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# INSTRUMENTAL DEVELOPMENTS IN MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY

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## SUMMARY

Two instrumental developments in micellar electrokinetic capillary chromatography are reported. A solvent delivery system capable of generating continuous linear-, concave-, and convex-shaped solvent gradients is shown to have a dramatic effect on the chromatographic profiles obtained in the separation of a mixture of fluorescently-labeled *n*-alkylamines. A versatile on-column flow cell that employs a unique laser-etched, on-column optical slit is described. This flow cell is adapted for photometric detection using a modified UV absorbance detector and spectrophotometric detection using a photodiode array detector.

## INTRODUCTION

As a consequence of the development of the field of biotechnology and advances in biomedical diagnostics and therapy, there are many new and demanding challenges for separation scientists. High sensitivity, large peak capacity, and exceptional resolving power are bioseparation requirements that are often encountered. Conventional liquid chromatography techniques, despite a variety of operational modes and the mature development of instrumentation, often fall short of satisfying these requirements. Conversely, electrophoretic separation methods have been very prominent in bioseparations[1]. Unfortunately, traditional electrophoretic techniques suffer from two "volume-related" problems in modern biotechnology/biomedical applications. The first problem relates to the scarcity and value of many biosamples, thereby precluding the use of large-scale (large sample volume) traditional techniques for their characterization. The other problem relates to the enormous number of samples that need to be analyzed as a result of the great amount of activity in the biotechnology area. Traditional electrophoretic techniques are labor intensive, slow, and not easily automated. However, the aforementioned problems have been addressed over the past several years by attempts to develop capillary electrokinetic methods of separation.

Capillary zone electrophoresis (CZE) has received the greatest attention for the separation of ionic species. Jorgenson and Lukacs[2] were the first to usher-in the "high performance" era of CZE by employing microcapillary columns (I.D. $< 100$ $\mu$m) that very efficiently dissipated the heat generated by the electrophoretic process, resulting in large plate counts. One approach to extend the applications of CZE to include

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neutral species involves the addition of surfactants, such as sodium dodecylsulfate (SDS), to the mobile phase to form charged micelles. Under these conditions neutrals are separated based on their differential partitioning between the electroosmotically-driven mobile phase and the micellar phase, which is moving at a different velocity (usually a slower velocity) from that of the mobile phase due to electrophoretic effects. Since the first reports of this micellar electrokinetic capillary chromatography (MECC) technique by Terabe and co-workers[3,4], fundamental studies that characterize the effects of experimental parameters on efficiency[5,6], retention[4,7–10] and selectivity[11,12] have been reported. Many compounds that are typically found in biosamples have been separated by the technique[3,7,10,11,13–19].

The development of instrumentation for capillary electrokinetic separation techniques is the focus of much research effort. Except for detection complication arising from the very small solute bands involved (typically $< 100$ nl), there do not appear to be any fundamental problems to limit the development of automated instrumentation for techniques such as CZE and MECC. We report herein the results of two instrumentally-oriented research efforts in our laboratory.

Retention programming is accomplished in MECC using a solvent delivery system that is capable of generating continuous linear-, concave-, and convex-shaped solvent gradients. Retention characteristics in MECC resemble that observed in reversed-phase high-performance liquid chromatography (HPLC)[11]. The effects of acetonitrile solvent gradients on the chromatography profiles for a series of fluorescently derivatized *n*-alkyl amines is demonstrated and the complex effects of incorporating organic solvents in MECC mobile phases discussed. The gradient system is not restricted to the MECC technique and the ability to continuously adjust pH with the apparatus is also demonstrated, indicating potential utility for CZE and capillary isoelectric focusing (CIF).

The small solute bands encountered in MECC and CZE preclude the use of commercial optical flow cells for detection. In most cases optical detection is performed on-column, often resulting in problems with short optical path lengths, large amounts of stray light, low throughput, and electronic noise from the large applied fields. A versatile on-column flow cell is described that is fabricated using a unique laser-etched slit and optically isolated from the detection electronics. The flow cell is adapted for use with two commonly available detectors. Using an external light source and fiber optics for light transmission the flow cell is adapted for use with a commercially available HPLC detector. Multidimensional detection is accomplished by combining the flow cell with a photodiode array detector that we used previously for laser spectrofluorometric detection in MECC[20]. The analytical characteristics of these flow cell-detector combinations is presented.

