conventional affinity probe assays, such as ELISA or RIA, CE-based methods offer a number of advantages including (1) higher mass sensitivity, (2) faster analysis for single assays, (3) potential for real-time monitoring, and (4) compatibility with multianalyte determination. CE-based assays are usually no better, and are often worse, than other types of affinity probe assays in terms of concentration detection limits. The use of preconcentration techniques in combination with APCE, however, may generate assays with comparable or superior concentration detection limits. With appropriate automation or parallel operation, the throughput possible with a CE-based assay is at least as high as conventional ELISA's; however, this has yet to be demonstrated.

**Noncompetitive Assays.** Noncompetitive assays are the preferred mode of assay since, compared to competitive assays, they have (1) larger linear dynamic range, (2) better detection limits, and (3) easier detection of cross-reactivity.[207] Despite these advantages, noncompetitive assays have not been as fully developed because of two problems that make them harder to implement. The first problem is that if the affinity probe is a large complex molecule, like an antibody or antibody fragment, it can be difficult to label such that a homogeneous product is formed. Inhomogeneous products lead to broad or multiple peaks which can be difficult to separate from the complex. The second problem is that separation may also be difficult if the AP is a large molecule and the target is relatively small since the electrophoretic mobility of the complex may not be significantly different from the AP. Despite these difficulties, a number of noncompetitive assays have been successfully demonstrated.

The most popular application of noncompetitive assays has been for determination of antibodies. In an early example, FITC−insulin was used as an affinity probe to determine anti-insulin with a 60 s separation in a simple demonstration of the concept.[209] Reif et al. developed a method for detecting human IgG using FITC-labeled protein G.[210] Protein G has a natural affinity to bind to the Fc region of antibody and can be used in the CE-AP assay to detect IgG. To perform the assay, the authors mixed previously labeled protein G with the human IgG and incubated for a certain amount of time. The mixture was then hydrodynamically injected on to a 27 cm long (20 cm effective) long, 50 $\mu$m i.d. capillary. Separation of the analytes was performed in less than 3 min using 15 kV applied across the capillary filled with 0.05 M borate buffer (pH 10.5). The authors indicated that if the separation took longer than 3 min, the majority of the complex would dissociate. Thus, separation time had to be minimized. Length of incubation had little effect on the complex, suggesting rapid formation of complexes; however, increase in the temperature of incubation significantly decreased the size of the complex peak. Using the above separation conditions and incubation at 20 °C, the authors were successful in measuring concentrations of IgG in serum. Furthermore, the authors developed a sandwich assay in which a mixture of protein G and anti-human $IgG_1$ has been added to the serum. After incubation, the mixture was sepa-

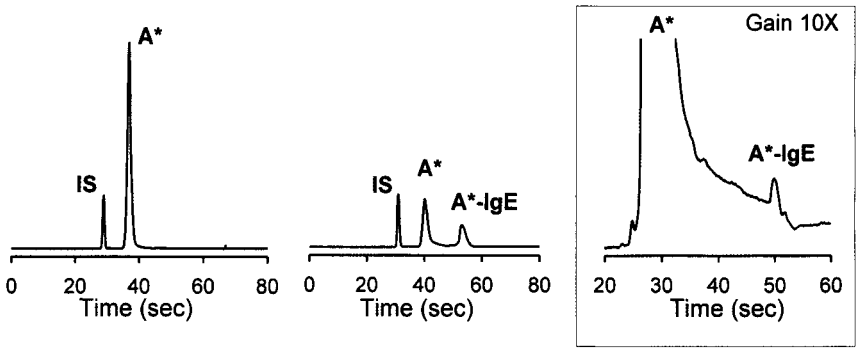


**Figure 27.** Determination of IgE using aptamer-based CE-AP assay. Solutions of 300 nM of A* with no IgE (left), 300 nM IgE (middle), and 1 nM IgE (right) were separated with injector to detector length of 7 cm. After 48 s of separation, a vacuum was applied to the outlet to rapidly pull the complex to the detector. A 5 nM concentration of 4(5)-carboxyfluorescein was used as internal standard. Fluorescent signal scale for the 1 nM IgE sample is expanded by 10-fold relative to the other electropherograms. (Reprinted with permission from ref 215. Copyright 1998 American Chemical Society.)

rated and revealed two bands corresponding to protein G−anti-human $IgG_1$ and to protein G−anti-human $IgG_1$−$IgG_1$. One of the disadvantages of this method was formation of excessively wide peaks due to inhomogeneity of FITC−protein G. This group was also able to use protein A as an affinity probe for IgG.[211] In this case, they were able to obtain a separation between the affinity complex and FITC−protein A in under 3 min.

Rapid CE-AP assays have been used to screen antibodies and evaluate their binding and cross-reactivity for the antigen. Evangelista et al. used Cy5-labeled morphine to determine the best commercially available antibody against morphine and to analyze its cross-reactivity with morphine analogues.[212] In this work, a 25 cm long, 20 $\mu$m i.d. capillary was used to obtain a complete separation of the complex in under 2 min. The authors used 0.01 M phosphate buffer at pH 7.0 as background electrolyte and applied 20 kV. Due to vast variation between commercially available antibodies, it is important to be able to screen for the best antibody for a given application. The authors have determined that for their desired application, the Biodesign (Biodesign International, Kennebunkport, ME) produced antibody performed the best. The authors also tested this antibody for cross-reactivity with nine morphine analogues and determined that the antibody has poor affinity for them. According to the authors, this antibody can be used for the practical drug screening and forensic analysis.

One of the problems often encountered during noncompetitive assays is little or no difference in migration between the complex and a free affinity probe. Chen utilized charge modification to change the electrophoretic mobility of FITC−anti-IgA used to determine IgA.[213] He used succinic anhydride to gain extra negative charge on the surface of the FITC−anti-IgA. Using a 27 cm long, 20 $\mu$m i.d. capillary filled with borate buffer (pH 10.2), he was able to obtain a separation between free and bound IgA in under 120 s (applied potential 20 kV). Again, the width of the peaks appears excessive due to inhomogeneity of FITC−anti-IgA. The reported detection limit for IgA was 66 nM.

While determination of antibodies is certainly an important application, the ability to expand this technique to other targets would be important for widespread acceptance and application. An elegant solution to the problems of antibody labeling, effective separation, and preconcentration has been demonstrated by Shimura et al.[214] In this report, a Fab′ fragment was labeled with iodoacetamidorhodamine at a free thiol formed at the hinge region away from the binding site, allowing formation of a singly labeled protein that formed a narrow band in separation. The separation was performed by isoelectric focusing which gave good resolution of bound and free AP (the target was human growth hormone). Furthermore, the IEF allowed a significant preconcentration for a detection limit in the low picomolar range. The only drawback of the method was the low yield in production of the labeled Fab′ and the slow speed of the IEF separation (several minutes).

In our laboratory, we have used high-affinity oligonucleotide ligands (aptamers) to produce fluorescently labeled homogeneous affinity probe. Aptamers are DNA- or RNA-based oligonucleotides that have been selected from a large initial pool to bind with high affinity and specificity to the target of interest by SELEX.[200] Although aptamers resemble antibodies in their ability to bind a specific target, several advantages make aptamers more favorable for affinity probe assays. First, production and fluorescent labeling of a sequenced aptamer is simple and inexpensive and can be accomplish using commercially available synthesizers. Second, development of aptamers does not require animals permitting aptamers for toxic compounds to be obtained. Third, aptamers possess a negative charge at physiological pH, leading to more predictable separations. To demonstrate the possibility of using aptamers for CE-based determination of analytes, we have developed an aptamer-based noncompetitive affinity probe assay for human IgE and human thrombin.[215] The assay for IgE shown in Figure 27 could be performed in less than 60 s and had a detection limit of 46 pM with a linear dynamic range of $10^5$.

Noncompetitive assays have also been used to study protein−DNA interactions involved in transcription. Such binding experiments have typically been performed as described above for noncompetitive APCE except the separation was done by gel electrophoresis and the technique called gel-mobility

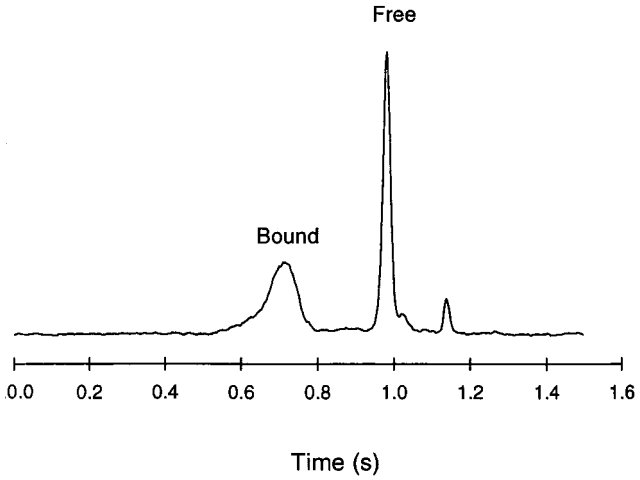


**Figure 28.** Electropherogram obtained using flow-gated interface for the described on-line system. The peak at 0.7 s is bound FITC−insulin, and the peak at 0.97 s is free FITC−insulin. Smaller peaks are contaminants of FITC−insulin. Effective length 8 mm, capillary i.d. was 5 $\mu$m, and electric field strength was 4000 V/cm. Injection time was 200 ms. (Reprinted with permission from ref 217. Copyright 1998.)

shift assay. When done by conventional gel electrophoresis, a dense gel must be used to allow "caging", an effect in which dissociated components of a complex are held in the pores of a gel and allowed to reform complex before separating. Using CE, the separation is fast enough that caging does not need to be used for detection of complexes.[216] As discussed below, this type of assay has been used for binding constant determination as well.

**Competitive Assays.** Although noncompetitive assays have analytical advantages as discussed above, competitive assays have been much more popular due to their ease of implementation, especially for small analytes. In this case, the small analyte can be simply labeled and the separation required (T* from T*−AP) is much simpler.

In our laboratory, we have worked extensively on a competitive immunoassay for insulin. In early versions, the assay was performed in a 4 cm separation distance (25 $\mu$m i.d.) at 3000 V/cm. Using these conditions, complete separation of bound and free FITC−insulin was achieved in about 7 s. Since our final goal was to develop a system for monitoring release of insulin by the single islet of Langerhans with high temporal resolution, the above assay was further improved to allow on-line reaction of insulin and the antibody. Additionally, the separation capillary was shortened to an injection to detector length of only 8 mm and the i.d. of the separation capillary was decreased to 5 $\mu$m to allow $E = 4000$ V/cm. The automated assay system incorporated a flow-gated interface with injection times of 200 ms. Such modifications resulted in complete separation between bound and free FITC−insulin in about 1 s, shown in Figure 28.[217] This system allowed continuous monitoring of insulin in sample streams for several hours, generating thousands of assays. This device was demonstrated for flow injection analysis and for continuous monitoring of the release of insulin from single islets of Langerhans.[22] These assays have a

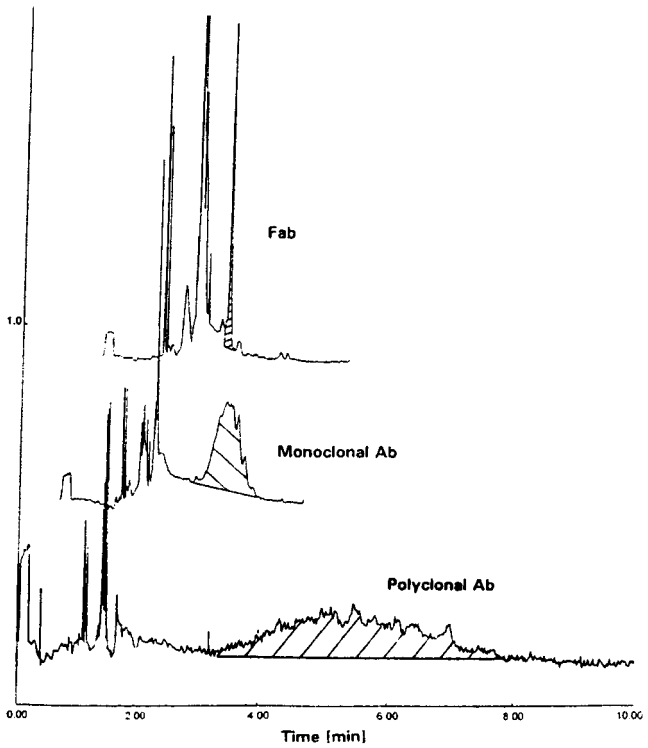


**Figure 29.** Comparison of the complex signal produced with three different antibody preparations for a cortisol CE competitive immunoassay. Conditions: 8 s pressure injection, coated capillary, 27 cm effective, 30 cm total length, 50 $\mu$m i.d., 20 mM TAPS/AMPD, pH 8.8, 30 kV. (Reprinted with permission from ref 218. Copyright 1995.)

concentration detection limit of 270 pM and a mass detection limit of 432 zmol.

Schmalzing et al. have developed a competitive immunoassay for determination of cortisol in serum.[218,219] In the assay, the authors used fluorescein-labeled cortisol molecules as the tracer which competed with analyte cortisol for binding the antibody. A 50 $\mu$m i.d., 20 cm long (27 cm total length) coated capillary filled with 20 mM TAPS/AMPD buffer (pH 8.8) was used to perform the analysis. The authors investigated possible use of polyclonal or monoclonal antibodies and antibody fragments for the analysis. Although use of antibody fragments produced the best complex peak shapes in a 2 min long separation, as depicted in Figure 29, the authors indicate that polyclonal antibodies can be successfully used for the assay when determination is made using free cortisol peak only. The same group was able to perform similar assays on a microchip, suggesting the possibility of a highly automated immunoassay.[220] In these experiments, a 2.2 cm long channel with cross-sectional area that is equivalent to that of a 44 $\mu$m i.d. capillary, and $E = 800$ V/cm, the authors were able to perform separations in less than 30 s. This work demonstrated repetitive injections of the same sample mixed on-line in a chip. Several other groups have investigated applications of microchips to the immunoassays.[221,222] A more complete review of microchip separations will be presented separately in this issue of *Chemical Reviews*.

Ye et al. have used competitive CE immunoassay to rapidly determine cyclosporine A (CsA).[223] To improve detection limits, the authors used a sheath

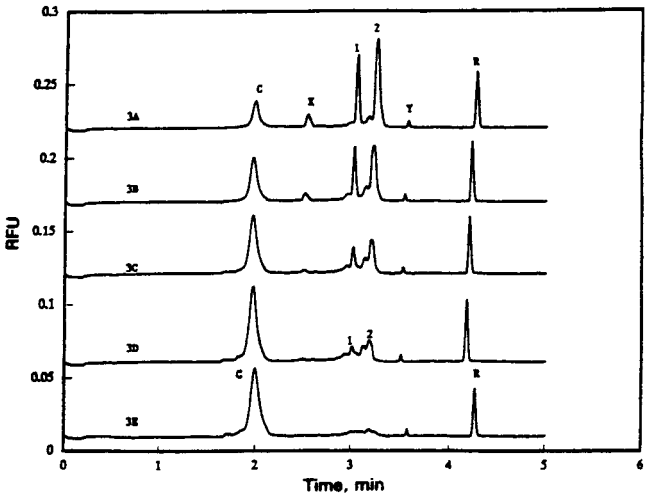


**Figure 30.** Simultaneous assay for PCP and morphine in urine by CE-LIF. (A) PCP 24.3 $\mu$g/mL, morphine 285 $\mu$g/mL; (B) PCP 4.3 $\mu$g/mL, morphine 57 $\mu$g/mL; (C) PCP 1.9 $\mu$g/mL, morphine 23 $\mu$g/mL; (D) PCP 0.98 $\mu$g/mL, morphine 11 $\mu$g/mL; (E) drug-free urine. Peaks: (1) Cy5.5-PCP; (2) Cy5-morphine; (C) Ab:Ag* complexes; (X) immunoreactive impurity associated with Cy5.5-PCP; (Y) nonimmunoreactive impurity; (R) Cy5 diacid internal standard. (Reprinted with permission from ref 224. Copyright 1994.)

