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Instrumental Development of Novel Detection and Separation Methods for
Capillary Electrophoresis

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Instrumental development of novel detection
and separation method for capillary electrophoresis

Tommy Wayne Garner

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In Section I the use of indirect fluorescence detection (IFD) is extended to the visible region. Indirect fluorescence detection is shown to be a universal and sensitive detection technique for CZE. Detection is based on charge displacement and is not based upon any absorption or emission properties of the analyte, therefore the need for chemical derivitization is eliminated. CZE-IFD can separate and detect almost any compound that contains a charge. This was demonstrated by the separation and detection of a mixture of sugars which are only weakly acidic. The mass detection limit of fructose was 2 fmol using a 5 μ m diameter capillary. This peak had an efficiency of more than 600,000 theoretical plates.

In Section II a novel detection system for CZE is explored. Normally, only highly fluorescent materials can be detected at low concentrations in the small detection volumes typical of capillary electrophoresis in a laser-based fluorometer. We report here the detection of absorbing but non-fluorescing analytes by laser excited fluorescence. This

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An Abstract of
A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
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DOCTOR OF PHILOSOPHY

Approved:

In Charge of Major Work

For the Major Department

For the Graduate College

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is possible if the excited analytes transfer their energy to a fluorescent additive in the running buffer to increase the background fluorescence level. Two different fluorophores and four different absorbing analytes were tested in this detection scheme. Concentrations as low as 1.5×10^{-8} M and amounts as small as 9 amol at injection are detectable. The data support a long range energy transfer scheme, but the transfer efficiency is much larger than those reported for other donor-acceptor pairs.

Section III describes a method for the CZE separation of compounds with similar mobilities. Increased selectivity is shown for compounds of this type when other modes of separation are added to the system. A capillary coated with a hydrophobic stationary phase is shown to be a dynamic ion exchanger when a quaternary ammonium compound is added to the running buffer. Compounds are shown to have a decrease in retention when the concentration of the running buffer is increased. The effect of adding an organic modifier and the effect on the concentration of the surface active reagent is also studied.

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GENERAL INTRODUCTION

Capillary electrophoresis (CE) is a family of related techniques that employ small diameter capillaries (5-200 μm i.d.) to produce highly efficient separations of large and small molecules. A high voltage power supply is connected across the capillary which produces electroosmotic flow and analyte migration. This simple instrumentation allows unique modes of liquid chromatography and electrophoresis separations. The most common mode of CE, Capillary zone electrophoresis (CZE), separates compounds based on their differential migration rates in an applied electric field.

The attractiveness of this technique comes from many factors. The efficiency of CZE separations often approaches 1 million theoretical plates with examples close to 2 million. A typical injection in CE is on the order of nanoliters, therefore, CE consumes only small amounts of sample. CE is also applicable to a wide range of analytes, from small inorganic or organic molecules to large biopolymers such as proteins and DNA. CE is also very fast, with typical run times measured in minutes.

This is all accomplished in a relatively simple instrumental format. The basic CZE instrument consists of a fused silica capillary, a high voltage power supply, two electrodes and two buffer reservoirs. A small section of the polyimide coating is removed from the capillary and aligned in

the detector to facilitate on-column detection. The basic instrumental configuration is shown in Figure 1.

The signal from the detector is collected either on a chart recorder or computer data acquisition system and is called an electropherogram, which is analogous to a chromatogram for an HPLC system. This simple system is easily automated. Most commercial instruments available today are under complete computer control.

Theory of Capillary Electrophoresis

Electroosmosis

When a voltage is applied to a capillary tube filled with a buffer a flow develops, called electroosmotic flow. The origin of this flow is often explained using the Stern-Gouy-Chapman model of the electrical double layer at a charged solid-liquid interface. At the solid-liquid interface of the capillary ions are not oriented as in the bulk of the solution. The capillary surface most commonly contains a negative charge due to ionized silanol groups. Immobilized counter charges to this are cations in adjacent layer often called the Stern compact layer. The counterions of these functional groups also extend into the diffuse Gouy layer. When a potential is applied across this capillary the hydrated cations in the diffuse layer, which are free to move, migrate towards the negative electrode. A plane of shear develops

Figure 1. Basic configuration of a capillary electrophoresis system.

between the bound solvent molecules on the surface of the capillary and the mobile solvent.

A graphical representation of this model is shown in Figure 2. Here, ψ_0 , represents the potential between the liquid and the capillary wall. As we move from the surface to the bulk of solution ψ decreases, linearly between the surface to the edge of the compact layer and exponential from the compact layer into the bulk of solution. The potential at the plane of shear is termed the zeta potential, ζ , and is given by Equation 1.

$$\zeta = \frac{4 \pi \eta \mu_{eo}}{\epsilon} \quad (1)$$

where η is the viscosity, μ_{eo} is the coefficient of electroosmotic flow and ϵ is the dielectric constant. The migration of these solvated cations towards the cathode is the driving force behind electroosmotic flow. The fact that flow originates from the capillary wall offers advantages over laminar flow and will be discussed later.

The linear velocity of electroosmotic flow, u , is given by Equation 2.

$$u = \frac{\epsilon \zeta}{4 \pi \eta} E \quad (2)$$

Figure 2. Potentials and relative ion positions in the double layer.

where E is the electric field strength (volts/cm). The magnitude of the zeta potential, and thus the electroosmotic flow, depends on, among other things, the nature of the solid surface, the concentration of ions and the pH of the electrolyte.

The electroosmotic flow velocity can be quite robust, with a linear velocity of around 1 mm/s for a pH 7.0, 25 mM sodium phosphate buffer under a field strength of 350 V/cm (1). As the pH of the buffer is lowered u decreases due to the decrease in zeta potential. The control of electroosmotic flow has been shown to be an important method to alter the selectivity in CE.

Efficiency and Resolution

In CZE efficiencies over 100,000 plates are routine. The two most important reasons for this result from the flat flow profile of electroosmotic flow and the lack of a stationary phase. In a pressure driven system the flow profile can be described by a laminar or parabolic flow profile. The flow in this system is highest in the center of the tube and approaches zero at the surface capillary. This gradient flow profile results in band broadening.

In an electrically driven system the flow originates from very near the wall of the capillary. This results in a flat flow profile that contributes very little to broadening. The

comparison of electroosmotic flow and hydrodynamic flow profiles is given in Figure 3.

To understand how broadening occurs in CZE we can start off with some basic operating principles. The velocity of a compound in a CE system with electroosmotic flow is given by Equation 3.

$$v_{eo} = (\mu_e + \mu_{eo}) \frac{V}{L} \quad (3)$$

where μ_e is the compound's electrophoretic mobility, v is the applied voltage and L is the capillary length. The time it would take this compound to migrate the length of the capillary is given by Equation 4.

$$t_m = \frac{L^2}{(\mu_e + \mu_{eo}) V} \quad (4)$$

The major contribution to band broadening in CZE is molecular diffusion. The amount of peak dispersion caused by diffusion is proportional to the amount of time the compound spends in the capillary and is given by Equation 5.

$$\sigma^2 = 2 D_m t_m = \frac{2 D_m L^2}{(\mu_e + \mu_{eo}) V} \quad (5)$$

Figure 3. Cross-Sectional flow profiles for electroosmotic and hydrodynamic flow.

**Cross-Sectional Flow Profile
Due to Electroosmotic Flow**

**Cross-Sectional Flow Profile
Due to Hydrodynamic Flow**

where σ^2 is the peak dispersion and D is the species diffusion coefficient. The expected number of theoretical plates is then given by Equation 6.

$$N = \frac{L^2}{\sigma^2} = \frac{(\mu_e + \mu_{eo})V}{2 D_m} \quad (6)$$

As can be seen from this result small diffusion coefficients and large applied potentials are favorable for efficient separation. There is, however, no dependence on the length of the column. Analysis times can be reduced, therefore, by decreasing the length of the column without degrading efficiency. There are practical limits to column length and applied voltage, however, due to the effect of Joule heating. The result for the number of theoretical plates shows that compounds with small diffusion coefficients, such as biopolymers, are favorable.