## EXPERIMENTAL

### *Reagents*

The *n*-alkylamines used to evaluate the gradient elution technique were obtained from Alltech Assoc. (Dearfield, IL, U.S.A.). HPLC-grade acetonitrile, the organic solvent employed in the gradients, was purchased from Mallinckrodt (St. Louis, MO, U.S.A.). Bromocresol green, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl), isoquinoline, and sodium dodecylsulfate (99% pure), were acquired from Sigma (St.

Louis, MO, U.S.A.). Mobile phase components, dibasic sodium phosphate, sodium borate, and citric acid, were obtained from Fisher Scientific (Springfield, NJ, U.S.A.). Sodium fluorescein was purchased from Eastman Kodak (Rochester, NY, U.S.A.). Fused-silica capillaries (50–100 cm × 50 μm I.D.) were supplied by Polymicro Technologies (Phoenix, AZ, U.S.A.) and used without chemical modification.

*Apparatus*

Gradient elution studies were performed using on-column, laser-based fluorescence detection, employing a Liconix (Sunnyvale, CA, U.S.A.) Model 4230 NB He-Cd laser (442 nm, 30 mW) for excitation. Fluorescence emission was isolated at 525 nm with a bandpass interference filter and an Instruments SA (Metuchen, NJ, U.S.A.) Model H-10 monochromator (bandpass approximately 10 nm), and detected with an RCA (Lancaster, PA, U.S.A.) Model 1P28 photomultiplier tube. Photocurrents were processed with a Pacific Precision Instruments (Concord, CA, U.S.A.) Model 8376-20 photometer and displayed with a strip chart recorder.

Electric fields were generated with a Hipotronics (Brewster, NY, U.S.A.) Model 840A high-voltage power supply. Most separations were performed at an applied potential of 20 kV. Except when gradients were investigated, the inlet of the column was maintained at a positive voltage and the outlet of the column at ground potential. For gradient work, the power supply was used in a negative polarity configuration (*i.e.*, the inlet of the column was at ground and outlet of the column was at a negative potential). To accomplish the negative polarity configuration the outlet of the column and the detection optics were electrically isolated from ground by anchoring them to a 1.5 in. thick slab of plexiglass.

The solvent gradient apparatus employed herein consists of an in-house built conical mixing chamber containing a micro stirring bar, as the inlet reservoir, and two stepping motor syringe pumps to deliver and withdraw reservoir solutions. Gradients are formed with this design by pumping, at a set rate, a mixed organic-aqueous mobile phase into the reservoir (which contains a measured volume of initial mobile phase) and pumping the contents of the reservoir to waste at another set rate. Adjusting the pump rates and initial volume of mobile phase in the reservoir produces gradients with various profiles (see eqn. 1). The inlet of the capillary column is positioned within the inlet reservoir to insure that a fully-mixed solution is electrokinetically pumped into the column.

A laser-etched flow cell (see *Procedures* for its construction) was employed in two detection schemes, both diagrammed in Fig. 1. The first, a single channel detector, utilized a Laboratory Data Control (LDC) UV III monitor, from which we removed the flow cells and source. A cadmium pen lamp, 229 nm output (UVP, Palo Alto, CA, U.S.A.) was employed as an excitation source. The lamp was placed adjacent to the flow cell. Light passing through the laser-etched slit was collected and delivered to the sample photodiodes of the detector by three 400-μm-diameter UV-grade optical fibers (General Fiber Optics, Cedar Grove, NJ, U.S.A.) arranged in a line (*i.e.*, the effective slit height was approximately 1.2 mm). A 200-μm-diameter UV-grade optical fiber was employed in the reference channel.