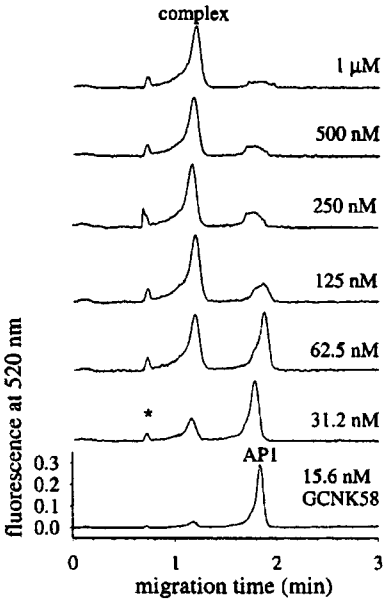


**Figure 31.** Electropherograms of a mixture of 1 nM double-stranded DNA and GCNK58 at indicated concentrations. Samples were made from freshly annealed at 90 °C for 5 min DNA in 50 mM MOPS/TEA buffer (pH 7.5), 50 mM KCl, 133 $\mu$g/mL poly(dAdT)poly(dA-dT), 100 $\mu$g/mL BSA, 5% glycerol. (Reprinted with permission from ref 225. Copyright 1998 American Chemical Society.)

flow cuvette with a fluorescence polarization detector. The assay performed using 10 mM phosphate buffer in a 40 cm long, 20 $\mu$m i.d. capillary was completed in less than 3 min and had a limit of detection for CsA of 0.9 nM (applied electric field was 500 V/cm). This method was successfully used to analyze whole blood samples from patients who were administrated the drug. This work presents another example of the clinical assay that has speed and detection limits better than that of any currently utilized method. The authors indicate that one possible problem that could be encountered during development of similar or more complex immunoassays is cross-reactivity of antibodies. One possible solution could be initial separation of the sample by other method such as HPLC followed by either on-line or off-line immunochemical reaction. Additionally, the analytes could be preconcentrated during the first separation step to improve limits of detection.

High separation power of CE allows for simultaneous multianalyte analysis by competitive immunoassay. Chen et al. have developed an assay for the analysis of urine for two drugs of abuse.[224] Morphine was derivatized with fluorescent label Cy5, and phenycilidine (PCP) was tagged with Cy5.5 for the analysis. In about 5 min, the authors were able to fully resolve all four peaks as demonstrated in Figure 30. The experimental conditions were 200 mM borate buffer (pH 10.2) and an untreated 27 cm long, 20 $\mu$m i.d. capillary. The authors indicated that they were able to perform the analysis routinely and reproducibly with detection limits of 4 nM for PCP and 40 nM for morphine.

**Binding Constant Determination by CE-AP Assay.** High speed and low limits of detection possible with CE-AP-LIF assays provide an opportunity for simple and rapid measurement of binding constants between AP and T. Flouds and Etzkorn have measured a binding constant between yeast transcription activator GCNK58 and the DNA sequence for which it is specific using CE.[225] In the experiment, fluorescein-labeled DNA molecule was mixed with the transcription activator and allowed to equilibrate. The mixture was than injected on to a 20 cm long (effective, 27 cm total), 75 $\mu$m i.d. capillary and separated in less than 2 min as shown in Figure 31. Separation was performed in 10 mM MOPS/TEA pH7.5 and a an applied voltage of 25 kV. By injecting a mixture containing several different concentrations of transcription activator and measuring the ratio between bond and free DNA, the authors were able to determine the binding constant of 35 nM.

In our laboratory, the system described above was used to determine the binding constant between insulin and anti-insulin.[22] Additionally, interspecies binding constants were determined by allowing monoclonal anti-insulin to react with insulin from several species. By measuring the ratios of bound and free FITC-labeled insulin peaks, the binding constants for ovine, equine, porcine, bovine, and human insulins were measured.

### *5. Rapid CE for in Vivo Chemical Monitoring*

A popular method of studying concentration dynamics in vivo is to sample the extracellular space by microdialysis and then assay the sample stream for target compounds to track concentration dynamics. Such methodology is used in pharmacokinetics and in neuroscience where the dynamics of neurotransmitters are followed to understand chemical signaling. High temporal resolution is of utmost importance in such studies, and this requires frequent sampling. Assays can be done off-line, in which case high temporal resolution generates many fractions to be analyzed and a fast separation is desirable

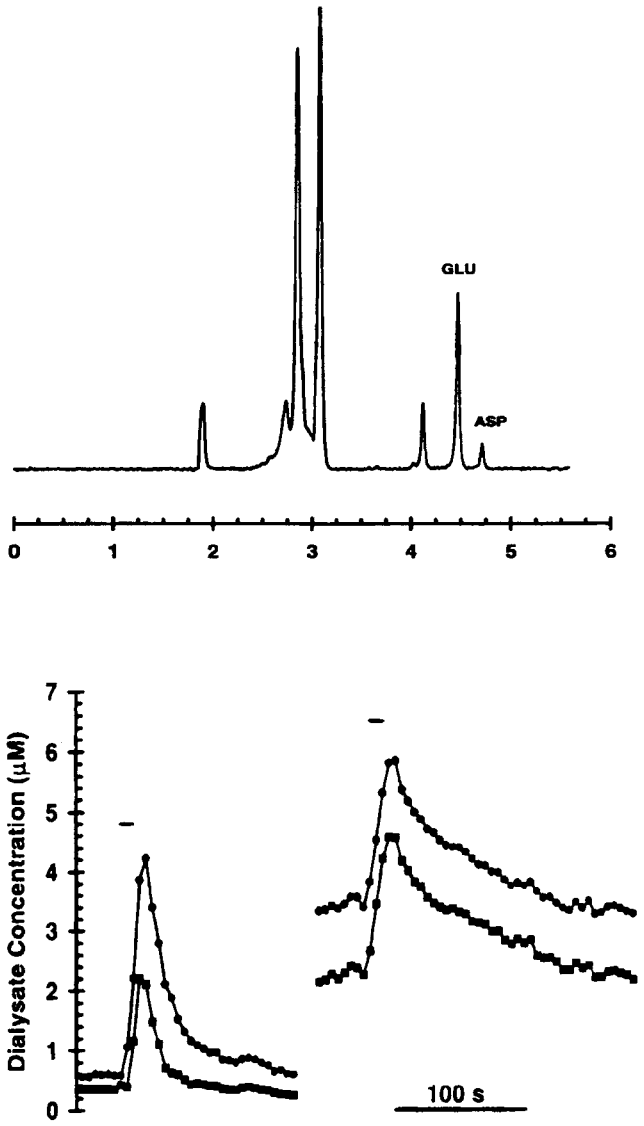


**Figure 32.** (top) Example of an in vivo electropherogram under basal conditions. (bottom) Effect of electrical stimulation on glutamate and aspartate in vivo. The bar indicates electrical stimulation. (■) aspartate; (●) glutamate. (Reprinted with permission from ref 72. Copyright 1997 American Chemical Society.)

to have reasonable turnaround time. If assays are performed on-line, then the speed of analysis is likely to determine the temporal resolution and fast separation is necessary to maintain temporal resolution. In both cases, high-frequency sampling results in small samples and requires high mass sensitivity. CE, with its high speed potential and high mass sensitivity, is ideal for this type of monitoring.

In an example of off-line sampling and fast separation, Bert et al. used rapid CE separation to detect subnanomolar concentrations of noradrenaline and dopamine.[226] Analytes were labeled with naphthalene-2,3-dicarboxaldehyde (NDA) prior to the injection. Separation parameters were a 23 cm long with a separation distance (25 $\mu$m i.d.) capillary, 200 mM phosphate buffer (pH 7.05) and 29 kV total voltage. To increase sensitivity and efficiency, the authors used sample stacking by injecting 60 pL of orthophosphoric acid (200 mM). Separation of dopamine and noradrenaline was complete in 82 s and produced

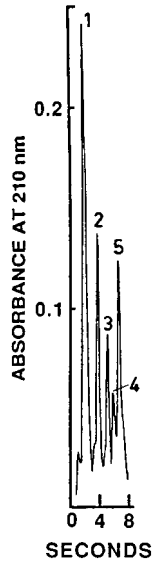


**Figure 33.** High-speed protein separation using 2 $\mu$m nonporous particles. This example combines high flow rates, steep gradients, and elevated temperature. (Reprinted with permission from ref 260. Copyright 1990.)

peaks of at least 6 million theoretical plates. The reported detection limit for both molecules was $8.6 \times 10^{-11}$ M.

It is also possible to directly couple the microdialysis eluent with an analytical system in an on-line approach for real-time chemical monitoring.[227−231] CE has also been coupled on-line with microdialysis sampling in a variety of systems for in vivo monitoring. In one system, illustrated in Figure 6, the sample stream from the dialysis probe flows through a length of fused silica capillary into a derivatization tee where it reacts with the fast-reacting, fluorogenic reagent *o*-phthalaldehyde/$\beta$-mercaptoethanol. Derivatized sample is then periodically introduced onto the electrophoresis column by means of a flow-gated interface (described in the instrumental section). The excellent mass sensitivity and separation efficiency of this high-speed CE system allows sampling rates of <5 s and resolution of glutamate and aspartate (excitatory neurotransmitters) with >200 000 theoretical plates[72,73] ($E$ = 4.2 kV/cm across 4.5 cm effective separation distances using 10 $\mu$m i.d. capillaries). Figure 32 is an example electropherogram taken from this work as well as a plot of dialysate concentration as a function of time. As shown, the rapid separations provide excellent temporal resolution in monitoring release of glutamate and aspartate elicited by electrical stimulation. This technique has also been used to characterize the regulation of neurotransmission in response to pharmacological manipulations.[73] In addition to these analytes, this system has also been applied to ultralow flow rate measurements of glutathione and cysteine using monobromobimane as the derivatization reagent[232] as well as ascorbate and lactate[74] (UV-absorbance detection) in rat caudate nucleus. (Ultralow flow rate microdialysis provides nearly 100% recovery of analytes, which allows more accurate quantification.)

Other groups have also reported the on-line coupling of microdialysis/CE systems for in vivo chemical monitoring.[228,233,234] While not as compatible with

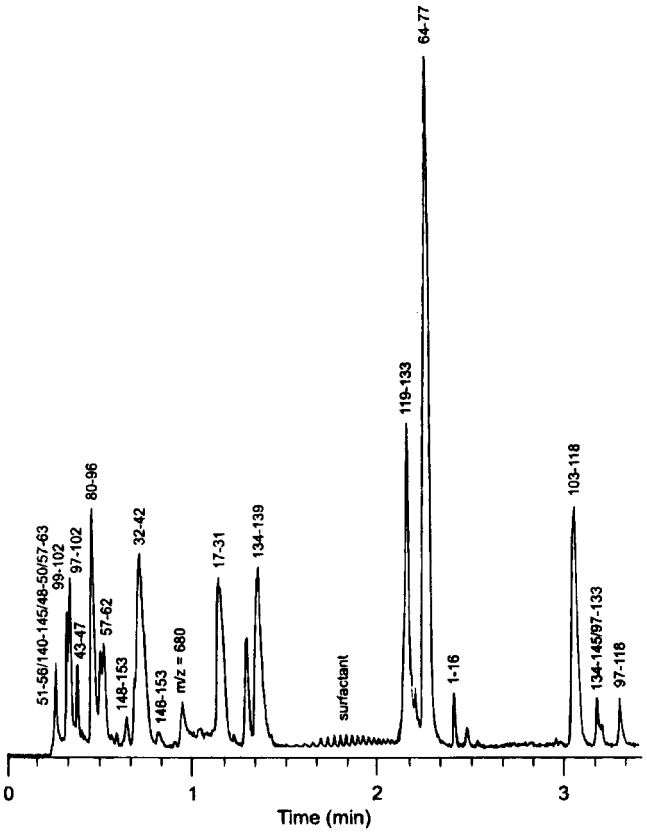


**Figure 34.** Total ion chromatogram from 300 pmol of trypsin-digested myoglobin. Flow rate 1 mL/min, gradient 5−90% ACN, 0.1% TFA in 5 min. (Reprinted with permission from ref 278. Copyright 1997 American Chemical Society.)

high-speed separations as the flow-gated system, these other systems, which utilize injections controlled by an HPLC valve as described above in the instrumentation section, have been able to provide high temporal resolution monitoring of a wide array of compounds including monitoring of neurotransmission and metabolism.[235−237] In addition, the neurotransmitters glutamate and aspartate were monitored with 120 s temporal resolution using an on-line NDA derivatization.

Restricted transport across the microdialysis membrane prevents larger molecules from being sampled; however, biological macromolecules (>20 kDa) may also have in vivo roles as chemical messengers and consequently are targets for chemical monitoring. Recently, in vivo sampling of proteins was demonstrated through the use of miniature ultrafiltration probes.[238] Ultrafiltration contrasts with microdialysis in that the external solution is pulled through the membrane. Since this method does not rely on diffusion, higher recoveries of larger molecular weight species is accomplished. This work reports recovery of >90% of a series of model proteins with molecular weights up to 68 000. In addition, Chen[239] demonstrates a CE separation of nine proteins in less than 200 s on a commercial instrument. These works suggest large molecular weight compounds can effectively be sampled and analyzed with acceptable temporal resolution.

Related experiments of chemical monitoring discussed elsewhere in this review include monitoring neurotransmitter release from single cells by channel electrophoresis and insulin release from single islets with a flow-gated system and fast immunoassay. These results show that fast CE offers a method to provide high temporal resolution measurements relevant to biomedical, pharmaceutical, and neuroscience applications. A comprehensive review of microdialysis coupled on-line with CE has been recently presented.[240]

## *III. Liquid Chromatography*

## A. Introduction

### *1. Background*

The speed of liquid chromatographic separations has been steadily increasing over the past 30 years due to advances in column technology which directly follow the well-developed theories of band broadening. Separations which took over an hour in the 1970s and 1980s can now be performed in minutes or even seconds. Excellent reviews of high-speed chromatographic separations have recently appeared.[241,242] At the time of these reviews, improvements in separation speed were shown to be largely the result of production of particles with diameters down to 1−2 $\mu$m, nonporous particles, superficially porous particles, and perfusion particles. In addition, elevated temperatures were illustrated to be effective in decreasing analysis times.[243] In the years since these reviews, the potential for fast separations has further increased with the use of even smaller particles (<1 $\mu$m) packed into capillaries with flow controlled by either ultrahigh-pressure pumps or electroosmosis. In addition, several other experimental systems such as monolithic columns, open tubular columns, and small diameter packed capillaries have been explored with some success for fast separations. In this section of the review, we will cover advances in high-speed chromatographic separations with an emphasis on advances since the last reviews and on techniques not covered in the previous reviews on fast LC.

### *2. Theory*

Before considering the recent advances in rapid technology and separations, it is useful to briefly consider the band broadening theory which has been used as the basis for the advances. The speed with which a chromatographic separation can be performed is limited by the sources of band broadening which become dominant at high mobile phase velocities. A useful method of evaluating band broadening is based on the Knox equation

$$h = Av^{0.33} + B/v + Cv \qquad (24)$$

where $h$ is the reduced plate height and $v$ is the reduced velocity.[244] $A$, $B$, and $C$ are system constants which are determined by the magnitude of band broadening due to eddy diffusion, longitudinal diffusion, and resistance to mass transfer, respectively. The Knox equation shows that increasing the mobile phase velocity, while decreasing the analysis time, will also increase the plate height and yield lower

resolution. This well-known problem is of course distinct from the situation with CE.

For well-packed columns using porous supports and unretained solutes, it is expected that $A = 1$, $B = 2$, and $C = 0.05$.[244] For a column with these figures of merit, the $C$ term will dominate the band broadening at high mobile phase velocities. For example, at $v$ of 150, the resistance to mass transfer accounts for over 60% of the total plate height. The $C$ term is dependent upon the capacity factor and can become even more dominant for retained compounds. The $C$ term is an aggregate term that includes effects of slow desorption−sorption kinetics, inhomogeneous flow, and slow diffusion out of the stagnant mobile phase in porous particles. It is frequently observed that the majority of the $C$ term, especially for large compounds like proteins, arises from slow diffusion in and out of stagnant mobile phase in pores. In cases where the $C$ term is sufficiently reduced, the $A$ term can dominate band broadening, even at high flow rates.[245] As discussed below, certain capillary column configurations can reduce or eliminate the $A$ term for improved performance at high flow rates. Since the $A$ and $C$ terms both increase with increasing particle diameter, it is also apparent that smaller particles will aid performance at high flow rates. This necessitates the use of higher pressures. These considerations will also be discussed in more detail below.