As an example of the high efficiencies possible in CZE one can do a calculation from tabulated results using Equation 6 (2). The mobility of sulfate is $8 \times 10^{-4} \text{ cm}^2/\text{V s}$ with a typical diffusion coefficient for a small molecule of about $10^{-5} \text{ cm}^2/\text{s}$. A typical electroosmotic flow is about $3 \times 10^{-4} \text{ cm}^2/\text{V s}$ but will be in the opposite direction of an anion. With a separation potential of 30,000 volts this calculates to be about 750,000 plates. Large molecules are expected to be

as good with the lower mobilities of these compounds offset by a smaller diffusion coefficient.

The resolution of two components in CZE is proportional to their difference in migration velocities and is given by Equation 7.

$$R = \frac{\sqrt{N}}{4} \left(\frac{\Delta v}{\bar{v}} \right) \quad (7)$$

where N is the average number of theoretical plates, Δv is the difference in migration velocities and $\bar{v}$ is the average velocity. Substituting Equations 3 and 6 into 7 and rearranging gives Equation 8

$$R = \frac{1}{4\sqrt{2}} (\mu_{e2} + \mu_{e1}) \sqrt{\frac{V}{D_m(\mu_e + \mu_{eo})}} \quad (8)$$

where μ_{e1} , and μ_{e2} are electrophoretic mobilities of species 1 and 2 respectively. As can be seen from this equation high voltages and low electroosmotic flow enhance the resolution for CZE. Differentially changing the mobilities of each species is a common way to enhance resolution for CZE and can be accomplished by adjusting the pH and through the addition of complexing agents or other additives.

Micellar Electrokinetic Chromatography

CZE is an excellent technique for the separation of ionic molecules that migrate through the capillary at different rates. Neutral molecules migrate with the electroosmotic flow and coelute, however. Tarabe et. al.(3) has extended the use of CE to neutral compounds by addition of micelles to the mobile phase. This method is termed micellar eletrokinetic chromatography(MECC).

When micelles are added to a CZE buffer they transform the system from a single phase to two phases. Although not stationary, molecules can partition in and out of the micellar phase. A commonly used surfactant for these applications is sodium dodecyl sulfate (SDS). Under the conditions used for MECC the micelles have migrations that are opposite in direction to that of the electroosmotic flow. The magnitude of this migration is smaller than that of the electroosmotic flow so that the micelles are carried along in the direction of the electroosmotic flow. As compounds have varying affinities for the micellar phase, their migration is impeded depending upon the magnitude of this attraction.

MECC has a limited "window" of elution times (4,5) with all compounds eluting between a totally unretained neutral compound and a totally retained compound that elutes with the same retention time as a micelle. It has also been found that a micelle system can add selectivity when compounds of different charges need to be resolved (6). Micelle systems

have also been shown to be useful in the separation of chiral compounds (7).

Capillary Gel Electrophoresis

Gels have been used on flat plates for electrophoresis as a means of reducing convection. The addition of gels to a CE column also reduces convection while introducing a unique mode of selectivity. The nature of gels produces a sieving effect in which large molecules will migrate slower the smaller ones. Proteins can be separated based on their size by SDS polyacrylamide (SDS-PAGE) gel electrophoresis. It has also been demonstrated that this method works in capillaries filled with polyacrylamide gel. A constant amount of SDS binds to the denatured proteins to form complexes with similar mass to charge ratios. The linear relationship between the log of MW and mobility allows the determination of molecular weights.

The electrophoretic mobility of double stranded DNA is not a strong function of size. DNA can be separated by the use of cross-linked gels which alter the frictional characteristics of DNA such a way as to show a molecular weight dependance (8). Capillaries have been used for single base resolution of synthetic nucleotides and for the separation of DNA restriction fragments (9,10). The high resolution achievable by slab gel electrophoresis is also present in capillary gel electrophoresis with the added advantages of short analysis times, minimal sample

requirements, real-time on-column detection and the ability of automation.

Detection

Perhaps the most demanding aspect of CE is detection. The most common detector for CE is UV absorption. The major advantage of this technique is its universal nature. Most commercial instruments available today rely on this mode of detection. The difficulties with this mode of detection, however, become apparent when one considers the amount of sample injected in HPCE. A common injection volume for CE is on the order of 500 pL for a 20 μm i.d. column, for the injection of a 10^{-4} M solution this would translate to 52 fmol (52×10^{-15} mol) of the species injected onto the column.

Two other commonly used modes of detection for CE are laser induced fluorescence (LIF) and amperometric detection. As a consequence of their light amplification properties, lasers produce spatially narrow, extremely intense beams of radiation. These properties allow the laser beam to be focused inside the small i.d. capillary columns and allow on-column detection. The image of the detection region is focused on to a photomultiplier tube after the fluorescence is isolated from the scatter of the excitation wavelength by emission and spatial filtering. LIF has been used to detect amounts of injected compounds to subattomole levels.

Ewing and coworkers have demonstrated that extremely sensitive amperometric measurements can be made in CZE. Basically, a small break is made in the capillary a few centimeters from the end of the capillary which is used to shunt the current from the CE separation system. A carbon fiber is inserted into the end of the capillary and the reference electrode is placed in the buffer at the end of the capillary. Detection limits in the subattomole levels were reported for indoles and catecholamines.

Detection limits for these methods are quite impressive for selected compounds. Unfortunately, the most sensitive detectors are also the most selective. For compounds that are not fluorescent or electrochemically active, these methods show no considerable sensitivity without derivitization.

Another mode of detection that requires only that the compound carry a charge is indirect fluorescence. In this detection scheme a compound that fluoresces is added to the buffer. This causes a continuously high signal at the detector. When compounds with the same charge as the fluorescent additive are injected onto the column, they displace the fluorophore causing a reduction in signal. As discussed before, in CZE a buffer is used to provide even conductance across the capillary. Because this buffer is basically inert, it should be of little consequence if we replaced it with a ionic fluorescent compound. This is in

fact true and indirect fluorescence detection has been shown to be a very good detection mode for CZE.

An Explanation of Dissertation Organization

This general introduction precedes three research papers which have been published in major analytical chemistry journals. The papers are followed by a general summary section. A references section following the general summary includes references for the general introduction and general summary sections. References for each of the three papers follow the paper in which they are cited.

**PAPER 1. INDIRECT FLUORESCENCE DETECTION OF SUGARS SEPARATED
BY CAPILLARY ZONE ELECTROPHORESIS WITH VISIBLE LASER
EXCITATION.**

INDIRECT FLUORESCENCE DETECTION OF SUGARS SEPARATED
BY CAPILLARY ZONE ELECTROPHORESIS WITH
VISIBLE LASER EXCITATION

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SUMMARY

This work extends the use of indirect fluorescence detection for capillary zone electrophoresis to the visible region. Detection is based on charge displacement and is not based upon any absorption or emission properties of the analyte, therefore the need for chemical derivatization is eliminated. Capillary zone electrophoresis-indirect fluorescence detection can separate and detect almost any compound that contains a charge. This was demonstrated by the separation and detection of a mixture of sugars which are only weakly acidic. The mass detection limit of fructose was 2 fmol using a 5- μ m diameter capillary. This peak had an efficiency of more than 600,000 theoretical plates.

INTRODUCTION

Capillary zone electrophoresis (CZE) is a separation technique that separates ionic compounds based on their differential migration rates in an electric field (1). CZE is known for its high separation efficiencies that can be more than 10^5 theoretical plates (2). The separation in CZE takes place in small capillaries with internal diameters often less than 50 μm . Detection of the pL volumes injected in this technique is a challenging problem. Fluorescence and electrochemical detection have obtained excellent results, but are limited to compounds that have the appropriate physical properties (3,4). These physical properties can be obtained by chemical derivitization, but derivitization is time consuming and inefficient. There exists a need for an all purpose detector for CZE.

Indirect fluorescence detection for capillary zone electrophoresis (CZE-IFD) has been applied successfully to many ionic compounds including amino acids (5), proteins, nucleotides (6), tryptic digests (7) and other ionic organic and inorganic compounds (8). Indirect detection is a universal detection scheme that can detect a wide variety of compounds without the need for chemical derivitization. CZE-IFD is also very sensitive with limits of detection (LOD) in the attomole range. Previously, CZE-IFD has been done in the UV region using salicylate as the indirect fluorophore.

Fluorophores that fit lasers in the visible region will aid detection by allowing the use of visible optics and more powerful visible light sources.

In CZE a buffer is necessary to establish the potential gradient across the capillary tube. This buffer, which is usually inert, can be selected to optimize detection. In indirect fluorescence detection a fluorescing buffer is chosen. This gives a continuously high signal at the detector. When a charged analyte molecule reaches the detector region it displaces the fluorescing buffer ions to maintain charge neutrality. Even though the analyte does not absorb or fluoresce it will give a signal by this displacement mechanism.