Multichannel detection was performed using a TN 6100 photodiode array (Tracor Northern, Middleton, WI, U.S.A.). Light passing through the laser-etched flow cell was collected and collimated by a 4-cm-diameter *f*/1 quartz lens and then

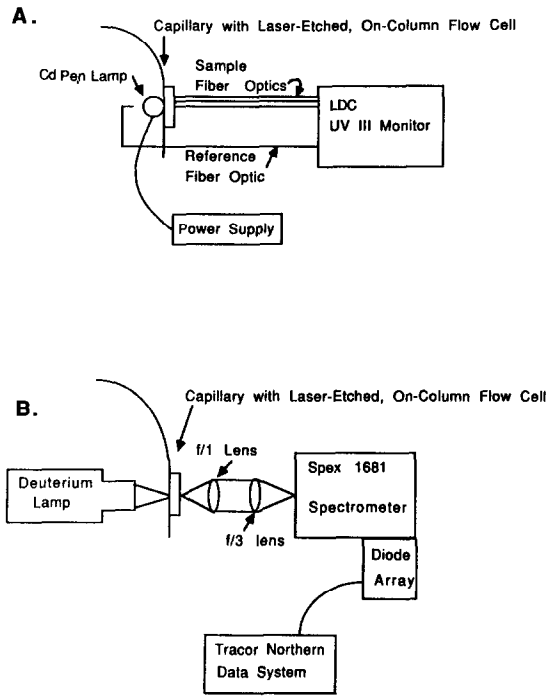


Fig. 1. Schematic diagrams of modular flow cell adapted for use with (A) HPLC UV absorbance detector and (B) multichannel photodiode array detector.

focused by a 4-cm-diameter *f*/3 quartz lens onto the entrance slits (0.2 mm) of a Spex (Edison, NJ, U.S.A.) Model 1681 spectrometer, which dispersed the light across the diode array. Photocurrents were processed by a TN 6500 controller/analyzer. To alleviate the need for large amounts of memory, the array was operated in its lowest resolution, which resulted in a spectral resolution of 4 nm.

*Procedures*

Stock solutions of the derivatized *n*-alkylamines were prepared by introducing an excess of NBD-Cl to a $10^{-3}$ *M* solution of the amines in ethanol (absolute). The reaction mixture was diluted by a factor of 100 with mobile phase and then refrigerated until needed. All solutions used for obtaining calibration plots were prepared in distilled, deionized water.

In an initial evaluation of the gradient system the solution being pumped into the inlet reservoir was doped with a fluorescent dye. Thus, it was possible to determine the shape of the gradient by the temporal change in fluorescence intensity at the inlet of the capillary. The solvent delivery system was also used to produce a pH gradient, which was created by titrating 54 m*M* citric acid with 0.1 *M* sodium hydroxide in the inlet reservoir. Visualization of the pH gradient was accomplished by doping the mobile phase with bromocresol green and using the photodiode array detection scheme to acquire on-column absorbance spectra.

Laser-etched, on-column flow cells were produced by first removing a small

section of the capillary column's polyimide coating and then applying epoxy to afix the exposed column between two microscope slides. The slides and column were attached to a square aluminum or plexiglass bracket, providing stability and allowing the flow cell to be held in place. The front face of the apparatus was painted black using water-based acrylic paint (Badger Air Brush, Franklin Park, IL, U.S.A.). Next, the beam of an argon ion laser (Coherent, 488 nm, 0.8–1.0 W) was focused onto the painted section of the capillary. The apparatus was translated horizontally until a symmetrical pattern was seen in the far field. This indicated that the beam was cleanly focused through the flow channel of the capillary. Following this the flow cell was translated vertically to create a slit along the flow channel of the capillary. The laser power and translation rate can be adjusted to produce a slit of the desired width. To complete the flow cell all but a small section (approximately 1.5 mm) of the slit was masked with electrical tape. Fig. 2 is a photograph of the flow cell assembly that also shows an exploded view of the slit.