All of the concerns with regard to extra-column band broadening that were discussed with capillary electrophoresis apply to fast chromatographic separations as well. The main difference is that fast chromatographic separations do not necessarily demand miniaturization; therefore, small volume, ultrahigh sensitivity detectors are not required. Many separations, for example, are performed using conventional UV-absorbance detectors with perhaps some modifications of the reduced cell and connecting tube volume. The main requirement of course is a fast responding detector. Also, the ease of preconcentration on HPLC columns lessens the requirements of small injection volumes that were discussed with fast CE. A frequently found limiting factor in the speed of the separations, especially for larger columns, is pumping capacity. In a conventionally sized HPLC column, a high-speed separation may require flow rates >10 mL/min. Such flow rates are only available with preparative-scale pumps which are not typically part of an analytical system.

## B. Nonporous and Superficially Porous Particles

One of the most common ways of obtaining improved performance at high flow rates in chromatography is the use of pellicular or nonporous particles. In nonporous particles, the stationary phase is coated on the outside of a solid microsphere, thus eliminating any stagnant mobile phase in the column. With no stagnant mobile phase, the $C$ term is significantly reduced compared to porous particles resulting in flat van Deemter curves and high efficiency at high flow rates compared to porous particles of the same size. Pellicular glass beads with large diameters (>40 $\mu$m) were used as stationary phase supports early in the development of liquid chromatography but they lost favor with the advent of porous silica particles with much smaller diameters <10 $\mu$m which provided high surface area (and therefore high capacity) and higher efficiency. Improvements in synthetic methods for pellicular particles have allowed production of monodisperse beads with diameters <3 $\mu$m.[246] This improvement, coinciding with increased demand for higher speed separation, has sparked renewed interest in nonporous particles.

### 1. Preparation of Nonporous Particles

The synthesis of nonporous particles has been reviewed for a variety of supports.[247,248] The most common synthetic method for nonporous silica involves hydrolysis of alkyl silicates and subsequent condensation of silicic acid in alcoholic solutions to produce highly mondisperse (2% variation in diameter) particles. [246,249] The production of nonporous zirconia beads coated with reversed-phase stationary phase has also been described.[250] These particles, with diameters <3 $\mu$m, were found to yield rapid, high-efficiency separations for a variety of analytes including polycyclic aromatic hydrocarbons, alkylbenzenes, oligosaccharides, amino acids, peptides, and proteins. With appropriate end-capping and use of polymeric surface coverage with stationary phase, the particles had little metallic interactions and yielded quasi-homoenergetic interactions. Alternative approaches to producing monodisperse, nonporous chromatographic supports include filling in the pores of porous particles with polymer [251] and rendering porous particles nonporous by sintering at high temperatures.[252] Nonporous supports based on organic polymers are produced by suspension polymerization techniques which allow control of particle diameter.[253,254] At present, nonporous particles in the size range of 1−3 $\mu$m can be obtained in prepacked columns from several vendors (see Table 1).

### 2. Characterization of Nonporous Particles

Unger's group performed an in depth evaluation of nonporous particles with a 1.5 $\mu$m diameter for the separation of proteins and peptides in a series of papers.[255−258] In a comparison of pellicular and porous particles for reversed-phase chromatography of peptides and proteins, several differences were observed. One difference was that the capacity factor of an analyte was much less sensitive to changes in the percentage of organic solvent modifier compared to porous particles.[255] Plots of log($k'$) versus organic phase ratio yielded slopes 10 times more shallow than would be expected with porous particles. This effect requires adjustment of gradient development and was attributed to the proteins having free access to the stationary phase on the surface rather than being required to diffuse into pores. We note, however, that for small molecules, nonporous columns have been reported to be more sensitive to organic content with the slope of log($k'$) vs organic content being twice as steep for nonporous reversed-phase columns.[262]

The main observable difference between nonporous and porous particles was an increase in peak capacity for small, nonporous beads compared to columns packed with porous particles.[256] For example, in one

**Table 1. Summary of Column/Particle Suppliers for Nonporous and Perfusion Particles**

| pore size (nm)[a] | particle diameter ($\mu$m) | base material[b] | manufacturer | trade name(s) | column dimensions (mm)[c] | modes available[d] |
|---|---|---|---|---|---|---|
| NP | 4 | PSDVB | Jordi | NPR-DVB | 30 × 4.6 | RP |
| NP | 5 | silica | Keystone Scientific | | 20 × 1.4, bulk | RP, NP, HIC |
| NP | 1.5 | silica | Kovasil | MS−C14, − H | 33 × 4.6 | RP |
| NP | 4 | PSDVB | Hamilton | PRP-Infinity | 30 × 4.1 | RP |
| NP | 1.5 | silica | Micra Scientific | NPS | 33 × 4.6 | NP (bare silica), RP |
| NP | 0.5, 1.0, 1.5, 2.5, 3.5, 4.5 | silica | Micra | NPS | bulk | NP (bare silica) |
| NP | 2.5 | polymer | TosoHaas | TSK gel, SP-NPR, butyl-NPR | 35 × 4.6 | RP, ion exchange, HIC |
| NP | 40 | silica | Supelco | Pelliguard | bulk | NP, RP |
| NP | 1.5, 6, 10, 50 | methacrylate | YMC | NP-C14, YMC-Pack | varies | RP |
| NP | 2 | silica | Glycotech | Hytach C18 | 30 × 4.6 | RP |
| NP | 2 | silica | Nomura | Develosil NP, ODS-2 | 10 × 4 | RP |
| NP | 5 | silica | Nomura | Deveolsil NP, ODS-5 | 30 × 4 | RP |
| NP | 10 | PSDVB | BioChrom Labs | Hydrocell DEAE, NP10, QA NP10, SP NP10, C3 NP10, C4 NP10 | varies | ion-exchange, HIC |
| NP | 7 | polymethacrylate | Bio-Rad Labs | Bio-Gel MA7P, MA7C, MA7S, MA7Q | 30 × 4.6, 50 × 7.8 | ion-exchange |
| NP | 2.5 | methacrylate | Waters | Gen-Pak FAX | varies | ion exchange |
| NP | 10, 13 | PSDVB | Dionex | ProPac PA1, Nucleopac PA-100 | varies | ion exchange |
| NP | 2.5 | methacrylate gel | Showa Denko | Shodex IEC QA-620N | varies | ion exchange |
| NP | 2.5 | PSDVB | Transgenomics | DNASep | | IP, RP |
| 6000−8000, 800−1500 | 10, 20, 50 | PSDVB | PerSeptive Biosystems | Poros | varies, bulk | ion-exchange, HIC, RP, affinity, activated affinity, metal chelate |
| 1000 | 5 | polymer | MCI GEL | ProtEx-SP, -DEAE | 50 × 4.6 | ion exchange |
| >1000 | 10, 13, 17 | polymer | TosoHaas | TSK GEL | varies | ion exchange, HIC, RP |
| 1000, 4000 | 7 | silica, polymer | Macherey-Nagel | Nucleosil, Nucleogen | varies | RP, HIC |
| 1000, 4000 | 5, 10, 20 | PSDVB | Phenogel | Phenogel | 300 × 7.8 | GPC |
| 1000, 4000 | 8, 10, 20, 5, 10, 20 | PSDVB | Polymer Labs | PL GEL, PL RP | varies | GPC |
| 1000, 4000 | 7, 10 | polymer | Synchrom | SynChropak | varies | GPC, ion exchange, SEC |
| 1000, 4000 | 7 | polymer | Alltech | Macrosphere | varies, bulk | GPC, SEC, RP, ion exchange |
| 1000, 4000 | 5 | PSDVB | Jordi | | varies | NP, RP |
| 1000, 4000 | 10 | PSDVB | YMC | | varies | SEC, GPC |

[a] NP (non-porous). Materials with a pore size designated are perfused phases (Perfusion or gigaporous-type materials) and the pore size refers to the flow-through pore. [b] Base material. Abbreviations used in this column are PSDVB (Polystyrene divinylbenzene). Information given is as specific as available in the literature. Thus, in some cases only "polymer" is given for a substrate that is organic polymer but not otherwise identified. [c] Dimensions given as length × inner diameter. Some manufacturers sell a variety of column sizes and are so designated. In addition, those that sell particles as separate bulk quantities suitable for packing by the user are also indicated. [d] Many manufacturers sell mutiple phases for a given mode such as phenyl, C8 and C18 for reversed-phase. Abbreviations used in this column include: RP (reversed-phase), NP (normal phase), HIC (hydrophobic interaction), SEC (size-exclusion), GPC (gel permeation), and IP (ion-pairing).

comparison, columns with the same surface area-to-volume ratio revealed peak capacities, defined as gradient time/peak width ($4\sigma$), that were typically 3 times greater on nonporous beads than porous beads for a given analysis time. Peak capacities as high as 94 were achieved for proteins with 10 min gradients on the 1.5 $\mu$m nonporous particles. Alternatively, peak capacities that were the same as that achieved on porous particles could be achieved in shorter times on the nonporous particles. A potential drawback of nonporous columns is the reduced amount of stationary phase resulting in reduced capacity. Unger's work showed a decreased capacity with nonporous columns; however, with a sample capacity of 1 mg of protein per column volume (column dimensions 36 long × 8 mm i.d.), the column was considered suitable for "micropreparative" and analytical work. Thus, it appears that the capacity issue is moot for analytical-scale separations on nonporous particles. Some workers however, have investigated the use of fimbriated phases and tentacle gels (these are "fuzzy" polymeric stationary phases that can increase the capacity 10-fold for a given surface area) as a way of improving the performance of nonporous particles.[259]

Studies comparing pellicular and porous particles with a variety of stationary phases and analytes have supported the initial observations made by Unger.[260−263] Many of these studies have emphasized the analysis speed possible with pellicular particles and consistently have shown equivalent resolution with 200−400% reductions in analysis time.[262,264] Several researchers have observed the need to use weaker solvents to obtain retention in nonporous materials that is similar to porous particles as a result of the lower capacity of the pellicular supports.[263,265]

### 3. Small Nonporous Particles and Ultrahigh Pressure

It is apparent from chromatographic theory that the most sure approach to achieving faster separations with high efficiency is to decrease the particle

size. Over the past 30 years, decreases in the particle size have indeed resulted in impressive decreases in the analysis time while maintaining comparable efficiencies. Decreasing the particle size requires increased pressures, and at present, most chromatographic equipment has approximately a 6000 psi limit. With this pressure limit, it is difficult to further improve separation speed by going to particles less than 2 $\mu$m because of the extremely high pressures required to achieve high flow rates. Another factor that limits the utility of smaller particles is the high temperatures generated due to the friction associated with flow through a packed bed. It is calculated that a 1 mL/min flow rate on a typical 4.6 mm column at 3500 bar (50 000 psi) will generate over 5 W of power, much more than could be dissipated.[266]

The temperature problem can be solved by using packed capillaries due to their small diameter and excellent ability to dissipate heat. A 30 $\mu$m i.d. capillary operated at 4100 bar (60 000 psi) is expected to generate approximately 0.4 mW of power, which can easily be dissipated across the capillary. A route to increasing the pressure limit has recently been reported with the development of high-pressure pumping equipment compatible with HPLC solvents consisting of a modified, three-stage pneumatic amplifier pump that can supply 4500 bar (65 000 psi). Special high-pressure fittings to connect capillary columns to the high-pressure system were also described. The pump was used with 50−70 cm long by 30 $\mu$m i.d. capillary columns packed with 1.5 $\mu$m nonporous particles. The optimum flow rate required 1300 bar (19 000 psi) of pressure for 30 min analyses and generated up to 330 000 plates (52 cm column) for a lightly retained compound. At 4 100 bar (59 000 psi), analysis times were less than 10 min and still produced 190 000 plates. More recently, the use of 1 $\mu$m particles yielded efficiencies of 200 000 plates in 6 min for a small solute with $k' \approx 1$.[267] Using an exponential dilution method to form a gradient, a different type of high-pressure pump (syringe design with maximum pressure of 130 000 psi) was used to generate a peak capacity of 300 in a 30 min analysis time on a 27 cm long column.

While emphasis of these initial reports using ultrahigh-pressure LC was on high efficiency and not speed, it is apparent that the availability of such high pressures could be used for fast separations in shorter, packed capillaries. Also, while all work published so far with ultrahigh-pressure columns has utilized nonporous particles, it is clear that similar equipment could be used with small, porous particles to achieve high flow rates and fast analysis.

### *4. Superficially Porous Particles*

Although nonporous particles are gaining popularity, some disadvantages can be noted. Most importantly is the lower capacity. Furthermore, the use of small particles, which is typical with the nonporous material, requires high pressures (which are still not commonly available), and the resulting shear forces between particles can harm delicate macromolecules. As a way to take advantage of the short diffusion paths associated with small particles, but at lower pressures and with higher capacity, Kirkland developed superficially porous particles.[268] These particles consist of a solid 5 $\mu$m core coated with a layer of porous microspheres (1 $\mu$m diameter with 300 Å pores). The particles are produced by spray drying a mixture of silica sol and pellicular particles. The efficiency of the columns was equivalent to columns packed with 1−2 $\mu$m totally porous particles but had the back pressure of 7 $\mu$m particles. Their application to high-speed separations was demonstrated by the separation of three polystyrene samples by SEC and five test proteins by reversed-phase HPLC, both in about 90 s.

### *5. Examples and Applications of Fast Separations on Nonporous Particles*

Numerous examples of impressively fast separations on columns packed with pellicular supports can now be found in the literature.[269−275] In the sections below we discuss some examples of these separations. Table 2 summarizes many other examples.

**Peptides and Proteins.** Certainly the most likely application for pellicular supports is in the area of large molecule separations since their efficiency is most often limited by the slow diffusion in and out of the stagnant mobile phase. In the early work of Unger, the capability of fast separations for proteins was shown by the separation of eight test proteins under increasing flow rate and gradient steepness. Even the highest flow rates (4 mL/min) and steepest gradients (2.5−0 mM $(NH_4)_2SO_4$ in 0.1 M phosphate in 2.5 min) allowed resolution of the eight proteins in 2.5 min.[258] In an example of pushing the limits, 2 $\mu$m diameter nonporous particles were used with a steep gradient and elevated temperature to separate five proteins in 8 s (Figure 33).[260] Several examples of separations of proteins and protein digests on 1.5 $\mu$m diameter reversed-phase particles were reported including one demonstration of separation of 79 peptides from a digest of bovine serum albumin in 22 min on a 3.3 cm long by 4.6 mm i.d. column.[276]

A whole cell lysate of human erythroleukemia cells was analyzed for its protein content using a column with 1.5 $\mu$m C-18 particles.[277] The column was held at 60 °C, and a multistep gradient was utilized to separate over 100 peaks in 30 min. In a few cases, fractions with coeluting peaks were subjected to a second chromatographic step with a shallow gradient to aid in identification. Fractions of each component were collected, enzymatically digested, and the resulting peptides were analyzed by MALDI. The resulting spectra were compared to a protein database to determine identity.