Sugars present especially challenging separation and detection problems in CZE. Sugars are only weakly acidic. They must be made very basic before any ionization will occur, as an example the pK for glucose is 12.35 (9). The sugars can then be separated by their differences in migration velocity. Sugars also show little if any ultraviolet-visible absorption or fluorescence activity. This problem is even more challenging due to the small volumes of analytes injected in CZE.

In this work we will introduce two new fluorophores that can be used for CZE-IFD. Fluorescein is a highly efficient fluorophore that matches very well with the argon ion 488 nm line. Coumarin 343 works very well with the 442 nm line of a

helium-cadmium laser. The separation of mixtures of amino acids, peptides and sugars will demonstrate the utility and sensitivity of visible indirect fluorescence detection.

EXPERIMENTAL

The CZE system is similar to that described previously (5). A high voltage power supply (Spellman, Plainview, NY; Model UHR50PN50 or Glassman, Whitehouse Station, NJ; Model MJ30P0400-11) was used to supply the electromotive force across the capillary. The anodic high voltage end of the capillary was isolated in a plexiglass box for operator safety while the cathodic end was held at ground potential.

Capillary columns of various lengths and diameters were used. Lengths ranged from 80 to 100 cm. The outer diameters of the columns were all 150 μm , while the inner diameters ranged from 5 to 22 μm . The polymer coating was burned off 10 cm from the cathodic end of the capillary to form the observation region.

A helium-cadmium laser (Linconix, Sunnyvale, CA; Model 4240NB) operating at 442 nm or an argon ion laser (Control Laser Corp., Orlando, CA; Model 554A) operating at 488 nm was used for excitation. The laser beams were stabilized within 0.04% with a laser power stabilizer (Cambridge Research Institute, Cambridge, MA; Model LS100). The laser was focused onto the capillary with a 1 or 0.5 cm focal length lens (0.5 cm for 5 μm capillary). The capillary was mounted at Brewster's angle to reduce scattered radiation.

The fluorescence was collected at 90 degrees with a 20X microscope objective. The fluorescent image was focused onto a PMT (Hamamatsu, Middlesex, NJ; Model R928). Stray and

scattered radiation were rejected by two spatial filters at either end of a blackened tube preceding the PMT. The fluorescence was further isolated with two color filters (Corning Glass, Corning, NY; Model 3-71 and Schott Glass, USA; Model GG475).

The PMT current was monitored with a picoammeter (Keithly, Cleveland, OH; Model 417). The output from the picoammeter was recorded on a chart recorder (Fisher series 5000) or on a personal computer after analog-to-digital conversion (Data Translations, Marlborough, MA; Model DT 2825 or DT 2827). Digital data was smoothed with a smoothing routine based on the Savitsky Golay smoothing algorithm. A 1 s time constant was added after the picoammeter.

The PMT voltage was adjusted to maintain a 1 μ A background current. Injections were made for 1 s at 30 kV and the voltage was held at 30 kV throughout each run.

Reagents

All chemicals were reagent grade unless otherwise noted. Fluorescein (Molecular Probes) and coumarin 343 (Eastman Kodak Company, Rochester, NY; laser grade) were prepared in deionized water (Millipore Corp., Bedford, MA; Milli-Q System) and the pH was adjusted with sodium hydroxide. Buffers were purged with nitrogen to remove carbon dioxide. The pH was checked and adjusted if necessary every three hours. All quantitative data were obtained with freshly prepared buffer

solutions. The tryptic digests were prepared by the standard procedures with modifications (6). All analytes were diluted in buffer before injection.

RESULTS AND DISCUSSION

There are several factors involved in the selection of a fluorophore used for CZE-IFD. First, the molecule must have a high molar absorptivity for the wavelengths available. Second, it must have a high fluorescence quantum efficiency, that is, a high value for the ratio of fluorescence photons emitted to photons absorbed. Third, it must be compatible with the solvent system used. Will the molecule dissolve to the levels needed? The molecule must also be charged, preferably a charge of one. This will ensure a value as close to unity as possible for the transfer ratio, TR. TR is defined as the number of fluorophore molecules displaced by one analyte molecule. The molecule must also must be well behaved in the system. Some molecules may adsorb to the column walls causing a nonequilibrium state to exist.

The first fluorophore studied in this work was disodium fluorescein, and is demonstrated by the separation of two amino acids shown in Figure 1. THAM was added to the buffer to increase the buffer capacity. Separation efficiencies were very high for this system with plate numbers for each amino acid exceeding 100,000. Although this system was not optimized for the best LOD high absolute sensitivities were obtained with a LOD for each amino acid of 2 fmol. Lower detection limits should be obtainable by lowering the concentration of buffer (6). As can be seen from these results, fluorescein worked well at pH 7. When the pH was

Figure 1. Indirect detection of glutamic and aspartic acid.
Buffer is 100 μ M disodium fluorescein and 50 μ M
THAM at pH 7.0. Each amino acid was injected at a
concentration of 20 μ M onto a 80 cm column (15 μ m
I.D., 150 μ m O.D.).

increased to 9, however, the baseline became very erratic. This was believed to be caused by the interaction with fluorescein with the wall of the capillary.

The next fluorophore studied in this work was coumarin 343. Coumarin 343 was selected because of its good solubility in water, high molar absorptivity and high fluorescence yield. Coumarin 343 has a molar absorptivity of 2×10^4 at 442 nm which matches very well with the 442 line of a helium-cadmium laser. The high fluorescence yield was evaluated experimentally by injecting coumarin 343 in the direct mode. The buffer used was 10 mM sodium bicarbonate at pH 10.7. The detection limit was 8×10^{-10} M (column 80 cm, 5 μ m diameter, 1 s injection at 30 kV, 30 kV separation).

The use of Coumarin 343 is demonstrated with the separation of a complex mixture of amino acids shown in Figure 2. The elution order agrees with previous work (5). The broad peak at 5.5 minutes is an unknown impurity. The separation efficiency is very good here also with an average plate number of 270,000.

The separation of an even more complicated mixture is demonstrated by the separation of the tryptic digests of β -casein and bovine serum albumin (BSA) shown in Figures 3 and 4. The samples were the same ones prepared for another experiment and are repeated here for comparison (6). Each digest mixture was diluted in the operating buffer one part in

Figure 2. Indirect detection of an amino acid mixture using coumarion 343 as the fluorophore. The buffer used was 1 mM coumarin 343 at pH 9.5. A 1 s, 30-KV injection of 75 μ M each was followed by electrophoresis at 30-KV on an 80 cm (18 μ m I.D., 150 μ m O.D.) column.

Figure 3. Indirect detection of the tryptic digest of β -casein. The buffer used was 1 mM coumarin 343 at pH 10. The column dimensions were 80 cm (22 μ m I.D., 150 μ m O.D.). Data were taken digitally at 7.5 Hz.

Figure 4. Indirect detection of the tryptic digest of bovine serum albumin (BSA).
Conditions are the same as in Figure 3.

ten. Peptides are expected to be almost fully charged at pH 10 so this was the pH selected for these separations.

There were 18 peaks detected for the BSA digest and 15 peaks detected for the β -casein digest. This agrees quite well with previous work. It is noteworthy that this system was not optimized for separation or detection. The total set of experiments for the tryptic digests was completed in less than three hours. This demonstrates that indirect detection for CZE is very easy to implement and can obtain highly efficient separations with little preparation time.

Sugars have been separated by CZE as their borate complexes (10). It is shown here that sugars can be separated and detected without any prior derivitization. CZE separates compounds based on their mobility that is related to the pK values at any given pH. Sugars have very high pK values starting at the low end of a pH around 12.

In indirect detection the sensitivity depends on the fraction of the analyte that is ionized. This means when using indirect detection for the detection of sugars the pH of the running buffer must be approaching 12 to have any of the sugars in an ionized form. This presents another problem. When the pH of the buffer solution gets this high the concentration of hydroxide ion is no longer negligible relative to the concentration of the indirect fluorophore. This results in the decrease of TR. These effects must be balanced to obtain the best sensitivity possible for the

system. In our system the mode of displacement is by charge neutrality. The fluorophore molecule would have a charge of -1, from the ionization of its carboxylic acid group, while the sugar would have an average charge somewhere between 0 and -1 depending on the pH. This means that the best possible value of TR will be less than unity. This has the effect of decreasing the sensitivity of the system.