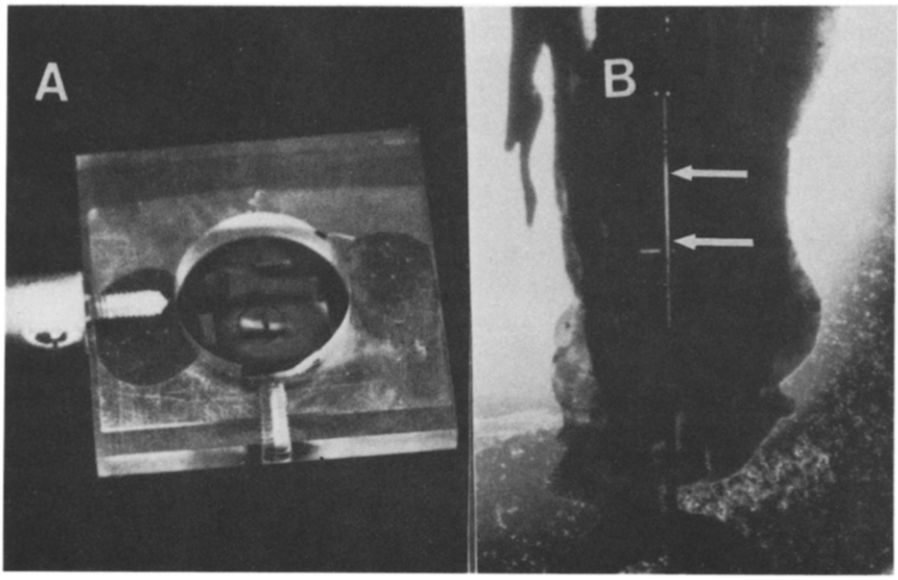


Fig. 2. Photograph of laser-etched, on-column flow cell. (A) Finished masked flow cell and (B) exploded view of slit region (denoted with arrows) of flow cell prior to masking.

Stray light measurements were performed by filling the flow cell with isoquinoline–acetonitrile (50:50, v/v). Signals observed under these conditions were assumed to be due to stray light. Minimum detectable concentrations were determined by filling the capillary with solutions of isoquinoline or sodium fluorescein. Data obtained using the photodiode array detection scheme was collected in the system's histogram mode which integrates diode signals over a previously designated spectral region and displays the integral versus time. Data for preliminary characterization of pH gradients was collected as a series of absorbance spectra obtained at 4 Hz.

## RESULTS AND DISCUSSION

### *Gradient elution*

The capacity factor, $k'$, of a solute in chromatography can be expressed as the product of the thermodynamic partition coefficient, $K$, and the phase ratio, $\beta$, the ratio of the stationary phase volume (micellar volume in the case of MECC) to the mobile phase volume. Gradient elution theory for conventional chromatography[21] can not be readily applied to MECC. This is because the presence of organic solvent in the MECC mobile phase affects not only $K$, but also the micellar volume (hence $\beta$) by altering critical micelle concentration (CMC), as well as the viscosity and dielectric constant of the mobile phase and the zeta potential at the capillary surface (and thus the electroosmotic and electrophoretic flow-rates).

The MECC technique exhibits a limited elution range that is determined by the relative magnitudes of electroosmotic and micellar electrophoretic flow rates[7]. This characteristic of MECC results in a maximum resolution for solutes with $k'$ values of approximately 2–5 (ref. 4). We have previously demonstrated that significant reduction in $k'$ can be achieved using mobile phases containing moderate concentrations of organic solvents such as 2-propanol or acetonitrile[7,10,12,22]. However, optimization of $k'$ values via the addition of organic solvents can be offset by dramatic increases in analysis time if the elution range is increased by an excessive amount[10]. Effective use of solvent gradients can provide a suitable compromise between the many divergent effects discussed above when separations of complex mixtures are performed.

A simple approach to generating solvent gradients in MECC is to alter the composition of the solution in the inlet reservoir during the course of the separation. Since reversed-phase elution characteristics are generally observed in MECC, this would normally involve increasing the organic content of the mobile phase during the separation. Our first attempts at gradient elution involved generating stepwise gradients by manually adding a mixed aqueous–organic solution to the inlet reservoir while the voltage was momentarily turned off[10,22]. This cumbersome approach was necessary because the customary grounded-outlet reservoir/hot-inlet reservoir arrangement resulted in shorting when solutions were delivered to the inlet reservoir with a pump while voltage was applied. In the work reported herein we electrically isolated the outlet reservoir and the detector components using a thick piece of plexiglass, permitting the reversal of the polarity and solving the aforementioned shorting problem. Using stepping motor-driven syringe pumps to deliver and withdraw solutions from a stirred inlet reservoir we were able to generate continuous linear-, convex-, and concave-shaped gradients that are described by eqn. 1[23]

$$\%_{out} = \left[1 - \left(\frac{V^\circ}{V^\circ + \Delta R t}\right)^{\frac{R_{in}}{\Delta R}}\right] \%_{in} \qquad (1)$$

where $\%_{in}$ and $\%_{out}$ are, respectively, volume % concentrations of organic solvent delivered to and withdrawn (to the capillary and to waste) from the inlet reservoir, $t$ is time after initializing the gradient, $V^\circ$ is the starting volume of the initial mobile phase (1–10 ml in this work) in the inlet reservoir, and $\Delta R$ is the difference in pump rate in,