As discussed in the electrophoresis section, increasing the speed of separation poses special problems when coupling the instrument to a mass spectrometer. Given the tremendous importance of LC-MS, especially for proteomic research, and the increasing speed of separations, it is clear that considerable effort will be needed to make fast separations compatible with MS. Banks and Gulcicek have explored the use of electrospray interfaced to a time-of-flight mass spectrometer for detection of peptides separated on nonporous media. Simple peptide mixtures were

**Table 2. Examples of Fast Separations with Nonporous Particles**

| analyte[c] | sample[b] | separation time | gradient | particle size[c] ($\mu$m) | mode[d] | temperature[e] | ref |
|---|---|---|---|---|---|---|---|
| 5 proteins | standard | 8 s | yes | 2 | RP | 80 °C | 260 |
| 6 peptides | standard | 35 s | yes | 1.5 | RP | r.t. | 278 |
| peptides | protein digest | 3.5 min | yes | 1.5 | RP | r.t. | 278 |
| 79 peptides | protein digest | 22 min | yes | 1.5 | RP | r.t. | 276 |
| small molecules | standard | 75 s | no | 5 | RP | r.t. | 265 |
| amino acids | brain homogenate | 1−2 min | no | 5 | RP | 45 °C | 286, 287 |
| hydrazones | reaction broth | 4 min (30 s sampling) | no | 1.5 | RP | r.t. | 288 |
| drug | rat plasma | 4 min | yes | 1.5 | RP | 40 °C | 289 |
| RNA/DNA | PCR products | 5 min | yes | | RP | 50 °C | 284 |
| DNA | restriction fragments | 25 min | yes | 2.3 | IP−RP | 48−63 °C | 285 |
| proteins | synthesis | 10−15 min | yes | 0.7−2.1 | affinity | r.t. | 280 |
| 8 proteins | standard | 2.5 min | yes | 1.5 | ion exchange | r.t. | 258 |
| oligomers | restriction fragments, synthesis products, standards | 2−5 min | yes | 3.3 | ion exchange | 40 °C | 282 |
| DNA fragments | restriction | 5−15 min | yes | 2.1 | IP | r.t. | 283 |
| amino acid devivatives | standard | 19 s | yes | | chiral | 41 °C | 290 |
| drugs | human serum | 4 min | no | 1.5 | chiral | r.t. | 291 |

[a] Numbers of compounds resolved are given where specifically discussed in the article. [b] Standards refers to mixtures made specifically for separation. [c] Particles sizes given when available in the reference. [d] Abbreviations used in this column are RP (reversed-phase) and IP (Ion-pairing). [e] r.t. = room temperature.

separated in 35 s (3.0 cm × 4.6 mm i.d. column) with 1 s wide peaks. Despite the extremely narrow peaks, the TOF analyzer, which acquired complete mass spectra at 16 Hz, was able to accurately record mass spectra on the eluting peptides. In an interesting demonstration of this combination, a peptide map of horse heart myoglobin was completely resolved in 3.5 min compared to a 20 min separation required on a conventional porous column (Figure 34).[278]

In one example of using ultrahigh pressure with LC-MS, octadecyl-modified 1.5 $\mu$m diameter nonporous silica particles were packed into 150 $\mu$m i.d. capillaries with lengths of 20 cm and used to separate proteins and peptides generated from enzymatic digests of proteins.[279] Gradients were produced using an exponential dilution method at pressures of 520 bar (7500 psi), and electrospray ionization mass spectrometry was used for detection. This system was found to yield similar results to packed capillary perfusion chromatography with respect to chromatographic resolution and analysis time and had a limit of detection comparable to traditional packed capillaries which use 5 $\mu$m porous particles. Previous work with perfusion columns had found that dilution on column resulted in poorer sensitivity for this type of analysis. The analyses required as little as 250 fmol of protein or 500 fmol of peptide on-column in approximately 30 min.

Nonporous particles have been used to advantage with affinity separations as first demonstrated by Anspach et al.[280] They describe an immobilization procedure using 3-isothiocyanatopropyltriethoxysilane as an activation reagent for a variety of stationary phases on nonporous 0.7 to 2.1 $\mu$m particles. Several affinity separations were demonstrated including isolation of bovine follicotropin, human chorionic gonadotropin, nucleotides, and nucleosides with phenylboronic acid as the stationary phase; thrombin and trypsin with benzamidine as stationary phase; pig pancreatic elastase and human leukocyte elastase with tri-L-alanine as the stationary phase; and horseradish peroxidase with concavalin A as the stationary phase. Improved chromatographic performance (recovery and separation speed) was seen in each case compared to results obtained with identical activation and immobilization procedures on a porous support. In each case, the separation was performed in 10−15 min and recovery over 95% was obtained under optimized conditions. Loss in activity was similar to or less than that on porous supports. These improvements are attributed to the high flow rates and short analysis times and a decrease in nonspecific interactions with the silica support due to short contact time.

**DNA.** The use of nonporous particles has recently become very popular in the analysis of DNA and has been recently reviewed by Huber.[281] Types of analysis include sizing, restriction, PCR products, and preparation and purification of synthetic oligomers. A few examples will be discussed here.

Nonporous ion-exchange media have been used for the rapid separation of oligomers of DNA and was demonstrated over a range of DNA types on a 3 or 10 cm × 4.6 mm DEAE modified poly(styrene−divinylbenzene) packed column utilizing linear gradients.[282] An advantage of HPLC over the standard gel electrophoresis method is that fragments of the same chain length but with different bases can be separated. In this example, two 22-mers with different base composition were separated in less than 2 min. Preparative work could be done because the column had a comparable loading capacity to macroporous (250 and 550 Å) columns. DNA fragments were separated in less than 5 min at an elevated temperature (Figure 35). A series of chromatograms are shown for the separation of double- and single-stranded, linear and supercoiled, nicked and supercoiled, and digestive fragments of DNA all in less than 4 min.

Sizing of DNA fragments by ion-pair chromatography with C-18-coated poly(styrene−divinylben-

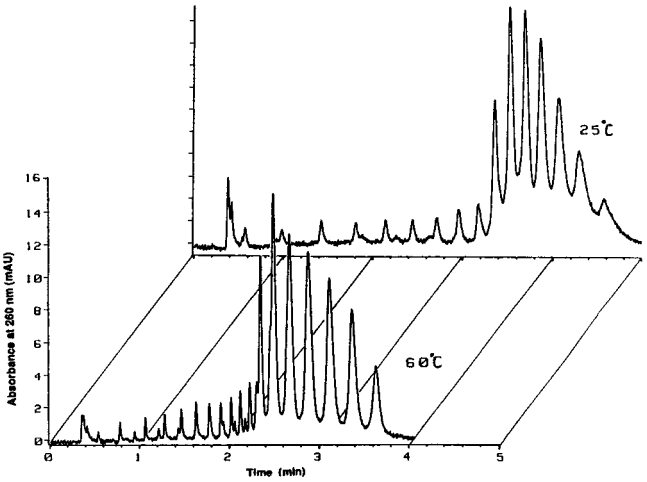


**Figure 35.** Chromatogram of the crude product of oligonucleotide synthesis on nonporous stationary phase. Note the effect of temperature on separation speed. (Reprinted with permission from ref 283. Copyright 1990.)

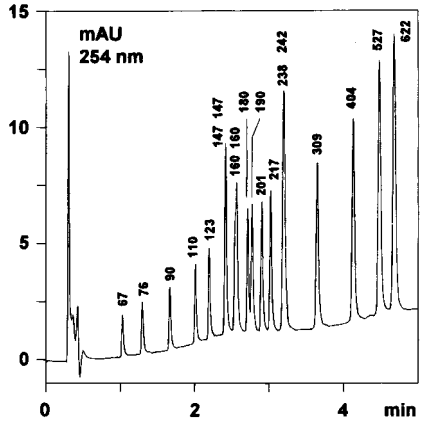


**Figure 36.** Separation of DNA restriction fragments. The number of base pairs are noted in the chromatogram. (Reprinted with permission from ref 283. Copyright 1995 American Chemical Society.)

zene) 2.1 $\mu$m particles has been shown to reduce analysis time from several hours to 5−15 min compared to gel electrophoresis (Figure 36).[283] The gradient method resolved restriction fragments differing by 2−3% in molecular weight without pretreatment prior to injection onto the column. Under these conditions, differences in sequence gave only small changes in migration of the fragments and were said to be caused by differences in secondary structure of the fragments.

In the development of a competitive reverse transcription PCR for the accurate quantification of gene expression in RNA, HPLC was integral because the reaction results in heteroduplexes of RNA which cannot be reliably separated by gel electrophoresis.[284] The nonporous column of C-18-bonded polystyrene−divinylbenzene particles allowed for the separation of fragments differing by 5% in size in 5 min with an acetonitrile gradient. This advantage allowed the researchers to use competitors that were 95% similar, allowing for a much more accurate competition. Because most other components of the PCR product mixture were virtually unretained on the column, direct injection of the reaction broth was possible. In addition, the use of HPLC allowed a general technique that could be easily modified for the analysis of PCR products of different lengths. The method could accommodate a size range from ~100 to 800 base pairs.

Temperature-dependent denaturation of DNA restriction fragments ranging in length from 46 to 910 base pairs was detected by ion-pair reversed-phase high-performance liquid chromatography using columns packed with 2.3 $\mu$m alkylated nonporous poly(styrene-divinylbenzene) particles.[285] The presence of acetonitrile in the mobile phase was found to decrease the melting temperatures of DNA fragments by 1.5−2 °C per percentage of acetonitrile in the eluent. Small fragments (<120 bp) were completely denatured between 53.6 and 63.5 °C, depending on their total GC content. Whereas retention times of completely helical DNA fragments increased gradually with increasing temperature, partial denaturation of larger DNA fragments (>150 bp) was found to reduce retention at temperatures above 53.6 °C. Inspection of the DNA sequences of partially denatured fragments revealed domains with repeating AT base pairs. Positions of partial denaturation within the pB*R*322 plasmid detected by chromatographic analysis were in good agreement with partial denaturation maps obtained by electron microscopy described in the literature. This effect is due to less negative charges available for interaction with the stationary phase because of single-stranded bubbles formed in denaturation.

**Small Molecules and Chiral Separations.** Although the advantages of nonporous particles are usually seen with large molecules, at sufficiently high flow rates the stagnant mobile phase term can affect small molecules as well. In one study,[265] alkylbenzenes (alkyl = $C_6$−$C_{18}$) were used as test analytes for packed columns (3 cm × 4.6 mm) of nonporous Develosil NP−ODS-5 and compared with those from similar columns containing microporous ODS-5 (5 $\mu$m) and macroporous ODS silica (5 $\mu$m). The effect of organic modifier was studied by the isocratic separation of 3 alkylbenzenes in 15 min. To obtain nearly identical separations, 35% acetonitrile was used for the nonporous column while 60% and 70% were required for the macro- and microporous columns, respectively. The combination of shorter analysis times and less organic modifier required for elution was seen as an advantage. High-speed separations of several other classes of analytes including anthryldiazomethane derivatives of fatty acids, phenylthiohydantoin derivatives of amino acids, lipophilic vitamins, and phthalate esters were also demonstrated. In each case, 5−9 analytes were isocratically separated in less than 75 s.

An interesting example of a fast separation of a complex matrix was the rapid determination of amino acids in brain homogenates. Following precolumn derivatization with *o*-phthalaldehyde/$\beta$-mercaptoethanol, samples were separated isocratically on a 4.5 cm × 4.6 mm column packed with 5 $\mu$m pellicular particles at 45 °C with a phosphate/methanol/THF buffer. GABA was resolved from the rest of the components in less than 1 min with a mobile phase composition ratio of 53.4/40/4.6.[286] While GABA was

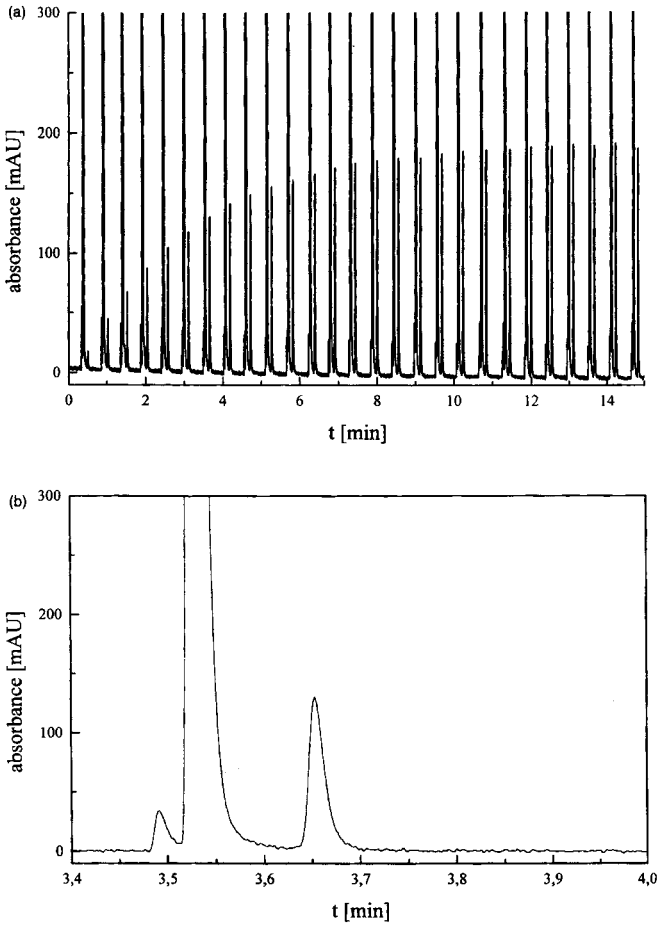


**Figure 37.** (a) Formation of formaldehyde 2,4-DNP-hydrazone monitored by multiple sample injection. (b) Individual chromatogram from a. (Reprinted with permission from ref 288. Copyright 1997.)

the amino acid of interest, alanine was also resolved from the other components. By manipulating the mobile phase conditions to a ratio of 39/6/5, it was possible to determine both taurine and 4-aminobutyric acid in 2 min.[287]

One potential application of fast separations is monitoring chemical reactions. One example that was reported was monitoring the production of formaldehyde 2,4-DNPhydrazone from formaldehyde by reaction with Brady's reagent. The isocratic separation required a total of 4 min; however, the analytes all eluted in a 30 s range; therefore, overlapping injections could be made for monitoring at 30 s intervals (Figure 37).[288]

A 1.5 $\mu$m nonporous column was compared to 3.5 $\mu$m porous (80 and 100 Å pores) columns for a pharmacokinetic study of drug dosage.[289] The small molecule drug, RO 48−3657, is metabolized into an inactive form and then into a second biologically active form. In this study, the inactive and active forms were assayed from rat plasma at different times after oral administration. The gradient method developed for the nonporous column required much less organic modifier than the porous columns (0.9% compared to 15% initial) as well as a lower flow rate to achieve a separation twice as fast as the porous column. They also found that the capacity factor was almost 3 times more sensitive to organic content in the mobile phase.

The combination of nonporous material with chiral stationary phases has allowed rapid separation of enantiomers.[290] With a novel chiral phase of *N*-(1-naphthyl)leucine, enantiomers of *N*-(dinitrobenzoyl) derivatives of amino acids and their esters and derivatives of ibuprofen were separated on a 1 cm long × 2 mm i.d. column. Separations at ambient temperature took approximately 150 s while increasing to 41 °C allowed separation in 19 s. The separation was used in an automated system for the determination of drugs in plasma.

In anther example of a chiral separation with 1.5 $\mu$m nonporous particles, $\beta$-cyclodextrin was added to the mobile phase to separate enantiomers of ketoprofen in human serum.[291] The enantiomers were baseline resolved in less than 4 min with an aqueous mobile phase with 2% acetonitrile and 10 mM $\beta$-CD. The use of a nonporous column made this separation possible because organic modifiers greatly decrease the solubility of cyclodextrins.

## C. Perfusion Particles

### 1. Introduction and Background

Another route to rapid chromatographic separations is the use of perfused particles or perfusion chromatography. As with pellicular particles, the advantage of perfusion chromatography is a reduction of stagnant mobile phase effects; however, the effect is achieved in a much different way. In perfusion particles, this effect is achieved by the presence of larger pores (>6000 Å) which transect the particles and allow flow through the particle, thus reducing the amount of stagnant mobile phase and the distance that analytes are required to diffuse. Perfusion particles behave much like regular porous particles at low flow rates but at sufficiently high flow rate, convection within the transecting pores becomes significant. Once convection reaches a critical level, further increases in flow rate have little effect on band broadening, resulting in van Deemter curves that are relatively flat at high velocities. As a result, flow rates 10−100-fold higher than typical can be used with little degradation in separation performance compared to particles of the same size. Compared to porous particles of the same size, perfusion particles offer improved performance at high flow rates. Compared to smaller porous particles, the performance can be comparable at high flow rates but with much lower back pressure. Perfusion media are generally produced from poly(styrene−divinylbenzene) by a suspension polymerization process.[292] Coatings for reversed-phase, ion-exchange, hydrophobic interaction, and affinity separations have been reported.