The effect of the hydroxide can be qualitatively described by Equation 1.

$$TR_{tot} = \frac{\alpha * [sug]}{[FL] + [OH-]} \quad (1)$$

In this equation TR_{tot} is the total transfer ratio. This includes the amount of fluorophore, [FL], and the amount of hydroxide ion, [OH-], displaced by the sugar molecules that are ionized. It can be seen that at constant fluorophore and sugar concentrations the numerator and the denominator are competing functions of pH. TR_{tot} goes through a maximum when it is plotted as a function of pH. The pH at this maximum is the most sensitive pH for detection.

The separation of a sugar mixture is shown in Figure 5. The optimum detection pH was found to be 11.65 based on tabulated literature values of the pk for each sugar and Equation 1 (8). A pH of 11.5 was used for detection, however, because the degradation of fluorophore was decreased without any appreciable decrease of detection efficiency. Table I is

Figure 5. Separation of a sugar mixture. Indirect detection of 640 fmol of each sugar in 1 mM coumarin at pH 11.5. The column was 90 cm (18 μ m I.D., 150 μ m O.D.).

a compilation of the sugars we detected using CZE-IFD and their corresponding migration times. Each of these sugars was injected at a concentration of 1 mM. This is compared to a previous report where the N-2-pyridylglycamine derivatives of sugars are injected in the 10-100 mM range and detected by UV absorbance (10).

In the analysis of biological compounds there is often a limited supply of sample, such as in the analysis of single cells (11). The analysis of such small volumes of analytes requires the use of smaller bore capillaries in the CZE system. The use of these smaller bore capillaries, however, requires the absolute detection limit of the detection method to decrease as the dimensions of the capillary decrease. The detection scheme should also be universal to detect the large number of biological compounds that lack analytically useful physical properties. CZE-IFD fulfills the requirements that are demanded by smaller capillaries. This was demonstrated in our lab by the detection of fructose after it was injected into a 5 μ m diameter capillary. The absolute limit of detection in this system was 2 fmol based on a S/N_{rms} of 3. The high separation efficiency offered by this system (over 600,000 theoretical plates) is partially responsible for the low detection limit obtained.

The detection limit of fructose was believed to be limited by the mechanical vibration of the system. This is

Table I. Sugars Detected by Indirect Fluorescence
Detection

Sugar	Migration time (min)
Raffinose	4.98
Sucrose	4.99
Maltose	5.35
Arabionose	5.47
Glucose	5.50
Lactose	5.61
Xylose	5.62
Fructose	5.66

because the capillary diameters are approaching the diameter of the focused laser beam (12). Small fluctuations in the capillary relative to the beam will cause large fluctuations in the signal. This can be partially compensated for by slightly defocusing the beam by moving the capillary away from the beam focusing lens. This problem will have to be studied further as capillary dimensions decrease.

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**PAPER 2. ABSORPTION DETECTION IN CAPILLARY ELECTROPHORESIS BY
 FLUORESCENCE ENERGY TRANSFER**

ABSORPTION DETECTION IN CAPILLARY ELECTROPHORESIS
BY FLUORESCENCE ENERGY TRANSFER

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ABSTRACT

Normally, only highly fluorescent materials can be detected at low concentrations in the small detection volumes typical of capillary electrophoresis in a laser-based fluorometer. We report here the detection of absorbing but nonfluorescing analytes by laser-excited fluorescence. This is possible if the excited analytes transfer their energy to a fluorescent additive in the running buffer to increase the background fluorescence level. Two different fluorophores and four different absorbing analytes were tested in this detection scheme. Concentrations as low as 6×10^{-9} M and amounts as small as 4 amol at injection are detectable. The data support a long-range energy-transfer scheme, but the transfer efficiency is much larger than those reported for other donor-acceptor pairs.

INTRODUCTION

Recent research efforts in microcolumn separations, particularly in capillary electrophoresis (CE) (1,2) and in open tubular capillary liquid chromatography (OTCLC) (3-8), have shown that very high separation efficiencies can be obtained. It appears that 2-10 μm i.d. open tubes provide the best performance. In CE, these small diameters restrict the current flow and thus reduce the amount of band broadening induced by joule heating. In OTCLC, small diameters are needed to maximize the interaction with the stationary phase. On the other hand, the small elution volumes and the short optical path lengths available across these columns put severe restrictions on the selection of detection schemes for the analytes.

The easiest way to detect analytes in capillary columns is by UV absorption (9-12). At 210 nm or so, most organic analytes have reasonable molar absorptivities. Indeed, the many commercial capillary electrophoresis systems on the market all rely on this detection mode. However, if one assumes an absorbance detection limit of 5×10^{-4} for the detector, for an analyte with a fairly high molar absorptivity of $10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$, the concentration limit of detection (LOD) at the detector can be estimated to range only from 5×10^{-5} to $1 \times 10^{-5} \text{ M}$ for path lengths of 10-50 μm , respectively. In fact, as one goes to small columns, the decreasing amount of

light available through the column degrades the absorbance LOD. The sensitivity of absorption detection can be enhanced by sophisticated techniques such as photothermal deflection spectrometry (13,14). The LOD is in the range $5-50 \times 10^{-8}$ M for dabsyl-amino acids injected into 50- μ m columns (14).

Very impressive detection limits have been achieved by laser-excited fluorescence (15,16) and by electrochemistry (17,18). The order of 10^{-10} M seems to be quite typical. One requires that the analyte fluoresce or be electrochemically active, respectively. For more universal applications, conductivity (19) and indirect fluorescence (20-23) have been developed. Conductivity has been shown to work in 50- μ m capillaries, and indirect fluorescence has been shown to work in even 5- μ m capillaries. The latter is based on the displacement of a fluorescing ion present in the electrophoretic buffer to create a negative-going peak. Concentrations down to 10^{-7} M or amounts to 50 amol have been detected.

The relative ease with which laser-excited fluorescence can be implemented for on-column detection in small capillaries raises the question whether there exist other detection schemes that are variations of direct fluorescence detection. The simplest idea is to monitor the fluorescence intensity of an additive rather than the transmitted light intensity for absorption detection. As long as the analyte

absorbs at either the excitation or the emission wavelength, a signal is generated. This is similar to the use of fluorescent thin-layer chromatographic plates (24), but the difficulties in achieving stable fluorescence levels and the extremely short path lengths prevent one from taking advantage of this scheme in capillaries. A special situation arises when the analyte quenches the fluorescence of the additive (25), but the interaction is nonlinear and not very general.

In this work, we explore the use of energy transfer as a detection scheme in capillary electrophoresis. A fluorophore is incorporated in the electrophoresis buffer at low concentrations. A steady fluorescence background is thus present at the detector all the time. When an absorbing analyte passes into the detector region, additional light energy will be extracted from the excitation laser beam. Normally, this extra excitation will be lost if the absorbing analyte does not fluoresce. However, energy (excitation) transfer can occur between the absorbing analyte and the fluorophore in the buffer. The fluorophore therefore receives additional excitation to produce a higher level of fluorescence, i.e. a positive peak. Detection is actually based on absorption by the analyte and not by converting the analyte to a fluorescent derivative. This then allows one to use the identical laser detector (21) to monitor fluorescing analytes (direct fluorescence), charged analytes with no absorption or fluorescence (indirect fluorescence or

displacement), or absorbing but nonfluorescing analytes (energy transfer).

EXPERIMENTAL

The CZE (capillary zone electrophoresis) system is similar to that described previously (21). A high-voltage power supply (Spellman, Plainview, NY; Model UHR50PN50) was used to supply the electromotive force across the capillary. The anodic high-voltage end of the capillary was isolated in a Plexiglas box for operator safety, while the cathodic end was held at ground potential. Injections were made for 1 s at 30 kV throughout each run.

The capillary columns (Polymicro Technologies, Phoenix, AZ) varied in length from 63 to 70 cm. The inner diameters of the columns were all 21 μm , while the outer diameters were 150 μm . The polymer coating was burned off 10 cm from the cathodic end of the capillary to form the observation region.

An argon ion laser (Control Laser Corp., Orlando, CA: Model 554A) operating at 488 nm with 15-mW power was used for excitation. The laser beam was stabilized to within 0.04% with a laser power stabilizer (Cambridge Research Institute, Cambridge, MA; Model LS100). The laser beam was focused into the capillary with a 1 cm focal length lens. The capillary was mounted at Brewster's angle to reduce scattered radiation.