$R_{in}$, and pump rate out, $R_{out}$. This equation assumes the initial mobile phase does not contain organic solvent. A linear gradient is generated when $R_{out} = 2R_{in}$, the slope of which is determined by $\Delta R$ and $V^{\circ}$. Concave and convex gradients are formed when $R_{out}$ is greater than and less than $2R_{in}$, respectively. The slope, duration, and final $\%_{out}$ for the gradient are limited by factors such as the volume capacity of the inlet reservoir, the available range of pump rates, and the increase of CMC with the addition of organic solvents. We have previously demonstrated that large reductions in $\beta$ can dramatically reduce efficiency[10]. In the case of acetonitrile this occurs at volume % values greater than about 20%.

By doping the mobile phase with a fluorophore and monitoring fluorescence intensity at the inlet of the capillary we were able to experimentally validate eqn. 1 for various shaped gradients. However, it should be mentioned that the equation can only be used to calculate the inlet mobile phase composition. Under the conditions employed to generate solvent gradients in this work, there is a several percent difference in volume % organic solvent across the length of the column. Thus while the velocity of a neutral solute band, $V_b$, in MECC can be expressed using eqn. 2[14],

$$V_b = \frac{1}{1 + k'} (k' V_m + V_{eo}) \quad (2)$$

where $V_m$ is the velocity of the micelles and $V_{eo}$ is the electroosmotic flow velocity, the actual value of $V_b$ can only be calculated by knowing the values of the parameters in the equation for the mobile phase composition in the region of the band (which can not be determined directly from eqn. 1). Although not studied in this work, it would be interesting to determine if the mobile phase gradient that exists in the region of a band produces a large enough variation in $k'$ (*i.e.*, $k'$ is greater before band center than after band center) to cause an appreciable concentrating effect.

Our initial evaluation of this solvent delivery system was performed on a homologous series of fluorescently-labelled *n*-alkylamines, ranging from *n*-propylamine to *n*-dodecylamine, using a variety of linear-, concave-, and convex-shaped acetonitrile gradients. Acetonitrile was chosen because it does not cause large changes in flow-rate and, hence, produces only a moderate extension of the elution range[22]. As far as general trends in the separation of these test compounds is concerned, our research to this point has indicated that a linear gradient is best for the separation of early-eluting components, a convex gradient provides the best separation of intermediate-eluting components (the earlier the elution within this group the steeper the initial slope of the gradient necessary for separation), and a concave gradient affords the best separation of late-eluting components (when compared to a linear gradient with the same initial slope). The effects of several gradients on the chromatographic profiles for the test compounds and three unidentified impurities is shown in Fig. 3, and the conditions for the gradients listed in Table I. Our ultimate goal is to determine band velocities for the components in a mixture using isocratic conditions, then develop computational procedures to determine the optimum gradient for the separation of the components using that information. An initial attempt at creating pH gradients with this apparatus is also demonstrated in the next section.