Several reviews have been written on the development, theory, and application of perfusion chromatography; therefore the main goal here will be to cover more recent work.[293−298]

### 2. Characterization and Theory

Perfusion particles have been the object of several characterization and theoretical studies since their initial introduction.[299,300] These studies have largely

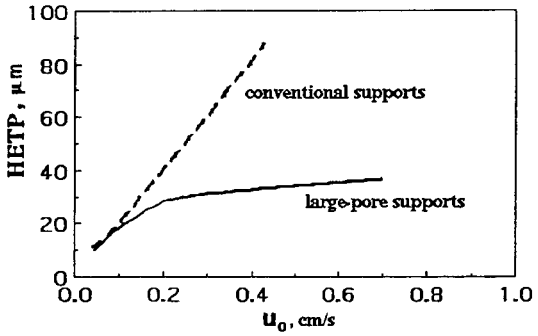


**Figure 38.** Plate height versus linear flow for a conventional support and perfusion support generated by eq 25. (Reprinted with permission from ref 293. Copyright 1997.)

confirmed that convective flow does occur and that the onset of such flow stalls the increase in band broadening with flow rate and enhances dynamic capacity relative to wholly diffusive supports.[300] The alteration of the usual band broadening effects in the column with the onset of substantial convective flow through the particles requires modification of the van Deemter equation for proper theoretical evaluation. Several studies have reported on variations of the plate equation for perfusive flow[301] (plus refs 10 and 11 therein). These equations focus on modification of the stagnant mobile-phase term, usually by incorporating the intraparticle Peclet number ($\lambda$) which is defined as the ratio of convective transport to diffusive transport within the particle. This ratio increases as intraparticle flow increases. The equation introduced by Rodrigues[300]

$$H = A + B/u + Cf(\lambda)u \qquad (25)$$

$f(\lambda)$ is approximately 1 at low flow velocity when there is little or no intraparticle flow and becomes $3/\lambda$ at high flow rates. A graphical representation of the effect on plate height is given in Figure 38. These modified equations successfully predict a linear dependency of plate height on flow velocity at low velocities which eventually converts to a flat curve, i.e., no dependency, at sufficiently high velocity. McCoy et al. used a modified van Deemter equation to characterize the particles with respect to various physical properties such as diffusivity and flow split ratio (the flow in the column is essentially split between the intraparticle and interparticle flow).[301] Their work demonstrated that the velocity at which the van Deemter curve becomes flat depends on the diffusion coefficient of the analyte such that larger compounds with smaller diffusion coefficients are more dramatically affected by the convective effect than smaller compounds. The flattening of the curve is also affected by the pore structure of the support. The support can be considered an agglomeration of microspheres with the large pores being found between the agglomerates and the smaller, diffusive pores, between the microspheres.[302] The relative sizes of the pores can be engineered, allowing different properties to be produced; therefore, it becomes important to be able to optimize the pore structure for performance. Larger pores and smaller particles were found to allow the convective effect to occur at a smaller flow rate range and give better performance.[297] Nash and Chase performed a similar study with a slightly different equation.[303]

Perfusive supports are considered to be especially useful for rapid, preparative scale separations. While this review is mainly concerned with analytical scale separations, this feature of perfusive supports deserves comment. The porous nature of perfusive supports gives them more surface area than what is found in pellicular particles; however, the larger pores also result in less surface area than that found in conventional microporous supports. The lower surface area translates into lower capacity for a given type of stationary phase.[303] Nash and Chase demonstrated that under conditions where all resolution was lost due to overloading on the perfusive support, resolution was still maintained on the diffusive support. At the same time, however, several studies have demonstrated that the capacity of perfusive supports eventually becomes independent of flow rate, much as plate height does. Furthermore, the larger pores of the perfusive supports mean that capacity is less dependent upon molecular size than "wide-pore" phases.[302] The use of "fimbriated" or fuzzy stationary phases have been explored as a route to increasing the capacity of perfusive phases without compromising the good flow effects.[259] As for nonporous particles, such chemistry seems to offer the capacity of microporous media and the kinetic properties of the support. The same study also demonstrated the relationship between pore size distribution to particle permeability and binding capacity and allowed identification of optimal pore size distributions. With optimal conditions, the perfusive supports have higher dynamic capacity at high flow rates than wholly diffusive supports.

### *3. Applications of Perfusion Particles for Fast Separations*

The initial reports of perfusion chromatography focused on demonstration of separations of simple mixtures in short times.[304,305] Perhaps the most dramatic was resolution of four out of five proteins in a mixture in ~12 s in a column packed with 20 $\mu$m particles.[304] Since these initial reports, however, a number of applications to real samples that have required high-speed capability have been reported. Most of these examples illustrate resolution of several proteins on the 1−5 min time scale. Several examples will be discussed below. Applications have included both analytical and preparative separations.

**Preparative.** A recent review of preparative-scale separations discussed the challenges of chromatographic separations of unstable proteins.[306] It is often found that in purification of sensitive enzymes, use of conventional chromatographic techniques results in a complete loss of biological activity of the target protein. Loss of activity is frequently attributed to the time in contact with surface supports and overall time required for separation. The review discussed several strategies for avoiding these problems including the use of rapid chromatographic separations.

Several reports of purifications that require <5 min even from crude mixtures have been reported using reversed-phase, ion-exchange, and affinity chromatography. Many of these reports have suggested that

the high speed of the separation, improved not only throughput, but recovery of active material. A few of these reports will be summarized here.

*Reversed-Phase.* In one example,[307] individual chains of human fibrinogen were purified by reversed-phase chromatography in <3 min. The chains were shown to retain their immunoreactivity by ELISA and immunoblotting and allowed characterization of the epitopes on fibrin. The throughput for purification was 5−10 times faster than that achieved with conventional porous media.

*Affinity.* Fibrinogen was also purified using a novel affinity phase consisting of a short-chain peptide (GPRP) with known affinity for fibrinogen.[308] The peptide was directly grafted onto an amine-functionalized POROS chromatographic resin. Fibrinogen from plasma bound to the $NH_2$−GPRP−POROS column at 15 mL/min flow rate for a total separation time under 5 min. Other examples of protein purification by this approach were also shown. Metal chelate affinity chromatography was used to purify histidine-tagged glutathione *S*-transferase fusion.[309] The combination of mild conditions and rapid flow allowed recovery with a high degree of biological activity. The rapid purification also assured minimum degradation or modification of the protein by naturally occurring proteases and other enzymes. The selectivity of the affinity process gave good purity, indicating the importance of selectivity for fast separations.

Large, "super-porous" agarose beads have been applied for the preparation of bovine lactate dehydrogenase and rabbit IgG.[310] Two sets of super-porous agarose beads (either 300−500 or 106−180 $\mu$m diameter) were prepared by a two-step emulsification resulting in flow-through pores of 50 and 30 $\mu$m, respectively, and diffusion pores of diameter typical of regular-sized pores. The particles were then derivatized with a $NAD^+$ analogue for purification of bovine lactate dehydrogenase and protein A for rabbit IgG. Compared to columns of regular-sized pores, the super-porous particles had nearly the same recovery and loading capacity even though the total porosity of the super-porous beads was 50%. This result could be explained in that normal diameter pores may become clogged with protein, thus eliminating a significant portion of the surface area. The super porous beads could also be operated at 3−5 times higher flow rate, cutting preparation time by that amount. The higher flow rates are presumably possible because of lower back pressures due to the large pores.

*Ion Exchange.* Ion-exchange chromatography has also been widely used for purification. A procedure to obtain large amounts of a chloroplast-localized heat shock protein (HSP21) using a purification procedure based on perfusion ion-exchange chromatography was reported.[311] After initial precipitation steps, the sample was applied to cation- and anion-exchange on two columns connected in sequence, which allowed rapid purification of HSP21 in one equilibration and one elution step. The purified recombinant protein appeared in assembled, oligomeric form (approximately 200 kDa) composed of 21-kDa monomers, similar to the native HSP21 protein. Ion-exchange perfusion chromatography was also applied to the isolation of hydrophobic membrane protein complexes from thylakoid membranes of spinach chloroplasts.[312] Pure reaction centers could be isolated from photosystem II core complexes after a chromatographic step requiring only 6.5 min, which was a substantial improvement in comparisons with previous procedures. This was the first example of purification of membrane proteins by perfusion chromatography. *endo*-$\beta$-*N*-Acetylglucosaminidase F3, an enzyme used for studies of the structure of asparagine-linked glycan, was purified intact and active from *E. coli* by perfusion chromatography.[313]

As mentioned above, the porous perfusive media is more adaptable to preparative-scale separations than pellicular separations because of the higher capacity. A direct comparison of the two types of media for purification of Pasteurella haemolytica leukotoxin (LKT) was reported.[314] While a short nonporous DEAE column allowed the partial microscale purification of the leukotoxin at pH 7.0, a high-capacity strong anion-exchange column of the perfusion chromatography type permitted the purification of LKT on a much larger scale. The anion-exchange stationary phases were employed to overcome difficulties of the relatively hydrophobic LKT interacting with other resins. The separation conditions allowed resolution of tetrameric and aggregate peaks of LKT from early eluting contaminant peaks in about 12 min.

Perfusion chromatography was also used in the preparation of supercoiled plasmid DNA.[315] Large-scale manufacturing of gene vectors such as plasmid DNA is an important issue in gene therapy, and anion-exchange chromatography is often used in the downstream processing of plasmids. Whereas Q-Sepharose was used as the stationary phase for actual purification, anion-exchange HPLC on a Poros QE 20 column was used to quantify plasmid yield.

### 4. Protein Analysis by Perfusion Chromatography

As mentioned above, initial demonstrations of perfusion media focused on separations of standards in relatively clean matricies. Since then, a variety of rapid separations and analyses of proteins in complex matricies have been reported in the literature. Several will be discussed here.

Reversed-phase separations were used for determination of soybean and whey proteins in commercial samples in a series of papers.[316−318] Linear binary gradients of acetonitrile−water−0.1% trifluoroacetic acid at 60 °C allowed separations in 1.5−3 min depending upon the sample. The method was validated and applied to the quantification of soybean proteins in infant formulas and powdered and liquid soybean milks. It also enabled the rapid detection of adulterations of powdered soybean milks by addition of bovine whey proteins. It was possible to detect about 1% and 1.3% of $\alpha$-lactalbumin and $\beta$-lactoglobulins, respectively, in a commercial powdered soybean milk in which these proteins were included in its formulation. Similar separations obtained by conventional reversed-phase high-performance liquid chromatography required up to ~4 times longer.

The high speed of perfusion chromatography separations of proteins may be of benefit in a clinical setting as well. One example was measurement of the Ggamma:Agamma ratio in fetal hemoglobin (Hb F). The Ggamma:Agamma ratio is around 70:30 at the time of birth and usually 40:60 in the trace amounts of Hb F found in the adult. Changes in this ratio are observed in several hemoglobin disorders, providing insights on the genetics and molecular pathophysiology of these diseases. Using perfusion chromatography, this parameter was measured ~10× faster than with conventional HPLC with similar quantitative results, suggesting a possible rapid clinical assay.[319−321]

Biotechnology depends on the ability to rapidly determine the quality of products produced in fermentation broths or other reactors, and the fast analysis possible with perfusion chromatography has allowed this to be done as reported in several examples. An automated reversed-phase high-performance liquid chromatography method for quantitative determination of recombinant apolipoprotein A-IMilano (r-Apo A-IM) in *E. coli* fermentation broth was developed and evaluated.[322] The use of a perfusion media (Poros IIR/H) made it possible to achieve rapid separation and good resolution at high pH. The r-Apo A-IM-containing fraction was well separated from other proteins, allowing a reliable quantification. The automation and high sample throughput of this method made it useful for routine determination of r-Apo A-IM in fermentation broth and in eluates from various purification steps. Similar success was found with a 3 min assay for recombinant acidic fibroblast growth factor in *E. coli* cell suspensions and lysate samples.[323] This experiment highlighted the importance of pH stability of the polymeric phase as part of the assay involved rinsing the column with NaOH solutions to maintain reproducibility.

### 5. Immunoassays

A popular application of the high flow rate systems has been immunoassay. Many different immunoassy formats have been described,[324−326] and just a few will be noted here. Since use of antibody−antigen interaction dramatically increases the selectivity of the assay, it appears that the major benefits of using perfusive supports for this application is the low back pressure at high flow rates and the high dynamic capacity, especially for large molecules.

Immunoassays have been used for the rapid determination of small molecules. In one example, a system called amplified flow immunoassay (AFIA) was developed for small haptens and applied to cocaine.[327] In the method complexes of enzyme labeled antibody with cocaine were separated from free enzyme by using a perfusion column with cocaine as the stationary phase. The enzymatic product formed in the eluent could be detected to quantify cocaine. The lower limit of detection was 380 pM (38 fmol) for cocaine with a sampling rate 26 $h^{-1}$ and with a recovery of (49 ± 3%). In another example, a competitive assay for phenytoin was performed by mixing sample with rhodamine-labeled phenytoin and antiphenytoin serum. The resulting mixture was injected onto a protein A column to allow separation of bound and free tagged antigen for quantification of phenytoin. The total analysis time was 3.5 min, and the detection limit was 0.8 ng/mL.[328]

In an example of determination of a protein, antigrowth hormone was added to sample containing hormone, and the subsequent complex and excess antibody retained on a protein G column. Desorption of the complex allowed rapid determination of the target.[329]

Antibodies have been directly determined by retention on a protein G stationary phase with a 5.5 min assay. Using UV detection, the minimum detectable concentration at 280 nm is about 2 μg/mL or 200 ng/mL at 214 nm. Accuracy of the quantification was independent of operator, solution composition, sample pH and the ionic strength. Since selectivity was based only on retention by protein G, however, antibody samples containing aggregated and fragmented Fc regions of the antibody will interfere with this method of quantification.[330] Perfusive immunoaffinity cartridges were explored for studying the interaction of antibody and growth hormone.[331] In one experiment, immobilized growth hormone was used to purify antigrowth hormone. It was shown that the immunoaffinity cartridges prepared by immobilizing the antigen offer more biological specificity for the purification of antibody than the protein G cartridges. The stability and lifetime of the immunoaffinity cartridges are mainly dependent on the stability of the biomolecules immobilized on them.

The use of immunoaffinity perfusion particles have been used for the fast preconcentration of insulin followed by a separation by either CE[332] or LC.[333] The low back pressures provided by the perfusion particles allowed very high flow rates (up to 100 μL/min) to be used on the 150 μm i.d. column. The insulin could then be desorbed from the column with a low flow rate (1 or 5 μL/min) giving up to a 1000-fold preconcentration in about 10 min.