The fluorescence was collected at 90° with a 20X microscope objective. The fluorescence image was focused onto a PMT photomultiplier tube (Hamamatsu, Middlesex, NJ; Model R928). Stray and scattered radiation were rejected by two

spatial filters preceding the PMT. The fluorescence was further isolated with two color filters (Corning Glass, Corning, NT; Model 3-69).

The PMT voltage was adjusted to maintain a 0.25- μ A background current, which was monitored with a picoammeter (Keithley, Cleveland, OH; Model 417). The output from the picoammeter was recorded on a chart recorder (Fisher Series 5000) or on a personal computer after analog to digital conversion (Data Translations, Marborough, MA; Model DT2827). Digital data were smoothed with a smoothing routine based on the Savitsky-Golay smoothing algorithm. A 2-s time constant was added at the picoammeter.

Reagents

The fluorescein used was laser grade (Eastman Kodak Co., Rochester, NY), while all other chemicals were reagent grade. All buffers were prepared in deionized water (Millipore Corp., Bedford, MA; Milli-Q System) and purged with nitrogen to remove carbon dioxide. The two dabsyl-amino acids were prepared by a standard method (26). All analytes were prepared in buffer solution. The fluorophores used in this work are fluorescein and riboflavin, and the absorbing analytes used are cresol red (CR), orange G (OG), dabsyl-phenylalanine (DAB-Phe) and dabsyl-glutamine (DAB-Glu). Their chemical structures are shown in Figure 1.

Figure 1. Structures of absorbers (A) and fluorophores (F)
used in this study.

Riboflavin

Fluorescein

Cresol Red

Orange G

Dabsyl Amino Acids

RESULTS

Normally, we use fluorescence spectrometry for the determination of analytes that "fluoresce". Clearly, every electronic transition that allows absorption also allows fluorescence. It is merely a matter of how much competition there is for the excited state to relax nonradiatively. When the fluorescence quantum yield is less than 10^{-3} , we normally will not select fluorometry as the detection method. Also, in the standard fluorometer it is difficult to distinguish between the observation of a weak fluorescence signal and a strong resonance Raman signal. The fact is, one can often detect "nonfluorescent" analytes by laser-excited fluorescence. This is illustrated in Figure 2a, which is the capillary electropherogram of the dyes CR and OG. For comparison purposes, a trace of fluorescein is also added to the sample. The native fluorescence yield of CR and OG is therefore some 4 orders of magnitude lower than that of fluorescein. The limits of detection for the two dyes are 1.5×10^{-6} and 5.6×10^{-6} M, respectively, which is comparable to or slightly better than the best one can do by monitoring absorption across these narrow capillaries. Since the resonance Raman spectra of CR and OG are not known, we cannot estimate what fraction of the signal in Figure 2a is actually due to fluorescence. In any case, this represents an unusual

Figure 2. Comparison of the detection of cresol red and orange G with and without fluorescein in the buffer: (a) 2×10^{-4} M CR, 4×10^{-4} M OG and 3×10^{-7} M fluorescein in 10 mM PO_4 buffer (pH 9.0); (b) 1×10^{-6} M CR, 2×10^{-6} OG, and 1×10^{-7} M fluorescein buffer (pH 9.0).

scheme for detecting nonfluorescent analytes in small capillaries.

On the addition of a small concentration of fluorescein in the running buffer, the same analytes produced the electropherogram in Figure 2b. Note that the concentrations of CR and OG have been lowered by a factor of 200. Depicted is a substantial enhancement in the fluorescence signal from CR and OG. The baseline is more noisy because there is now a constant large background fluorescence due to the fluorophore added to the buffer. An offset has also been applied to the electropherogram in Figure 2b.

The enhancement of the native emission from absorbing but nonfluorescing compounds opens up the possibility of detecting them in small capillaries with good sensitivity. An important question is how general this phenomenon is and therefore how broadly applicable the detection scheme might be. We tested four different analytes and two different fluorophores in such a detection mode. Figure 3 shows the electropherogram of two dabsyl-amino acids in a plain buffer (Figure 3a) and then in one with a trace of fluorescein added (Figure 3b). Note the samples are at the same concentration but there is a scale change in the two parts of Figure 3. The results are essentially identical with those in Figure 2. The dabsyl derivatives normally are used for the absorption detection of the amino acids but are detected here by the enhanced fluorescence emission. Similar results are obtained when

Figure 3. Separation of DAB-Phe and DAB-Glu with and without fluorescein in the buffer: (a) 10 mM PO_4 buffer (pH 9.0); (b) 10 mM PO_4 and 1×10^{-7} M fluorescein buffer (pH 9.0). The amino acids are at approximately 1×10^{-6} M.

riboflavin is used instead of fluorescein as the trace additive. A direct comparison is given in Table I. The enhancement factor is defined to be the ratio of peak heights for each analytes in the presence vs in the absence of the added fluorophore. Peak areas were not used because of the influence of retention times (migration velocity past the detector) and because of baseline noise. Table I shows generally large enhancement factors, which are beneficial to analytical applications. The enhancement factors for the analytes are surprisingly similar for a given fluorophore. This must be considered fortuitous since the native emission efficiencies (weak fluorescence or resonance Raman) of the analytes are highly variable.

A different comparison among the test compounds is also given in Table I, which lists the LOD for each analyte at the individually optimized fluorophore concentrations. Optimization was not universal because larger fluorescence background levels produce larger absolute noise levels, even though the dynamic reserve (baseline stability, refs 20 and 21) is held constant. The enhancement factors on the other hand, depend on the fluorophore concentration; vide infra. The best LOD is for CR with fluorescein as the additive. The 6×10^{-9} M (injected) level is substantially better than that detected with standard absorption and is comparable to that from photothermal methods in larger capillaries (14). The

Table I. Influence of fluorophore on enhancement factors and detection limits

analyte/ fluoro- phore	enhancement factor		LOD, M	
	fluorescein	riboflavin	fluorescein	riboflavin
cresol red	280	40	6×10^{-9a}	2×10^{-7c}
orange G	330	140	3×10^{-8a}	5×10^{-7c}
DAB-Phe	290		3.4×10^{-7b}	
DAB-Glu	310		2.5×10^{-7b}	

^aBuffer: 10 mM PO_4 and 2×10^{-7} fluorescein. ^bBuffer: 10 mM PO_4 and 1×10^{-7} fluorescein. ^cBuffer: 10 mM PO_4 and 2×10^{-6} riboflavin.

mass LOD is 4 amol on the basis of an estimated injection volume of 9.6 nL. The LOD is not nearly as impressive as regular fluorescence detection (e.g. dansyl-amino acids), but there is no reason to expect comparable LODs. The LOD is also better than indirect fluorescence detection via charge displacement (21) by 1 order of magnitude. There is no simple relationship between the LOD and the molar absorptivities of the analytes. We independently measured these as dissolved in the running buffer at 488 nm and found that $(CR) = 1.15 \times 10^4$, $(OG) = 2.45 \times 10^4$, and $(dabsyl) = 2 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$. By direct injection of a mixture of fluorescein and riboflavin in a clear 10 mM PO_4 buffer, the relative excitation-fluorescence efficiency was found to be 5 times higher for the former. This is consistent with the poorer LOD when riboflavin was used as the fluorophore.

DISCUSSION

The analytes and fluorophores were selected for this work on the basis of their similarity in molar absorptivities and fluorescence characteristics, respectively.

However, they represent very different structural classes (Figure 1) and electronic configurations. Included are both ionic and neutral species at the pH used. This indicates that the observations are not peculiar to certain chromophores or specific resonance structures. Naturally, more studies are needed to confirm the generality of this fluorescence enhancement effect. The difficulty in finding relatively nonfluorescent but strongly absorbing analytes at the wavelength used limits the present data set. Extension of these results to the UV region will shed new light on the structural requirements for the analytes and the fluorophore for this phenomenon.