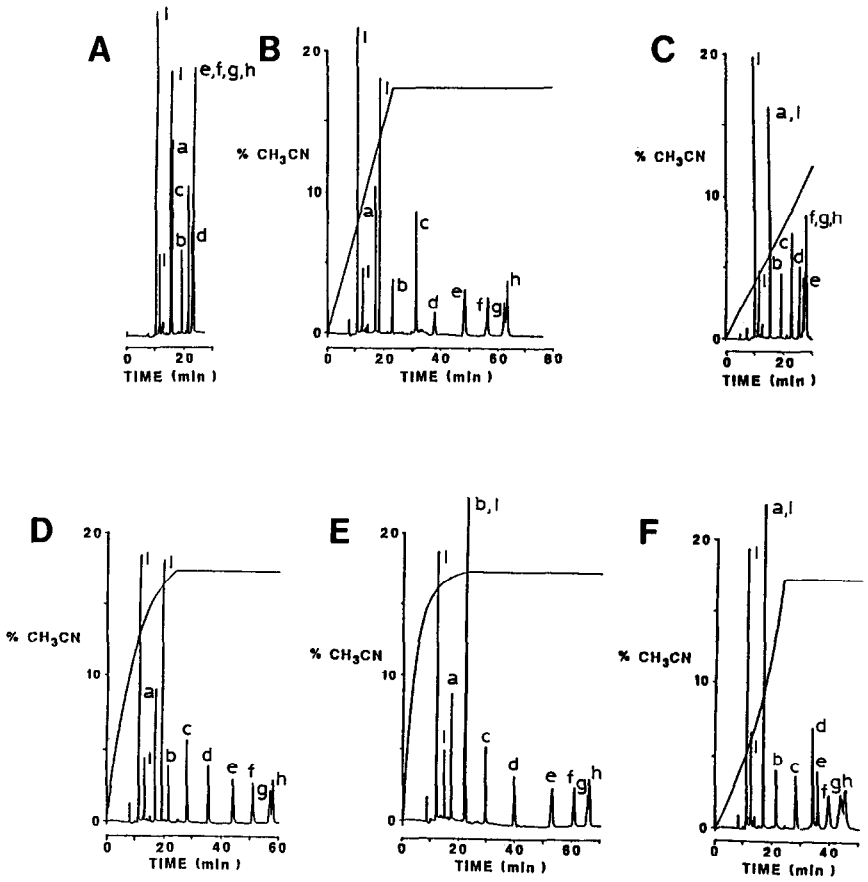


Fig. 3. Separation of NBD-derivatized (a) *n*-propylamine, (b) *n*-butylamine, (c) *n*-pentylamine, (d) *n*-hexylamine, (e) *n*-heptylamine, (f) *n*-octylamine, (g) *n*-decylamine, (h) *n*-dodecylamine and three impurities (I) using a mobile phase consisting of 0.01 *M* $Na_2HPO_4$, 0.006 *M* $Na_2B_4O_7$, 0.05 *M* SDS with (A) no organic solvent; (B) a linear gradient; (C) a less steep linear gradient; (D) a moderately convex gradient; (E) a more extreme convex gradient; and (F) a concave gradient.

TABLE I

PARAMETERS FOR THE SOLVENT GRADIENTS DEMONSTRATED IN FIG. 1

| *Gradient* | *V° (ml)* | *$R_{in}$ (ml/min)* | *$R_{out}$ (ml/min)* | *Duration (min)* | *Final %* |
|---|---|---|---|---|---|
| B | 6.0 | 0.22 | 0.44 | 23 | 17.5 |
| C | 10.0 | 0.20 | 0.40 | 30 | 12.0 |
| D | 4.0 | 0.38 | 0.35 | 23 | 17.5 |
| E | 1.0 | 0.25 | 0.23 | 23 | 17.5 |
| F | 9.0 | 0.10 | 0.45 | 23 | 17.5 |

*Optical detection using laser-etched, on-column flow cell*

Detection for electrokinetic capillary separations, as well as other microscale separation techniques, remains a formidable challenge. Commercial optical detectors that are employed extensively in liquid chromatography can not be used directly. In general, the very small solute band volumes that are involved necessitate that optical detection be performed on-column. In one exception, a post-column mixing chamber permitted off-column detection based on fluorescence[24]. This is possible when fluorescence detection is performed, particularly laser-excited fluorescence, because the high sensitivity of the technique[20,22] can compensate for the dilution that occurs with post-column mixing. However, absorbance-based detection, which is much more versatile, does not exhibit the sensitivity required for that approach.

On-column absorbance detection is usually performed by passing light through the column along its diameter using small pinholes or slits to restrict light to the flow channel. Thus, optical path lengths are very short and sensitivity suffers. Moreover, the fact that the flow channel of the column is typically only 50 $\mu$m in diameter results in significant problems with throughput and stray light. Low throughput often produces a noisy, drifting baseline and excessive stray light can distort spectral bands and result in nonlinear calibration plots at the high concentration end. The optical focusing effect of the capillary does provide some compensation for these problems[25].