### 6. On-Line Monitoring/Process Monitoring and Control

High-speed separations are especially important in situations where rapid feedback is necessary. One example of this situation is in process monitoring and control. In one example of this type of application, the production of IgM was monitored in hybridoma supernatant using perfusion chromatography. The sample was rather complex and required a two-column procedure in which sample was first injected onto an anti-IgM column and the eluent onto an ion-exchange column. This chromatogram was compared to that obtained without the antibody step. The subtraction of the IgM peak from the ion-exchange chromatogram allowed determination of IgM in <5 min for process monitoring. The same article also covered purification of IgM from ascites and hybridoma supernatant.[334]

Another example of process monitoring used multidimensional separations with perfusion media to follow the production of both glycosylated and nonglycosylated forms of γ-interferon from recombinant Chinese hamster ovary cells. For this application, the

forms of interferon were first captured on an immunoaffinity perfusion phase, then eluted into an ion-exchange column for further separation detection. The non-glycosylated form of I adsorbed on a cation exchanger with 20 mM Tris (pH 7) as the mobile phase and was eluted with a 10 min gradient to 1M NaCl in 20 mM Tris (pH 7). Glycosylated interferon was retained on the anion-exchange column using a mobile phase containing borate at pH > 9. Using this approach, the production of the proteins by the cells was monitored every 2 h over a 60 h fermentation period. This application demonstrates the versatility of the chromatographic approach for monitoring multiple proteins in a complex media.[335]

## D. Capillary Columns

### 1. Packed Capillary Columns

In the past, capillary columns were not considered to be compatible with rapid separations; however, this was largely due to two factors. The first, was that initial columns were not well-packed and exhibited rather high *A* and *C* terms. The second reason was that a perceived advantage of capillary columns was the ease of preparing long columns for generating high theoretical plates, even if at the expense of analysis time; thus, most work initially emphasized producing columns >50 cm long. In 1988 however, Karlsson and Novotny demonstrated that decreasing the column diameter from 250 $\mu$m to 44 $\mu$m resulted in improved performance with decreasing column diameter including reduced *A* terms (as small as 0.49) and *C* terms.[336] As a result, the 44 $\mu$m column, packed with 5 $\mu$m particles, was able to generate 226 000 plates in 33 min for a lightly retained compound. This work was confirmed and extended to columns as small as 21 $\mu$m i.d.[337] and 10 $\mu$m,[338] where it was shown that *A* terms as small as 0.15 could be achieved.

The potential benefits of reduced column diameters for fast separations were evaluated in a study of columns with 50−250 $\mu$m i.d. packed with nonporous and perfusion particles.[245] This work was based on the idea that with nonporous and perfusion media the *C* term becomes so small that the *A* term is the dominant form of band broadening, even at high flow rates. In fact, using typical values of *A* and *C* for conventionally sized, nonporous columns, the *A* term can contribute as much as 90% of the total plate height even at high velocities; therefore, reductions in the *A* term associated with packed capillaries may have substantial benefit. This study confirmed a combined reduction of *A* and *C* terms with the nonporous particles. This allowed generation of ~700 plates/s for unretained species using 8 $\mu$m particles packed into a 75 $\mu$m capillary. With this column, four catechol compounds were resolved in ~40 s isocratically.

### 2. Open Tubular Liquid Chromatography (OTLC)

Another type of capillary column, the open tubular column, may also offer a route to fast separations. In this type of chromatography, the stagnant mobile phase and eddy diffusion terms are eliminated.

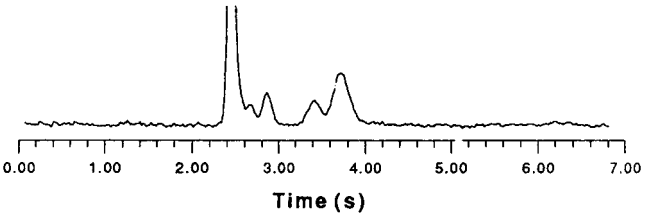


**Figure 39.** OTLC separation with optical gating with ensemble averaging (30 scans). Column length 0.5 cm. The first peak in an unreacted reagent peak; analytes are butylamine, amylamine, and cyclohexylamine. (Reprinted with permission from ref 339. Copyright 1991 American Chemical Society.)

Without these band broadening effects, the potential exists for fast separations; however, numerous difficulties arise from the need to have columns with i.d.'s of 10 $\mu$m or less to achieve reasonable results. Difficulties with implementing OTLC with such small columns include injection, detection and fabrication of columns with good phase ratios and stability. A handful of reports have been described utilizing OTLC for rapid separations. In the earliest example of a fast OTLC separation, columns with 1.7 $\mu$m i.d. were prepared by coating OV-17 onto the inner wall.[339] Using an optical-gating injection technique (see CE section), FITC-labeled amines were separated in as little as 5 s (Figure 39). With a column length of 0.5 cm, 1000−2000 theoretical plates were obtained with 5 s separation times. Analysis time and efficiency increased linearly with increasing column length as expected from theory.

OTLC columns have been prepared with a 5 $\mu$m i.d.[340] by coating using an in situ photopolymerization to apply fairly thick films of stationary phase with phase ratios as high as 1.3. These columns exhibited high efficiencies and were demonstrated for a variety of separations. Examples include separation of 19 phenylthiohydantoin amino acid derivatives in 10 min using a step gradient after the first 9 analytes had eluted. Up to 310 000 plates/meter were generated at 1200 plates/s (Figure 40). In addition, seven *o*-phthalaldehyde aliphatic amine derivatives were separated in 5 min, generating 400 000 plates/m and 1500 plates/s.

Research on the fabrication of open tubular columns, such as use of sol−gel technology for production of superficially porous capillaries,[342] continues to improve the state-of-the-art. These new procedures, combined with optical gating or other injection techniques used for fast separations in miniaturized CE systems, may ultimately allow practical, rapid separations by OTLC.

In addition to the properties mentioned above, capillary columns have been used with ultrahigh pressure and small particles because of their heat dissipation properties, with CEC for heat dissipation purposes, and with temperature gradients because of their low thermal mass. Thus, for a variety of reasons, capillary columns are part of the future of fast separations.

## E. Monoliths

In a monolithic or continuous bed column, the separation path consists of a network of pores defined

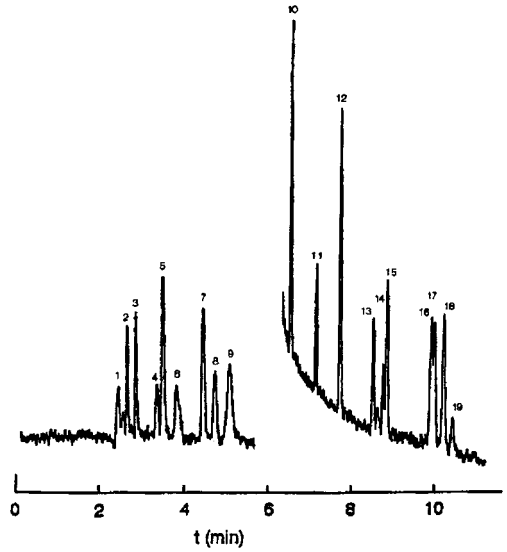


**Figure 40.** Separation of 19 PTH−amino acids with OTLC. A step gradient from 14% to 50% acetonitrile is applied at the break in the baseline. (Reprinted with permission from ref 340. Copyright 1995.)

by a continuous polymeric network of support instead of a bed of discrete particles. Three distinct approaches to the preparation of monolithic columns have been described: (1) polymerization of a rigid stationary phase support within a separation tube, (2) polymerization of a compressible gel network within a tube followed by compression, and (3) sintering of discrete particles packed into a tube. (Microfabricated "monoliths" have also been described; however, they will be considered under electrochromatography.) Monolithic columns are more frequently used with electrochromatography than pressure-driven chromatography because frequently the small pores prevent flow; however with sufficient control in the formation of the monolith, large pores are available to allow flow at reasonable back-pressures.

### *1. Rigid Polymer Monoliths*

Rigid polymer networks have been formed from both organic polymers[342−348] and silica networks[349−352] with both having advantages. Organic polymer networks offer selectivity and relatively simple preparation because the separation characteristics of the column are due to the functional groups and backbone of the polymer itself. A change in monomer composition will change the separation performance. Silica-based monoliths allow the use of previously developed derivatization chemistry for conventional particles and can handle higher pressures than most polymers.

In an early example of fast separation by rigid polymer networks, columns were synthesized by free-radical polymerization achieved by mixing initiator, monomer, and porogenic solvent within a tube (stainless steel with 5 cm length by 8 mm i.d.). The reaction created a macroporous polymeric stationary-phase rod for reversed-phase HPLC that contained a large volume of pores with diameters of 0.1−1 $\mu$m. The large pores allowed flow through the bed, while smaller pores provided surface area and capacity.

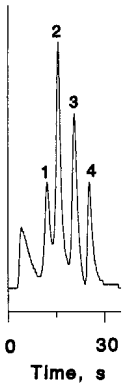


**Figure 41.** Fast separation of ribonuclease A, cytochrome *c*, myoglobin, and ovalbumin by RPLC on a monolithic poly(styrene-*co*-divinylbenzene) column. (Reprinted with permission from ref 354. Copyright 1993 American Chemical Society.)

Interestingly, with this pore geometry it was found that in the flow velocity range of 500−2000 cm/h, the plate height was independent of flow rate for proteins. The column was tested by separating four model proteins using an aqueous trifluoroacetic acid/acetonitrile gradient with mobile phase flow rates from 5 to 25 mL/min (Figure 41). At the highest flow rates, the compounds could be resolved in 30 s, thus demonstrating the capability of rapid separations with this type of media.[354] Thus, the effect appears to be similar to what has been observed with perfusion chromatography in which the presence of flow through pores allows high efficiency at high flow rates. Similar monolith technology was also evaluated for separation of polystyrene and alkylbenzene standards by both reversed-phase and size-exclusion chromatography.[347] In reversed-phase mode, three polystyrene standards could be separated with a MeOH/THF (gradient of increasing THF) within 4 s. The performance of the columns was compared to columns packed with porous and nonporous particles made of similar material. It was found that the monolith had superior performance at high flow rates compared to the porous column. The column with nonporous particles had too high of a back-pressure to be used at comparable flow rates.

Since these initial descriptions of molded polymer technology, the approach has been extended to hydrophobic interaction chromatography (HIC) and ion-exchange chromatography. For the HIC approach, the polymer was a rigid porous polyacrylamide-based material.[342] This material was thoroughly characterized with respect to loading, back-pressure, and effect of mobile phase on retention. The flow through pores allowed high efficiency at high flow rates so that five proteins were resolved in 3 min. For ion-exchange chromatography, porous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monoliths were modified with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) to form a strong anionic exchange column.[346] This media was demonstrated with separation of three proteins in 1.5 min.

Hjerten's group has explored the preparation of microcolumns based on monolithic media for reversed-phase and ion-exchange chromatography. Reversed-

phase columns as small as 25 μm i.d. were prepared by (1) synthesis in the column tube of a continuous bed matrix from a monomer solution (piperazine diacrylamide, methacrylamide) containing allyl glycidyl ether and 2-hydroxyethyl methacrylate and (2) linking of C-18 ligands by reacting 1,2-epoxyoctadecane with the epoxy and hydroxy groups in the matrix.[355] This continuous network showed good performance in the separation of proteins and peptides and analysis times as short as 100 s for the resolution of five standard proteins. Ion-exchange columns as small as 10 μm i.d. were prepared by a similar fashion.[356] With this synthetic procedure, the porous rod is attached directly to the walls, obviating the need for frits and resulting in a stable bed. Interestingly, it was also claimed that the covalent linkage of the bed to the tube wall also suppresses the zone-broadening "wall effect" which can occur with columns in the 100−300 μm i.d. range.

Reversed-phase monolithic columns have also been interfaced to mass spectrometry.[343] While in this example the potential for rapid separations was not explored, the convenience of being able to cast the column inside an electrospray needle was seen as an advantage. Interestingly, the monolith column was reported to give a lower background in the mass spectrometer than conventionally packed capillaries.

Porous, monolithic silica columns have been prepared by hydrolytic polymerization of tetraalkoxysilanes accompanied by phase separation in the presence of water-soluble polymers The resulting porous networks have silica skeletons 0.3−5 μm thick, 0.5−8 μm through pores, and 2−20 nm diffusive pores. Several direct comparisons of the silica rods with columns packed with 5 μm porous particles have been made. In one example, van Deemter plots for amylbenzene and insulin were compared on the two types of columns. In both cases, it was found that silica rods performed better with flatter van Deemter curves for higher performance at high flow rates. The differences in performance were also greater for larger, slower diffusing species. In one example, a plate height of ∼30 μm was achieved for insulin at 5 mm/s on the porous silica column compared to over 100 μm on a packed-bed column. The improved performance at high flow rates was affected by the geometry of the porous network so that thinner skeletons enhanced kinetic performance.[351] The good efficiency at high flow rates for these columns allowed isocratic separation of six alkylbenzenes in 35 s.[354] Another group made a direct comparison to columns that had the same surface area and pore diameters.[357] They also found better performance at high flow rates, while the selectivity and retention were similar to a conventional packed bed.

### 2. Sintered Packed Beds

Another approach to preparing silica-based monoliths is based on sintering porous particles into a 75 μm capillary. Columns prepared in this fashion with 6 μm particles had improved column stability compared to a normal packed-bed capillary; however, no improvement at high flow rates was noted.[358] It did not appear, however, that these columns had a fundamentally different performance than the packed bed.

### 3. Compressed Monolithic Beds

A third approach to forming monolithic columns, described by Hjerten's group, involves compressed-gel porous networks as separation media.[359−362] Such columns have been prepared with reversed-phase, ion-exchange, and affinity stationary phases. In an early disclosure of this approach,[360] the procedure for column synthesis was described as polymerization of an agarose support within the column to form a network that could be compressed with either pressure or a plunger in the column. The compressed bed had flow-through channels that formed a continuous porous network. Several impressive examples of rapid separations were shown including reversed-phase separation of five proteins in 3 min, cation exchange separation of six proteins in 30 s, and resolution of an enantiomeric pair in 40 s. Subsequent work has shown continued improvement in column fabrication as well as a number of applications of the technique. This technology also evolved into the rigid polymers for microcolumns described above.

**Applications of Compressed Monoliths.** Since these early reports, a number of examples of both preparative and analytical applications of the compressed beds have been described. Several examples will be given here.

*Preparative.* Several preparative-scale separations have been described. Ion-exchange beds were used to purify γ-globulin from human serum[363] and myelin basic protein.[364] In the latter example, the rapid separation allowed recovery of the protein in a fully active form as demonstrated by enzyme assay on the collected fractions.

Dye-affinity ligand chromatography on compressed, nonporous agarose beads has been used for purification of various dehydrogenases.[365,366] In one example, Cibacron Blue F3G−A was used as an affinity ligand for purifying glucose-6-phosphate dehydrogenase. It was found that the compatibility with high flow rates was essential as the recovered enzyme activity improved with separation speed. Procedures for preparing and utilizing immunoaffinity retention have also been described.[367] In this case, nonporous agarose beads were derivatized with human growth hormone via carbonyldiimidazol for the purification of antibodies against human growth hormone from antiserum. These columns yielded 100% recovery of antibody, and the column had a 50 μg of antibodies per gram of sedimented agarose beads.

*Analytical Applications of Compressed Beds.* In one application,[368] aminophenylboronic acid was attached to epoxy-activated nonporous agarose beads with diameters of 12−15 μm and was used for the fractionation of glycosylated from nonglycosylated hemoglobin. At a flow-rate of 4.0 mL/min, an analysis was finished within 2 min on a 2.5 cm × 0.6 cm i.d. column. The total time of an analysis was also short because a sample from a droplet of blood could be applied directly onto the column after haemolysis for

1 min without removal of cell debris by time-consuming centrifugation.

### 4. Summary of Monoliths

The results presented so far suggest that monolithic columns may hold promise for rapid, pressure-driven separations. By far the most activity has been with rigid polymer networks, and it appears that a great deal of versatility and control are possible in terms of pore design, skeleton thickness, and stationary-phase attachments. At present, however, a well-developed theory to guide experimenters in devising the appropriate properties of the porous network is lacking. In addition, while several groups have compared the performance of the monolithic columns to columns packed with conventional porous particles, more relevant comparisons would be with perfusion, pellicular, and superficially porous packed beds. Comparisons to these types of columns allow better evaluation of the true benefits of these types of columns for fast separations.