Even though it will require extensive work to establish a clear mechanism, there is sufficient information to draw some conclusions based on the observations presented here. The enhanced emission is a direct result of absorption by the analyte, since the injection of nonabsorbing analytes did not produce any peaks, even at high concentrations. The mechanism is not related to indirect fluorescence detection (20,21) although the experimental arrangement is almost identical. The reason is that both neutral fluorophores and neutral analytes can produce enhancement. Displacement by charge was

necessary in indirect fluorescence detection. The running buffer used here is also at substantially higher concentrations and the fluorophores are at substantially lower concentrations than those needed for sensitive indirect fluorescence detection. A corollary is that one does not need to deviate from the standard electrophoresis conditions to produce enhanced emission. The scheme should therefore also be applicable to other systems, such as open tubular capillary liquid chromatography. We can also rule out specific compound formation or ion pairing between the analytes and the fluorophores on the basis of the applicability to diverse structural classes. The retention times of the analyte peaks also remained constant (taking into account the slight variability of electroosmotic flow rates for different capillaries and different pH) regardless of whether the fluorophore is present or not. Furthermore, the enhancement was found to be a monotonic function of concentration, with no noticeable stoichiometric constraints. Finally, for samples containing a mixture of CR, OG, and fluorescein, the latter produces peak heights and retention times identical with those of the samples containing fluorescein alone.

The data are consistent with an energy-transfer mechanism whereby the excitation in the analyte, A, is transferred to the fluorophore, F, enhancing its fluorescence. This is analogous to fluorescence quenching, in which a nonfluorescing additive competes for the excitation of a fluorescing analyte.

Here, a fluorescing additive competes for the excitation of a nonfluorescing analyte. Energy transfer has been studied extensively before (ref 27 and reference therein). Almost all reported work involves molecules in the millimolar range and up. The potential for using energy transfer to detect nonfluorescing species at the submicromolar level has not been suggested previously. This is because instrumentally it is difficult to maintain a steady background fluorescence so that small enhancements can be recorded. In fact, it is our ability to stabilize the large background fluorescence level (created by the added fluorophore) that led to the impressive detection limits listed in Table I. Clearly, one cannot simply insert and replace a sample cell in a fluorometer to look for small differences in signal levels. Also, if the fluorophore is at the millimolar level, one cannot expect to detect micromolar levels of an analyte even if the energy transfer is 100% efficient. The fractional change in signal will be too small. There is one literature precedent for energy transfer at micromolar concentrations (28), but that is due to complexation between the donor and the acceptor, drawing them much closer together than the concentrations would suggest.

In the diffusion-controlled regime one has the equivalence of Stern-Volmer kinetics (29). A possible scheme is

where the asterisks represent the excited forms of the two species. k_1 is the rate of excitation of the analyte, k_{-1} is the nonradiative decay rate of A^* , k_2 is the self-quenching rate of A , k_3 is the rate of excitation transfer to the fluorophore. On the basis of our experience with fluorescein as a mobile-phase additive in indirect fluorescence detection, quenching of F^* by analytes (reverse of k_3) is unlikely at concentrations below 1mM. So, the rate of formation of the excited analyte is

$$\begin{aligned} d[A^*]/dt \\ = k_1[A] - k_{-1}[A^*] - k_2[A^*][A] - k_3[A^*][F] \end{aligned} \quad (4)$$

Using the steady-state approximation, one obtains

$$[A^*] = \frac{k_1[A]}{k_{-1} + k_2[A] + k_3[F]} \quad (5)$$

What is observed is proportional to $[F^*]$. Equation 3 gives

$$\begin{aligned} \text{relative signal} &= k_3[A^*][F] \\ &= \frac{k_3k_1[A][F]}{k_{-1} + k_2[A] + k_3[F]} \end{aligned} \quad (6)$$

For a fixed fluorophore concentration, one can see that at high analyte concentrations the enhanced emission reaches a maximum value. At low analyte concentrations the enhanced emission intensity is proportional to the analyte concentration. Figure 4 shows the experimentally observed concentration dependence of the enhanced emission for the two dyes CR and Og. To provide a valid comparison, a fixed concentration of fluorescein in the sample mixture is used as an internal standard. For clarity, we did not plot the data points corresponding to 1×10^{-4} M CR and OG, which are at 2.0 and 1.13, respectively. Figure 4 shows that the data fall within the nonlinear region between the two extremes, although the lowest values are in the linear region and the highest value of CR is approaching a maximum.

The above energy-transfer scheme also predicts that for a fixed analyte concentration, the enhanced emission intensity should be independent of the fluorophore concentration as long

Figure 4. Peak height as a function of analyte concentration in 10 mM PO₄ and 2 X 10⁻⁷ M fluorescein buffer (pH 9.0): (□) CR; (◇) OG. The curves extend to 10⁻⁴ M, where the intensities are CR =2.0 and OG = 1.3.

as it is large. When the fluorophore concentration is low the signal should show a linear dependence. Figure 5 shows the experimental dependence of the enhanced intensity on the concentration of fluorescein. This is a difficult measurement because the fluorescence background level changes as the fluorophore concentration changes. For a fixed phototube gain setting, we can only cover the concentration range between 3×10^{-8} and 1×10^{-6} M to avoid saturating the phototube at the high end and to magnify the display sufficiently at the low end. The background fluorescence intensity was found to be linear with respect to the fluorescence intensity was found to be linear with respect to the fluorophore concentration in this range Figure 5 shows that we are at the low end of the fluorophore concentration. For OG the plot is linear For CR, there is some noticeable curvature.

Equation 6 also predicts that the relative signal should be proportional to excitation intensity if all concentrations are held constant since $[A^*]$ is proportional to excitation intensity. This is confirmed over the 3X laser power used here. At very high laser powers, one expects that bleaching will occur to cause nonlinearity.

It is also interesting to compare the observations here with kinetics (30). Essentially, that is based on resonant dipole-dipole interaction. The probability of energy transfer is proportional to R^{-6} , where R is the intermolecular distance.

Figure 5. Peak height as a function of fluorescein concentration in 10 mM PO_4 and fluorescein buffer (pH 9.0). 10^{-6} of CR ($\square$) and 3×10^{-6} M of OG (+) were injected in each case.

A characteristic distance, R_0 , can be defined as that which produces a 50% probability for energy transfer. For a 10^{-6} M solution of CR, we can estimate that the average inter-molecular distance is about 120 nm. So, if the fluorescein molecules are randomly distributed, the furthest away any one is from a CR molecule will be 60 nm. We found that the peak height of 10^{-6} M CR is equal to that produced by 2.3×10^{-8} M fluorescein when a buffer containing 3×10^{-8} M fluorescein was used. If one assumes that the fluorescence quantum yield of fluorescein is unity, then the energy-transfer efficiency is $2.3 \times 10^{-8}/10^{-6} \text{ M} = 2.3\%$. So, $(60/R_0)^6 = (50\% / 2.3\%)$.

Therefore, $R_0 = 36 \text{ nm}$. A similar calculation based on the observation that a 2×10^{-6} M CR sample produces a peak equal in height to that of 10^{-7} M fluorescein gave $R_0 = 32 \text{ nm}$. These are extremely large R_0 values, exceeding typical values by 1 order of magnitude. In kinetics, one requires that (1) there is substantial overlap between the fluorescence spectrum of the donor and the absorption spectrum of the acceptor, (2) the molar absorptivity of the acceptor is large, and (3) the fluorescence quantum yield of the donor is large. Only criterion 2 is satisfied here. So, it is not surprising that our results cannot be readily explained by kinetics.

The long-range interaction that makes detection possible at such low concentrations suggests that dipole-dipole

interactions are responsible. It is possible that the excited states of the absorbers are highly polarized due to effective charge separation. If so, the dipole moments are much larger than those predicted from absorption coefficients alone, as in kinetics. R_0 can then be larger than expected, but probably not by 1 order of magnitude. Still and dipole-dipole mechanism will exhibit an R^{-6} dependence. To test this, we plotted log (peak height) vs log (concentration) for both CR and OG for a fixed fluorescein concentration. The data are identical with those in Figure 4. The slopes were found to be 0.55 ± 0.08 and 0.45 ± 0.09 , respectively, with correlation coefficients $r^2 = 0.92$ and $r^2 = 0.90$. An R^{-6} dependence implies a (concentration) $^{-2}$ dependence. However, there is significant curvature in the log-log plots, showing that the data really do not follow a simple (concentration) $^{-2}$ dependence.

It is also possible that ion-ion interaction (R^{-1} dependence) or ion-dipole interaction (R^{-2} dependence) may be responsible. However, riboflavin is neutral while fluorescein is ionic at the operating pH. The fact that very similar enhancements are seen here argues against these schemes. It appears that a likely mechanism is the existence of a long-lived intermediate state for the excited analytes. There is then sufficient time for collisions to occur with the

fluorophore, even at these low concentrations, to allow efficient energy transfer. Further studies are needed to clarify this behavior.