Many commercially-available absorbance detectors can be adapted for use with electrokinetic capillary separation techniques, provided on-column flow cells can be produced and conveniently mated with the instrument. We report herein (see Experimental) the preparation of a laser-etched, on-column flow cell and procedures for adapting it for use with an inexpensive UV absorbance LC detector (LDC UV III monitor) and a photodiode array detector (Spex/Tractor system). Although it requires some practice to develop the proper technique for fabricating the flow cells, once acquired, several flow cells can be prepared in a couple of hours. Because the slit is an integral part of the flow cell there is virtually no optical alignment involved in its use (we routinely switch flow cells between detectors). By adjusting fabrication parameters such as laser power, it is possible to create slits that eliminate stray light entirely or compromise some stray light in favor of greater throughput. We generally find it beneficial to isolate the detector from the large electric field applied across the column using fiber optics or a lens imaging assembly (see Fig. 1).

Employing the LDC detector, calibration plots for solutions of isoquinoline ($\varepsilon = 1.8 \cdot 10^4$, $\lambda = 229$ nm) were obtained using laser-etched, on-column flow cells exhibiting low, $<4\%$ stray light (the minimum amount of stray light we can measure with the LDC detector by the procedure given earlier), and moderate (7%) stray light. The 7% stray light flow cell was also used with the photodiode array detector, under optimized conditions (see below), to obtain a calibration plot for sodium fluorescein ($\varepsilon = 2.4 \cdot 10^4$, $\lambda = 490$ nm) in the visible spectral region where the array has good response characteristics. In all cases a linear response, with a correlation coefficient greater than 0.999, was observed over three decades in concentration. The slopes of the isoquinoline calibration plots, expressed as change in signal (mV) per change in molar concentration, were 2.16 and 1.23 for the low- and moderate-stray light flow cells, respectively. The limits of detection (signal-to-noise ratio (S/N) = 2) for the isoquinoline were $1.6 \cdot 10^{-5}$ *M* and $8.0 \cdot 10^{-6}$ *M* for the low- and moderate-stray light flow cells, respectively. The lower detectability for the moderate-stray light flow cell is

due to a slightly smaller baseline level (assuming an effective pathlength of 44 $\mu$m for the 50 $\mu$m I.D. column[6] the baseline noise was $2 \cdot 10^{-4}$ absorbance units). The greater sensitivity for the low-stray light flow cell, as indicated by the larger calibration plot slope, can probably be attributed to a longer effective path length. Although these measurements were made by hydrostatically pumping the test solutions into the column, we do not observe any change in baseline noise with our system when electric fields are applied.

In addition to providing an added dimension of spectral selectivity[20], multichannel detection permits substantial flexibility in the acquisition of absorbance data. For example, with the photodiode array employed in this work it is possible to sum the acquired signal intensities over a preset number of diodes (this is, in effect, adjusting the spectral bandpass for data acquisition) then calculate absorbance from the summed intensities. As expected, increasing the bandpass around band center decreases absorbance as diodes that probe spectral regions with smaller molar absorptivities than at band center are utilized. However, by including more diodes in the calculation the observed noise also decreases. Fig. 4 is a plot of S/N for a sodium fluorescein solution *versus* bandpass which illustrates that these effects produce an optimum bandpass in terms of detectability (20 nm centered at 490 nm in this case). Since detectability is often marginal when absorbance detection is employed in CZE and MECC, this capability can be important. The baseline noise using the optimum bandpass was $3 \cdot 10^{-4}$ absorbance units and, as the linearity of the aforementioned sodium fluorescein calibration plot indicates, there was no apparent failure of Beer's Law due to the use of polychromatic light.