## F. Temperature

Temperature control is an underused variable in LC separations as evidenced by the paucity of commercial chromatographs equipped with temperature control units; however, it has been established that elevated temperatures improve separation speed in several ways. Increasing the temperature decreases the mobile phase viscosity which decreases the pressure required for high flow rates, potentially allowing smaller particles to be used, and increases the diffusion coefficient of the analyte in the mobile phase. In addition, increasing the temperature can accelerate the sorption−desorption kinetics of the analyte, thus reducing this band broadening effect. In a demonstration of utilizing higher temperature for fast separations, four standard proteins (ribonuclease A, cytochrome *c*, lysozyme, and $\beta$-lactoglubulin B) were separated in 12 s at 120 °C with a 10−90% acetonitrile gradient (flow rate 5 mL/min) on a 2 $\mu$m ODS-silica column (3 cm × 4.6 mm). An important feature of this system was that the mobile phase was preheated prior to pumping into the column, eliminating the problems associated with poor heat transfer through the packed bed that would result in uneven temperature profiles and associated band broadening.[369]

Precisely controlled temperature is important if highly reproducible chromatograms are to be obtained, especially when dealing with steep gradients, which is often required for fast analyses. In one example, it was found that a water bath provided a much more stable temperature than a convective air heater for the separation of proteins and oligonucleotides.[370] Seven test proteins were studied with respect to retention and resolution over a range of temperatures from 30 to 80 °C on a 2.1 $\mu$m diameter nonporous poly(styrene−divinylbenzene) 50 × 4.6 mm column. It was found that most of the proteins' chromatographic behavior changed equally as temperature increased. One protein (catalase), however, was slightly more sensitive to temperature. It coeluted with $\beta$-lactoglobulin A at 30 °C but was

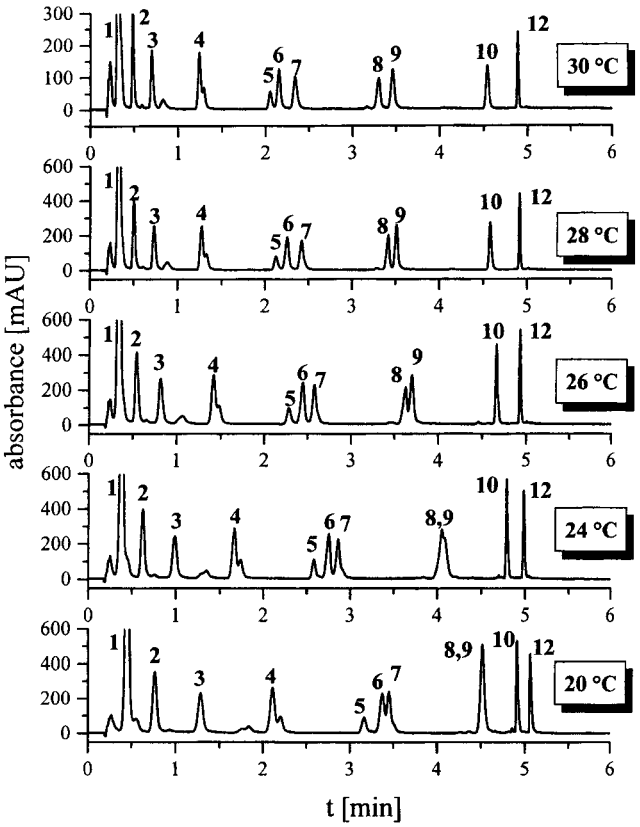


**Figure 42.** Temperature effect on resolution of hydrazones. Note resolution between peaks 6 and 7 and between 8 and 9. Column: 1.5 $\mu$m nonporous silica, 33 × 4.6 mm. (Reprinted with permission from ref 288. Copyright 1997 American Chemical Society.)

baseline resolved at 80 °C. The same study was carried out for the separation of oligonucleotides which reached maximum resolution at 40−50 °C.

The temperature change does not have to be a drastic one in order to see significant improvements in the separation. A 5 min gradient separation of 11 hydrazones at 20 °C did not allow for the complete resolution of all the components; however, increasing the column temperature by just 10 °C allowed all the components to be baseline resolved (Figure 42).[288]

The good heat transfer properties of small diameter fused silica capillaries make them more amenable to temperature-controlled LC and can even be used with temperature programming. This concept has been demonstrated with separations of mixtures of alkylbenzenes (AB) and PAH on a fused-silica column (50 cm × 180 $\mu$m i.d.) packed with 6 $\mu$m ZorbaxSB ODS-silica. The retention of the alkylbenzenes was evaluated as function of acetonitrile concentration at a fixed temperature and as function of temperature at a fixed acetonitrile level.[371,373] These studies showed that a 5 °C temperature change had the same effect on retention as a 1% change of acetonitrile concentration. Thus, it was possible to perform mobile phase programming and temperature programming to achieve identical separations. Temperature programming was also used for separating simple protein mixtures. Finally, a combination of temperature and mobile-phase programming was found to be effective for optimizing separations in less than 2 min (Figure 43).[373] The results from these studies show that

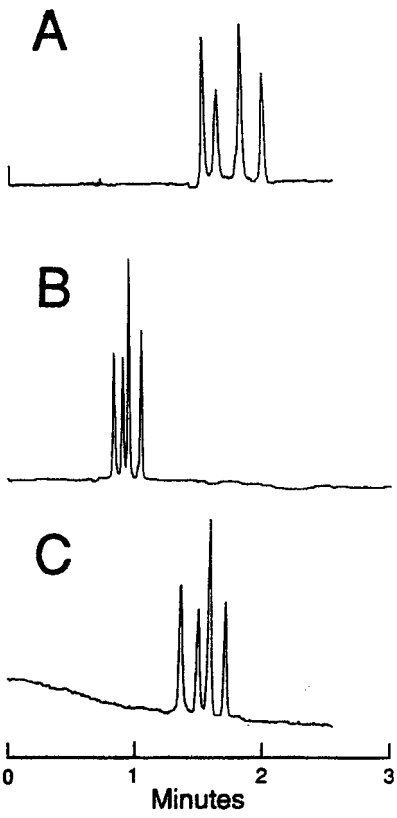


**Figure 43.** Chromatograms of protein standards by gradient elution at different temperatures and combination of mobile phase and temperature gradients. (A) 30 °C gradient, 0−70% acetonitrile in water, (B) 80 °C, same gradient, and (C) same gradient with 10 °C/min temperature gradient starting at 30 °C. (Reprinted with permission from ref 373. Copyright 1997.)

packed capillaries with elevated temperatures may offer a convenient route to faster separations. Limitations of this approach include the instability of stationary phase and the stability of delicate analytes at elevated temperatures.

One potential drawback of using high temperatures for fast separations is that columns become less stable at higher temperatures, resulting in changes in capacity factor and loss of resolution. A way to avoid this problem is to use a stationary phase or support material that is more stable than regular silica. This has been addressed by using 2.5 or 4.5 $\mu$m polybutadiene-coated zirconia particles.[374] The columns (100 × 4.6 mm) were evaluated for stability at temperatures up to 200 °C and were found to have a constant $k'$ for at least 1300 column volumes at the highest temperatures. An isocratic separation of test compounds at 100 °C maintained baseline resolution of all the compounds with a 5-fold increase in flow rate and 18-fold reduction in separation time.

The effects of temperature on efficiency and speed in open tubular columns have also been studied in the separation of steroids.[375] Air convection was used to thermostat commercially available OTLC columns of 50 or 100 $\mu$m diameter. As a result of pressurization, temperatures well above the normal boiling point for the solvent could be used. Increasing the temperature of the column from 100 to 150 °C gave a 3-fold improvement in efficiency. Both step and linear gradients were applied during the separation. With a linear gradient of 60 °C (90−150 °C), the analysis time decreased by 40% compared to an isothermal run at 90 °C and maintained baseline resolution of the six model compounds.

## G. Electrochromatography

In capillary electrochromatography (CEC), mobile phase is pumped electroosmotically instead of by pressure as is typical for HPLC. While the concept of CEC was introduced many years ago, it has recently received an explosive growth in interest. Several reviews of CEC[376,377] have appeared in the past few years as well as a series on the practical aspects of CEC.[378−381] Given the presence of these reviews, this work will emphasize CEC from the vantage point of fast separations.

### *1. Principles of CEC for Fast Separations*

CEC is an attractive possible route to fast separations. The plug flow profile associated with EOF decreases band broadening associated with resistance to mass transfer in the mobile phase allowing flat van Deemter curves as demonstrated in several studies.[382−386] The flat curves could potentially allow high efficiency at high flow rates and short times. Electroosmosis may also enhance intraparticle mass transfer due to flow within particles; however, this is still under investigation.[387−389] In addition, pressure differentials are not used to drive flow; therefore, it should be possible to use particles smaller than 2 $\mu$m without the problems associated with the pressure limits of current instrumentation. Flow rates of 1−3 mm/s are typical and can be easily obtained with 30 kV power supplies.

An instrumental advantage of CEC is that it can be carried out on most commercial CE instruments so no additional equipment must be purchased. The "short end injection" technique common in CE can be applied to CEC.[390] Commercial CE instruments typically require at least 30 cm of capillary, 8−10 cm of which is postdetection. For "short end injection", the portion that is normally after the detector is used as the inlet end, giving a separation distance of 8−10 cm. In CE, this arrangement "wastes" most of the voltage that is applied; however, in CEC, since the resistance is greater across a packed bed than open tube, it is possible to drop the majority of voltage across a short portion of the capillary that is packed so that only a small portion of the applied voltage is wasted across the empty tubing. A 4-fold reduction in analysis time was demonstrated by decreasing the separation length from 25 to 7.5 cm while maintaining a constant applied voltage.

A significant issue in fast CEC separation is control of EOF and obtaining EOF that is fast compared to what may be obtained by high pressures. EOF was studied according to type and percentage of organic modifier and demonstrated that as percent organic increases, electroosmotic flow decreases until very near 100% when the flow drastically increases.[391] Acetonitrile gives the fastest EOF followed by water and then methanol. These results suggest potential difficulties in developing mobile-phase gradients. The ability to apply a gradient to CEC would allow fast

separations of analytes with widely different hydrophobicities. Yan et al. have used two computer-controlled high-voltage power supplies to form gradients for CEC.[392] Potentials are applied to two separate reservoirs connected by a tee with a common ground at the outlet of the capillary. By maintaining the overall voltage and changing one voltage relative to the other, a gradient is produced. Huber, Choudhary, and Horvath[393] describe a gradient system in which the mobile phase (0.1−0.2 mL/min) was input from two reciprocating displacement pumps via a 10-$\mu$L mixer and a 17 $\mu$L cavity housing the column inlet and an electrode. This system provided reproducible gradient profiles. A test mixture of 12 phenylthiohydantoin derivatives of amino acids was separated at 10 kV using a mobile phase (100 nL/min) of 5 mM phosphate of pH 7.55/acetonitrile (7:3/2:3). The performance was compared with that of isocratic elution at constant and different temperatures.

Column design is still being actively explored in CEC. Designs have included packed columns, monolithic columns, open tubular columns, microfabricated open tubular columns, and microfabricated monoliths. No particular design seems to have a theoretical advantage in terms of speed of separation; however, the microfabricated columns do provide a convenient means for rapid injection which may be useful for separation times <10 s.

A microfabricated monolith[394] on a quartz wafer was constructed with the following dimensions: 4.5 cm long by 150 $\mu$m wide with 5 × 5 × 10 $\mu$m collocated monolith support structures and 1.5 $\mu$m channels and coated with poly(styrene sulfate) for reversed-phase separations. To evenly distribute the mobile phase and analyte across the entire width of the column, a binary splitting pattern was used requiring a $2^n$ number of channels. This feature allowed for identical flow paths to be maintained so that band broadening and nonlinear flow was kept to a minimum.

An open-channel electrochromatography system was developed with a channel 25 mm long, 33−50 $\mu$m wide, and depths from 2.9 to 10.2 $\mu$m.[395] The more shallow depths gave higher efficiencies but were more difficult to operate. The chip arrangement allowed for easy manipulation of linear gradients with different slopes, start times, duration, and percentages of organic modifier. This ability to easily tune selectivity allowed for a separation of four neutral coumarin dyes in under 25 s with a channel depth of 5 $\mu$m.

### *2. Examples and Applications of Fast CEC Separations*

Up to this point, most work on CEC has emphasized the additional efficiency possible from reduced band broadening and smaller particles for separation of complex mixtures rather than rapid separations; however, a number of impressive rapid separations have been reported. In one example, highly porous 500 nm silica beads were synthesized using a modified Stober process.[396] In this synthesis, *n*-alkyltrimethoxysilane was added to the reaction causing formation of a nonporous bead with *n*-alkyl groups chemically bonded throughout. Pores were generated by calcination at 550 °C, which burned out the alkyl chains leaving a highly porous particle with the following specifications: particle diameter = 0.56 $\mu$m, pore diameter = 3.4 nm, and specific surface area = 534 $m^2/g$. The particles were derivatized with *n*-octyldimethylchlorosilane and slurry packed into a 100 $\mu$m i.d. capillary with a 38 cm total length and 8.5 cm packed length. This column allowed flow velocities of 2 mm/s with a mobile phase of 80% acetonitrile:20% 25 mM Tris-HCl and 790 V/cm applied. Under these conditions, six *n*-alkylbenzenes were separated isocratically in less than 60 s with 83 600 theoretical plates for a virtually unretained solute, corresponding to $h = 3.5$ and $\nu = 1.2$ (Figure 44). On the basis of calculations by Knox,[397] the minimum diameter particle that can still support electroosmotic flow is 0.4 $\mu$m. These particles still support roughly the same flow as 3 $\mu$m diameter particles.

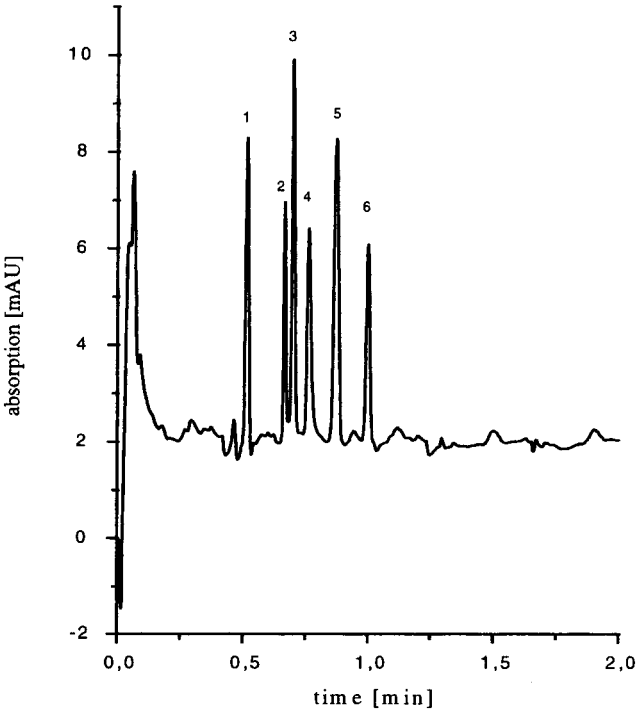


**Figure 44.** Electrochromatogram of *n*-alkylbenzenes on 0.5 $\mu$m C8 packed capillary. Electric field = 790 V/cm. Analytes: (1) thiourea, (2) benzene, (3) ethylbenzene, (4) propylbenzene, (5) *n*-butylbenzene, (6) *n*-pentylbenzene. (Reprinted with permission from ref 397. Copyright 1997.)

The combination of pellicular particles with EOF should generate high efficiencies in short separation distances as demonstrated recently. 1.5 $\mu$m reversed-phase particles were packed electrokinetically into 100 $\mu$m i.d. capillaries with a 6.5 cm packed-bed length. The packed bed was doped at 10% with 1 $\mu$m diameter silica gel particles to facilitate packing and to enhance EOF. With this system and a 65% acetonitrile/35% $H_2O$ mobile phase, a linear velocity of 18 mm/s was achieved at 2500 V/cm. With these conditions, five PAH's were separated in <5 s with more than 16 000 theoretical plates (reduced plate height = 3.9) as illustrated in Figure 45.[398] With 20 cm long columns, it was possible to generate 300 000−400 000 plates/m and 16 priority pollutant PAH's were separated in under 10 min.