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PAPER 3. INCREASED SELECTIVITY FOR ELECTROCHROMATOGRAPHY BY
DYNAMIC ION EXCHANGE

INCREASED SELECTIVITY FOR ELECTROCHROMATOGRAPHY BY
DYNAMIC ION EXCHANGE

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SUMMARY

Ions not resolved by free zone capillary electrophoresis are separated by CE with the addition of other separations modes to the system. A capillary electrophoresis column is coated with a reverse phase polymeric layer. This adds a component of reverse phase to compounds that have hydrophobic character. Dynamic ion exchange sites are also added to the column. This will allow retention of compounds with a high affinity for these sites to be retained. Therefore, compounds can be separated based on their differences in retention rather than their differential migration rates.

INTRODUCTION

Capillary zone electrophoresis (CZE) has been demonstrated to provide highly efficient separations of many charged species including small organic molecules, amino acids, peptides and nucleic acids(1-5). Because CZE separates compounds based on their differential migration rates, compounds with similar mobilities pose resolution problems(6,7). The most common optimization techniques for CZE are changing the pH of the running buffer, coating the column with a hydrophobic stationary phase and using additives to decrease the electroosmotic flow.

The addition of hydrophobic ion interaction reagents (IIR) to a mobile phase to enhance retention and resolution has been widely used in the liquid chromatographic (LC) separation of charged species on reverse-phase columns(8-13). The mechanism for this increased retention is not fully understood. The ion pair model presumes that an ion pair between the IIR and the sample ion is formed in the mobile phase. The ion pair then partitions into the stationary phase. This model most closely predicts the behavior in non-bonded reverse-phase columns.

When the reverse-phase is chemically bonded to the column, however, the retention is best explained by a dynamic exchange model(14). This model presumes that the IIR is adsorptively bound to the column where it behaves as an ion exchanger. The retention and separation in this mode is very

similar to ion exchange chromatography. There is, however, added flexibility in dynamic ion exchange due to the many parameters that effect the retention of a species. Another deviation is the ion-interaction model where the IIR is dynamically adsorbed to the column forming a charged double layer. Sample ions are then retained due to their columbic attraction to the double layer. The actual mechanism of ion pair chromatography is probably a mixture of these models depending upon the specific conditions.

In this paper, compounds with similar mobilities are separated using a reverse phase coated capillary with dynamic ion exchange sites. An IIR, cetyltrimethylammonium bromide, is added to the running buffer which is sorptively bound to the hydrophobic stationary phase to provide the ion exchangers. The results of these experiments support the dynamic ion exchange model for retention and separation.

EXPERIMENTAL

The buffers used in these experiments were prepared in deionized water (Millipore, Bedford, MA, U.S.A.; Milli-Q system). Cetyltrimethylammonium bromide (CTAB) solutions were prepared by diluting with buffer solution and adjusting to pH 7 with a 0.1 M solution of NaOH. The anions, 4-amino-1-naphthalenesulfonic acid (4A1N), 5-amino-2-naphthalenesulfonic acid (5A2N), 8-amino-2-naphthalenesulfonic acid (8A2N) (all from Aldrich) were prepared in running buffer prior to injection.

The fused silica capillaries (Polymicro Technologies Inc., Phoenix, AZ) were 50 cm and nominally 10- μ m in i.d. These capillaries were used as supplied or coated, and the coating later cross-linked with 10.0% (w/w) OV-17v (Alltech Associates, Inc., Deerfield, IL) as described in reference 7. The polyimide coating was removed with hot sulfuric acid 40 cm from the injection end of the capillary to facilitate on-column detection.

The CZE/electrochromatography system is similar to one described previously(15). The electric field is supplied by either a positive or negative high-voltage supply (0-30 kV, Glassman High Voltage Inc., Whitehouse Station, NJ; Model MJ30P0400-11 or MJ30N0400-11).

Sample introduction was accomplished by using electromigration in all cases. Detection was accomplished by UV (240 nm, ISCO Model 270, Omaha, NB) or by using a laser-

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based fluorescence detector similar to one described previously with the exception being that the laser line used was the 325 nm line of a He-Cd laser (Linconix, Sunnyvale. CA. U.S.A.; Model 4240NB).

All data were collected on either a strip chart recorder or subjected to analog to digital conversion (Data Translation, Inc., Marlborough, MA; Model DT 2827, 5-10 Hz) and later stored on a personal computer (IBM PC-AT).

DISCUSSION

The resolution of compounds separated by CZE is given by

$$R = \frac{1}{4\sqrt{2}} (\mu_{e,1} - \mu_{e,2}) \sqrt{\frac{V}{D (\bar{\mu}_e + \mu_{eo})}} \quad (1)$$

where $\mu_{e,1}$ and $\mu_{e,2}$ are the electrophoretic mobilities of the solutes, $\bar{\mu}_e$ is the average electrophoretic mobility, μ_{eo} is the electroosmotic flow, V is the applied potential and D is the diffusion coefficient. As can be seen from this equation, resolution is directly proportional to the difference in mobilities of the two species of interest.

The separation of compounds with similar mobilities is expected to be difficult with CZE(7). This is demonstrated in Figure 1 with the attempted separation of positional isomers, which would be expected to have similar mobilities. As can be seen, there are only two partially resolved peaks for the 3 compounds injected. Compounds with very similar mobilities will not separate, however, unless $\mu_{eo} \approx -\bar{\mu}_e$, which can be done through the addition of modifiers to the buffer. This condition leads to analysis times that approach infinity, however.

Another way to enhance the selectivity of this separation would be to change the pH of the buffer which may change the charge, and therefore the mobility, of each species to

Figure 1. Elution profile for the attempted separation of 4A1N and 5A2N. Buffer Conditions: 10 mM sodium phosphate, pH 7.0. The separating column is 60 cm X 10 μ m i.d., 350 μ m o.d. with the detection window 40 cm from the injection end. Absorption detection at 240 nm. Separation voltage -25 kV.

different degrees. This, however, would not be expected to work very well on positional isomers due to the small range of pK 's for compounds so similar.

Selectivity can also be enhanced by adding another mode of separation to the system. One way to do this for the compounds we have selected is to add anion exchange sites to the column. Compounds with larger affinities for the ion exchange sites would be impeded and would have larger migration times.

This can be accomplished by first coating the column with a polymeric stationary phase(16). This would have two effects on the column. First, the charged silanol groups on the surface of the column would be shielded, thus drastically reducing electroosmotic flow. Second, the surface of the column would be changed from a polar to relatively non-polar, hydrophobic surface. Compounds with a hydrophobic character could, therefore, partition with the stationary phase.

For the separation of anions, the dynamic ion exchange sites are added to the column by the addition of a surface active quaternary ammonium compound to the buffer. The hydrophobic end of this molecule is expected to adsorb to the hydrophobic surface of the coated capillary allowing the charged end of the molecule to be exposed to the polar aqueous mobile phase. The surface of the capillary would therefore be expected to carry a positive charge with a loose association of anions in the double layer region that extends to the

diffuse layer. This is in contrast to a bare fused silica column where under normal aqueous conditions the solid surface has an excess anionic charge resulting from ionization of surface functional groups. The counterions in the diffuse layer in this case would therefore be positive. The migration of hydrated counterions in the diffuse layer is the propulsive force behind electroosmotic flow(17). In the case of a bare fused silica capillary we would expect the flow to be towards the cathode where in the case of the dynamic exchange mode the flow would be in the direction of the anode.

The fact that the electroosmotic flow is reversed when a surface active quaternary ammonium compound is added to the buffer supports the dynamic ion exchange model for ion pair chromatography. This is the process where the surface active agent is first adsorbed to the surface of the column, and the sample ion is then retained by an ion exchange mechanism. The mechanism is more complex than ion exchange, however, because the ion exchange site is sorptively bound, while in an ion exchanger the ion exchange site is covalently bound.

The C₁₈ chain of the CTAB molecule is expected to be readily adsorbed onto the surface of a reverse phase column by hydrophobic attraction and would tend to behave as an ion exchange site. Since conductance data indicate that R₄N⁺ salts are highly dissociated under similar conditions it can be assumed that ion pair formation is negligible(12). The

adsorption of the CTAB to the column was confirmed by washing the column with ACN and the returning of the electroosmotic flow then returned to its original direction, towards the cathode.