It has been suggested that in certain situations adjusting pH during CZE separations could maximize solute mobility differences and thereby improve their separation[26]. The results of a preliminary experiment exploring the utility of the

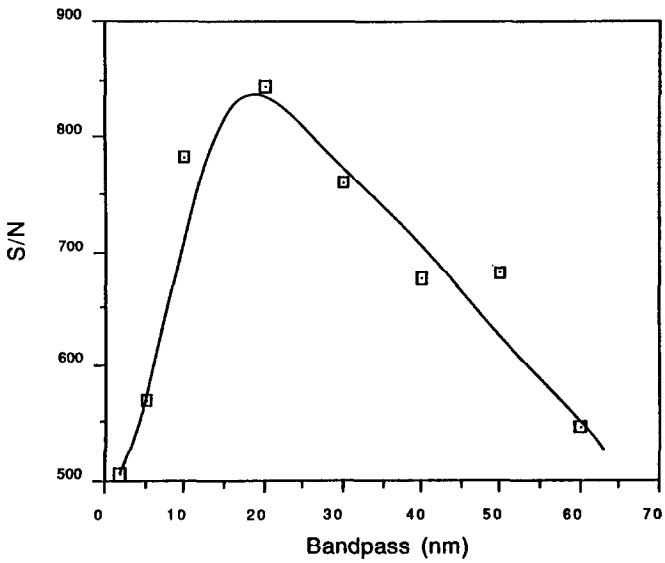


Fig. 4. Plot of S/N *versus* effective bandpass for a $10^{-3}$ *M* solution of sodium fluorescein.

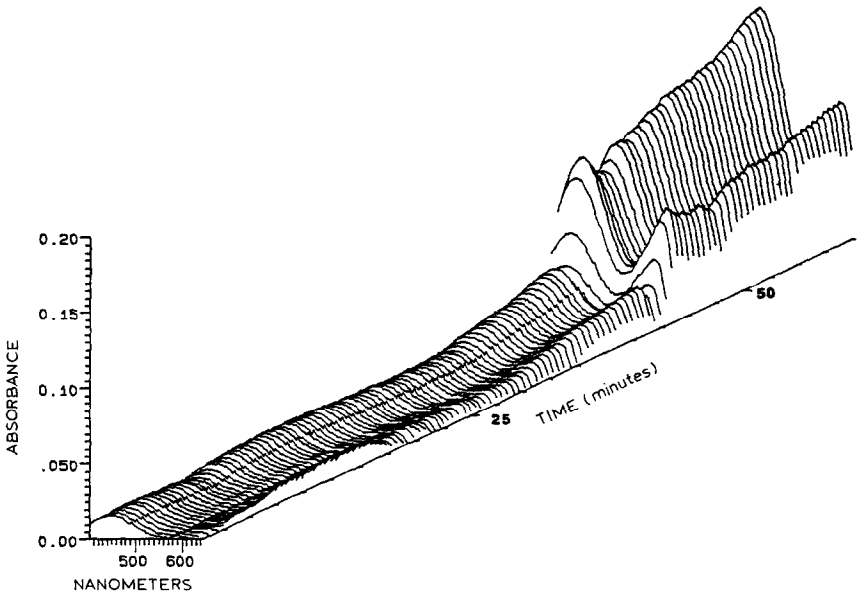


Fig. 5. Absorbance spectra of bromocresol green ($pK_a = 4.7$; $\lambda_{max}$ acid-form 444 nm; $\lambda_{max}$ base-form 617 nm) acquired with laser-etched, on-column flow cell/photodiode array detector during a pH gradient generated using the solvent delivery system and conditions described in the text.

previously described solvent delivery system for generating pH gradients for CZE and CIF is presented in Fig. 5. The capillary column and reservoirs were filled with a solution containing citric acid and bromocresol green indicator. The solvent delivery system was used to titrate the citric acid with sodium hydroxide to effect a 2.5–6.0 change in pH in the inlet reservoir over a period of 10 min. Because electroosmotic flow is very slow at low pH values, hydrostatic flow (the inlet reservoir was raised above the outlet) was used in conjunction with an applied voltage of 15 kV to drive the mobile phase through the column. At about 30 min spectral changes in the bromocresol green were observed, indicating that pH was changing. No attempt was made to calibrate the pH change in this experiment (obviously electrophoretic mobility of the buffer components will influence the pH gradient in the column) since, in so far as this report is concerned, the purpose of the experiment was to demonstrate the spectral acquisition capabilities of the flow cell/photodiode array detection system. Spectra were obtained with good S/N at a rate appropriate for electrokinetic capillary separations.

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