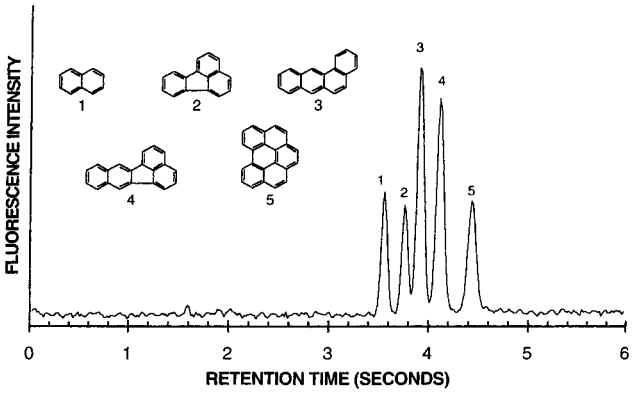


**Figure 45.** Fast electrochromatogram showing the separation of five PAHs on 1.5 $\mu$m nonporous ODS silica. Electric field = 3300 V/cm, linear velocity = 20 mm/s. (Reprinted with permission from ref 398. Copyright 1998 American Chemical Society.)

Open tubular CEC has been used in an interesting separation of proteins. In this example, OT-CEC was coupled on-line to an ion trap storage/reflectron time-of-flight mass detector for the analysis of peptide mixtures.[399] Reversed-phase columns prepared by the sol−gel process were coated with an amine that greatly enhanced the electroosmotic flow in acidic buffer and also reduced nonspecific adsorption of the peptides to the wall. The mass spectrum sampling rate was 8 Hz, which was sufficient to analyze the peptides without loss in resolution. Six model proteins were baseline separated in 3 min (Figure 46), and a tryptic digest of horse heart myoglobin was separated with a gradient in 6 min.

## H. Other Applications

Throughout the chromatography section of the review we have given examples and applications of fast separations that utilize the newer technologies that are emerging such as small monodisperse nonporous beads, CEC, and monoliths. Two applications, multidimensional separations and on-line monitoring, did not fit into these categories and will be discussed here.

### *1. Two-Dimensional Separations*

Most two-dimensional separations involving only chromatography are quite time consuming; however, they are included here because, as discussed in the electrophoresis section, the second step of separation must be fast enough to adequately sample the first dimension. Thus, the speed of the second dimension is important. In addition, the high resolving power of a 2D system typically allows a high peak resolution-to-time ratio.

A formal study of the effect of sampling rate on resolution in comprehensive two-dimensional liquid chromatography using reversed-phase HPLC as the first dimension and gel permeation chromatography as the second dimension has recently been performed.[400] Eluate was continuously sampled from the HPLC column via a computer-controlled eight-port valve with two identical sample loops filled alternately. A mixture of seven polymers was first fractionated by HPLC on a column (15 cm × 3 mm i.d.) of Zorbax SB18 (5 $\mu$m, 80 Å pores) (separation time 35 min.) and further separated by SEC on a column (5 cm × 8 mm i.d.) of styrene/divinylbenzene polymer (3 $\mu$m, 100 Å pores) (separation time 0.8 min). The effect on the separation of sampling time across a peak in the first dimension (achieved by varying the split ratio at constant flow), flow rate, and sampling phase (in-phase when sampling starts at the exact moment when a peak arrives) was studied. From the results, in-phase sampling (rare) required at least three samples across a peak whereas out-of-phase sampling required at least four. It was also found that the shortest sampling time into the second dimension gave the best resolution for the first dimension.

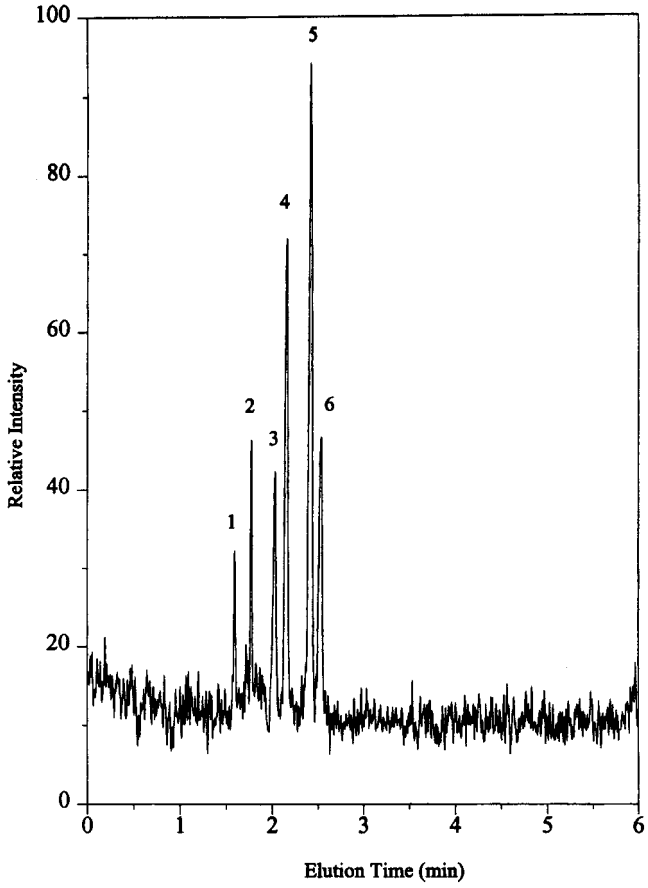


**Figure 46.** Total ion electrochromatogram of open tubular capillary electrochromatographic separation of six peptides. Analytes: (1) methionine enkephalin, (2) bradykinin, (3) angiotensin III, (4) methionine enkephalin-Arg-Phe, (5) substance P, (6) neurotensin. (Reprinted with permission from ref 399. Copyright 1997 American Chemical Society.)

Lundell and Markides give an optimization strategy for the separation of a small number of peptides from a complex biological sample by two-dimensional LC involving an ion-exchange separation followed by a reversed-phase separation.[401] The ion-exchange separation was performed with a step gradient which admits a high sample load and simplifies instrumentation; the reversed-phase separation uses a linear gradient which gives higher resolution for a wide range of hydrophobicities. The method was applied to the optimization of the determination of angio-

tensin II in rat brain tissue, resulting in fast separation with both high load capacity and high sensitivity.

Bushey and Jorgenson describe an automated two-dimensional system using cation exchange and gel permeation as the two separation methods.[402] A computer-controlled eight-port valve with two 30 $\mu$L loops was used to collect eluent from the first column and then inject onto the second column. The second column separation was 6 min, and with this sampling rate, all of the eluent from the first column could be re-injected on to the second column. Using this approach, a peak capacity of 126 was achieved in 150 min.

More recently, SEC has been coupled to RPLC with 1.5 $\mu$m nonporous particles used for the second separation and then coupling the system to an electrospray mass spectrometer for peak identification.[403] An interface placing the columns in parallel makes it possible to use conventional diameter columns for a more rugged system. The first dimension required a 150 min separation which was then followed by a 6 min reversed-phase separation. This system had a peak capacity of 495.

A cation exchange and reversed-phase two-dimensional system with perfusion particles for the second reversed-phase separation has also been shown for the analysis of an *E. coli* cell lysate.[404] The first separation step used a 750 $\mu$m × 12.5 cm column, while the second column had dimensions of 500 $\mu$m × 10 cm. Utilizing a gradient in each step and a 150 s separation for the RPLC step, this system had a peak capacity of 2500. The resolving power of this technique was demonstrated on an anion exchange/reversed-phase two-dimensional system for the separation of a crude brain extract.[405] The fully automated system utilized two 3.5 cm × 4.6 mm columns. The first step used a complex chloride gradient to maximize resolution followed by a steep acetonitrile gradient. Each separation took 10 min and was applied 5 times each for a total analysis time of 80 min, separating over 150 peptides (Figure 47).

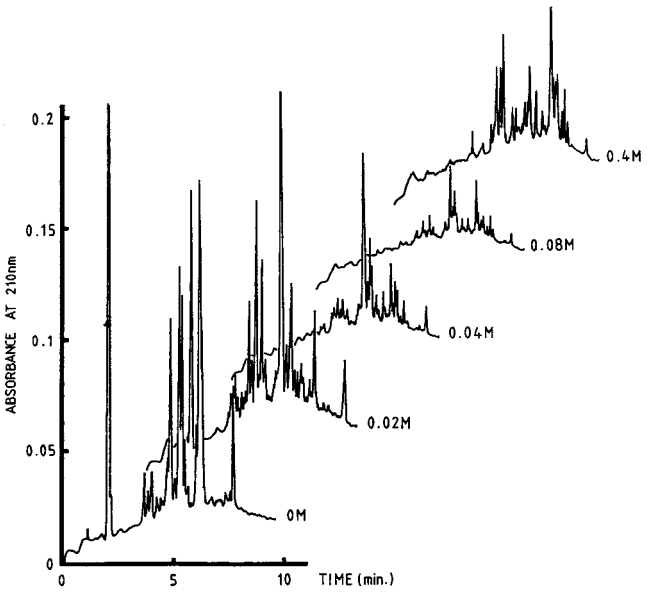


**Figure 47.** Two-dimensional separation of crude peptide extraction from brain tissue. The first phase is anion exchange, and the second is reversed phase. Over 150 components are detected in 80 min. (Reprinted with permission from ref 405. Copyright 1990.)

### 2. On-Line Monitoring

As discussed in the electrophoresis section, fast separations coupled on-line with sampling approaches is a powerful approach to chemical monitoring. Microdialysis with LC, for example, has been used extensively for monitoring.[406−409] A limiting factor in coupling LC with microdialysis is the injection volume required with standard bore columns; with typical microdialysis flow rates of 1 $\mu$L/min, several minutes are required to collect enough dialysate to inject. Several examples of high temporal resolution monitoring using LC have been reported.[410,411]

An on-line system has been developed for the *in vivo* monitoring of biological compounds, in this case dopamine with 1 min sampling.[412] In this system, microdialysate was pumped directly to the four-port injection valve fit with a 0.5 $\mu$L injection loop. The 10 cm × 0.5 mm column (5 $\mu$m $C_6$ particles) was coupled directly to the injection valve utilizing an isocratic separation. With this system, the effects of cocaine administered through the microdialysis probe could be monitored. A similar system has been used more recently to measure paracetamol and its metabolites or caffeine and its metabolites.[413] Microdialysate from the jugular vein of a rat was injected directly on a column (14 mm × 1 mm i.d.) packed with ODS silica (3 $\mu$m). The separation of paracetemol was performed in less than 30 s, while caffeine was separated in less than 60 s.

## IV. Concluding Remarks

Both CE and LC have seen dramatic increases in speed of separation over the past decade. For CE, such advances can be tied most directly to novel injection technology and high sensitivity detectors. These advances have allowed simple mixtures of amino acids, dyes, proteins, and antigen−antibody complexes to be separated in <1 s. More complex mixtures of DNA fragments, proteins, peptides, amino acids, carbohydrates, inorganic ions, and organic acids have all been separated in a few minutes. This capability has been used in a few applications such as analysis of protein conformers, in vivo monitoring, and two-dimensional separations. Future directions will likely include refinement and possibly commercialization of technologies, especially microfabrication, that enable such fast separations. In addition, integration of other aspects of the analysis such as sampling, derivatization, deproteination, preconcentration, and filtering will be required to reap the true benefits of fast separations. In addition, improvements in electrochemical, UV, refractive index, and mass spectrometry detection are required to allow a wider variety of mixtures to be rapidly separated and analyzed. While it is unlikely that the near future will bring routine separations substantially faster than the 1 s separations already seen, it will be important to expand the possibilities to other classes of chemicals. Successful development of commercial instruments and novel methods will open up

other areas of application for high-speed separation including process monitoring, clinical analysis, genetics analysis, portable analytical systems, chemical "sensing", and high-throughput screening for drug discovery.

For liquid chromatography, the advances in separation speed have been most related to particle and column technology such as smaller particles and monolithic columns. These advances have allowed numerous examples of separations of proteins, amino acids, inorganic ions, and small organics in less than 60 s. The speed of LC separations is likely to see substantial improvements over the next decade as novel pumping capabilities based on high pressures and electric fields are coupled with smaller particles, monolithic columns, highly selective stationary phases, microfabricated columns, and effective use of higher temperatures. To date, little has been done to combine these various innovations for optimal performance. As these systems are developed, the commercialization and applications will be similar to that for CE.

## V. Acknowledgments

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## VI. List of Symbols

| | |
|---|---|
| $\alpha$ | fraction of molecules which stick to the capillary surface on collision |
| $\Delta T$ | temperature difference between capillary wall and any internal radius |
| $\lambda$ | thermal conductivity of the solution (also Peclet number in chromatography section) |
| $\mu$ | electrophoretic mobility |
| $\mu_{app}$ | apparent mobility $\mu_{app} = \mu_{EOF} + \mu$ |
| $\mu_{EOF}$ | electroosmotic mobility |
| $\sigma^2_{DAQ}$ | variance due to data acquisition |
| $\sigma^2_{ED}$ | variance due to electromigration dispersion |
| $\sigma^2_{EF}$ | variance due to the electronic filter |
| $\sigma^2_{Ads}$ | variance due to sample adsorption |
| $\sigma^2_{Det}$ | variance due to detection |
| $\sigma^2_{Diff}$ | variance due to diffusion |
| $\sigma^2_{Heat}$ | variance due to effects of Joule heating |
| $\sigma^2_{Inj}$ | variance due to sample injection |
| $\sigma^2_{Tot}$ | total variance |
| $\tau$ | thermal coefficient of the solute mobility |
| ANTS | 8-aminonaphthalene-1,3,6-trisulfonate |
| AP | affinity probe |
| AP* | fluorescently labeled affinity probe |
| APCE | affinity probe capillary electrophoresis |
| CDCE | constant denaturant capillary electrophoresis |
| CE | capillary electrophoresis |
| CI | chemical ionization |
| $D$ | diffusion coefficient |
| $d$ | one-half of the intermicelle distance |
| $D_{aq}$ | analyte diffusion coefficient in the aqueous phase |
| $D_{mc}$ | analyte diffusion coefficient in the micellar phase |
| $E$ | electric field strength |
| EOF | electroosmotic flow |
| ESI | electrospray ionization |
| $f$ | sampling frequency |
| $G$ | heat generation rate |
| $G_v$ | difference in velocity between the axis and the inside wall of the capillary |
| $H$ | plate height |
| $h$ | initial width of sample plug |
| $H_{ads}$ | adsorption contribution to the plate height |
| $H_{aq}$ | intermicelle diffusion contribution to plate height |
| HDB | hexadimethrine bromide |
| HEC | hydroxyethyl cellulose |
| $H_{ep}$ | plate height contribution due to polydispersity of the micelle size |
| $H_{heating}$ | Joule heating contribution to the plate height for CE |
| HIBA | hydroxyisobutyric acid |
| $H_{mc}$ | plate height contribution of sorption−desorption kinetics in and out of the micelle phase |
| HPLC | high performance liquid chromatography |
| $H_t$ | Joule heating contribution to plate height for MEKC |
| i.d. | inner diameter |
| $k'$ | retention factor |
| $K_1$ | $6.5 \times 10^{-4}$ |
| $K_2$ | $4.34 \times 10^{-5}$ |
| $k_d$ | desorption rate constant |
| $l$ | separation length |
| $L$ | total column length |
| LIF | laser induced fluorescence |
| MEKC | micellar electrokinetic chromatography |
| MS | mass spectrometry |
| $N$ | number of theoretical plates |
| $n$ | number of molecules per unit volume inside the capillary |
| NDA | naphthalene-2,3-dicarboxyaldehyde |
| NDP | nucleoside diphosphate |
| o.d. | outer diameter |
| PCR | polymerase chain reaction |
| PEO | poly(ethyleneoxide) |
| $R$ | fractional concentration of free solute |
| $r_c$ | inner radius of the capillary |
| $R_s$ | separation resolution |
| $R_t$ | rate at which the sampling capillary is brought across the entrance of the channel in continuous channel electrophoresis |
| $r_x$ | distance from the capillary wall |
| $S$ | specific conductance |
| S/N | signal-to-noise ratio |
| T | target molecule |
| T* | fluorescently labeled target molecule |
| $t_0$ | insolubilized solute retention time |
| $t_{mc}$ | micelle retention time |
| $t_{mig}$ | migration time |
| TOFMS | time-of-flight mass spectrometry |
| $T_R$ | electronic filter rise time |
| $V$ | voltage |
| $v$ | linear migration velocity |
| $W$ | width of an individual separation lane in continuous channel electrophoresis |
| $Z$ | number of molecules striking a unit surface area per second |

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