The retention of a compound by dynamic ion exchange, as discussed above, is complex. Some of the parameters that can influence the dynamic ion exchange retention of compounds in HPLC are the size of the surface active agent, pH, type and concentration of organic modifier and the stationary phase characteristics(13,14). These parameters would be expected to have similar effects in electrochromatography.

In capillary zone electrophoresis the time a compound takes to migrate to the detection region of the capillary is given by

$$t_m = \frac{L}{(\mu_e + \mu_{eo})E} \quad (2)$$

where t_m is the migration time, L is the distance from the injection end of the capillary to detector, E is the electric field strength, μ_e is the electrophoretic mobility of the compound and μ_{eo} is the electroosmotic flow. In a modified CE system where the walls of the column contain ion exchange sites retention would lead to a reduction in μ_e , thus increasing t_m . The mobility of a species would be decreased by an amount depending upon its affinity for the ion exchange

sites. If the positional isomers shown in Figure 1 were retained to different degrees, there would be an increase in resolution based on Equation 1.

This is what is seen experimentally based on Figure 2. The two compounds here, 4A1N and 5A2N, are separated on a column coated with 2% OV-17v. The buffer consists of potassium phthalate, a common eluent used in ion exchange chromatography, and 200 μ M CTAB. The column was equilibrated with the buffer for two hours to increase the stability of the migration times. There is a reduction in efficiency in this system based on the number of theoretical plates in each peak. This would be expected due to the increased contribution of mass transfer in the stationary phase to the calculation of plate height(21).

The equilibrium describing the anion exchange of an anion A and the eluent E would be

and the equilibrium constant would be given by

$$K = \frac{[A]_s [E]_n^s}{[A]_{aq} [E]_s^n} \quad (4)$$

where the subscripts s and aq refer to the stationary and mobile phases respectively.

Figure 2. Elution profile for the same ions as in Figure 1,
a) 4A2N and b) 5A2N. Buffer Conditions: 32 mM
potassium phthalate, 400 μ M CTAB, pH 7.0.

Models for dynamic ion exchange based on HPLC experiments predict that increased eluent concentration will have two effects on retention of sample ions. The increased concentration of the counter ion increases the amount of the surface active agent adsorbed to the surface of the capillary(11). This is greatly offset, however, by the increased competition for the exchange sites as can be seen from Equation 3. Pfeffer et. al. have shown that the addition of a surface active reagent, such as CTAB, to the hydrophobic surface of a coated capillary column has the effect of increasing electroosmotic flow(18,19). A maximum in flow rate is reached when charge repulsion prevents additional CTAB adsorption and the hydrophobic phase is essentially saturated. With a 10 mM phosphate, 10% acetonitrile buffer this maximum was reached in the 20 μ M CTAB range. With no organic solvent in the mobile phase the saturation point would be expected to be even lower.

In our system we used a CTAB concentration of 400 μ M to ensure that the walls of the capillary were saturated. The net effect, therefore, of an increase in buffer concentration should be a decrease in migration time of the sample ions. This was confirmed with our experiments as shown in Figures 3. Another interesting result is that 4A1N, the least retained compound, showed very little change in retention at the highest concentration of buffer. This is believed to be because this compound is only slightly retained at the high

Figure 3. Buffer concentration dependance on migration times for 4A2N (■) and 5A2N (□). Buffer conditions are the same as in Figure 2.

buffer concentrations. Therefore, its migration time depends almost entirely upon its mobility and very little due to its retention on the column at the high buffer concentration. 5A2N, on the other hand, showed a decrease in retention at all buffer concentrations.

In addition to the cation exchange sites, there would also be a reverse phase character to this column due to the hydrophobic nature of the polymeric film coating. This reverse phase character can be examined by adding an organic component, acetonitrile, to the mobile phase and observing its effect on the migration times in the absence of CTAB.

Fujiwara et al. has shown that the addition of acetonitrile to a CE buffer can increase the velocity of electroosmotic flow by almost 30%(20). We would expect, therefore, that the addition of acetonitrile would decrease the migration times of non-retained compounds by the increase in flow rate and decrease the migration times of retained compounds by a larger amount, due to the decrease in retention. As seen from Figure 4, the addition of acetonitrile to our system did have an effect on the migration times of these species. In our experiment the retention time of 4A1N decreased about 40%, indicating that there is some reverse phase retention of this compound. The 5A2N migration time decreased about 200%, indicating that this compound has a strong reverse phase component. Because the amount of organic modifier added to the system also affects the adsorptivity of

Figure 4. Dependence of migration times for 4A1N (■) and 5A2N (□) on the concentration of organic modifier. Buffer Conditions: 32 mM potassium phthalate, pH 7.0, no CTAB.

the CTAB to the surface of the capillary, this could be a straight forward way to adjust retention in this system.

The contribution of these two retention mechanisms to the separation can be measured somewhat by the dependance of the concentration of the ion exchange sites on the surface of the column. As noted before, as the CTAB concentration is increased the velocity of electroosmotic flow also increases up to the point of saturation of the column wall with the CTAB molecules. The addition of CTAB would, therefore, shield reverse phase sites on the column up to the point of saturation. A compound with a stronger reverse phase component would have a decreased migration time with an increase in CTAB concentration, where a compound with a stronger ion exchange component would be expected to have an increased migration time as the number of ion exchange sites were increased.

Figure 5 and 6 shows the effect of CTAB concentration on the migration times of 5A2N and 4A1N. The compound with the strongest reverse phase component, 5A2N, did show a decrease in retention as the CTAB was increased. The 4A1N migration time, however, shows a sharp increase in migration time between 50 and 100 μ M CTAB and then slowly increases as the CTAB concentration is increased further. The diminished effect of the addition of CTAB can be explained by the saturation of CTAB on the walls of the column. 4A1N,

Figure 5. Dependence of 5A2N migration time on the CTAB concentration. Buffer
Conditions: 8 mM potassium phthalate, CTAB, pH 7.0.

Figure 6. Dependence of 4A1N migration time on the CTAB concentration. Buffer
Conditions: 8 mM potassium phthalate, CTAB, pH 7.0.

therefore, has a stronger ion exchange component, where 5A2N has a stronger reverse phase component.

It has been shown that manipulation of the surface of a capillary electrophoresis column can add selectivity to separations. The retention of the sample ions can be varied in this system by changing the concentration of the buffer, the addition on an organic modifier and by changing the concentration of the IIR. The addition of the dynamic ion exchange mode to electrochromatography extends its use while adding flexibility. The system can be returned back to the reverse phase mode by simply washing the column with a strong mobile phase which removes the IIR adsorbed to the hydrophobic surface.

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GENERAL SUMMARY

Capillary electrophoresis provides a rapid and highly efficient separation of many types of compounds. Compounds that prove to be difficult to separate may be separable using CE with a coated column. The electroosmotic flow provides the driving force of the separation and the stationary phase adds selectivity. This is an advantageous mechanism due to the small amount of broadening introduced by this flat flow profile of the electroosmotic flow.

CE is destined to be an important separation method where small volume samples are involved, because of its requirement of only ultra-low sample volumes. This is advantageous when only limited supplies of the sample exist (i.e single cells), but can be a disadvantage because of the increased requirements of detection.

The miniaturization of common LC detectors, Absorbance, fluorescence and electrochemical, are of some benefit to CE detection. Absorbance detection is the most common detector for commercial CE instruments. The conditions for optimum absorbance detection are necessarily contradictory to the for high separation efficiencies, however. Small diameter capillaries increase separation efficiency due to the increased heat dissipation while increasing detection limits because of reduced path length. Because of the ability to miniaturize fluorescence and electrochemical detectors, they readily lend themselves as detectors for very small diameter

capillaries. These detectors however, can only be used on compounds with very specific properties, they must fluoresce or be electrochemically active. Unfortunately, only a few compounds have these properties.

Because of the disadvantages of other detection methods this leads us to explore other detection alternatives. Some of the characteristics of an "ideal" detector for CE would be 1) a very low limit of detection, subattomole would be convenient for many applications 2) universal, because of the high separation efficiencies offered by CE, addition selectivity from the detector is not required.

The two alternative detection methods explored in this work, indirect fluorescence and absorbance by energy transfer, do not achieve the requirements of an "ideal" detector. They do, however, increase the number of choices available to the analyst in search of a CE detector.

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