PRESSURE-DRIVEN AND ELECTROOSMOTICALLY-DRIVEN LIQUID CHROMATOGRAPHIC SEPARATIONS IN PACKED FUSED SILICA CAPILLARIES

by

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(ABSTRACT)

Means of achieving rapid, efficient separations of analytes are explored in detail, with particular emphasis on the use of chromatographic and electrophoretic theory as an aid in system design and optimization. The benefits of miniaturization of chromatographic systems are assessed.

First, the utility of semi-micro Ion Chromatography is explored by the manufacture of 2mm ID analytical and suppressor columns and a micro-conductivity cell. The quality of the columns and detector cell are evaluated by the separation of a test mixture and the calculation of peak variance contribution of the detector cell. The use of readily available analytical scale instrumentation for semi-micro IC is demonstrated.

Next, a further downsizing of the IC system is described, in which 530μm ID fused silica tubing is utilized for column manufacture. In this case, a suppressor column is not used and UV detection is employed in the analysis of nucleoside monophosphates. Again, column performance characteristics are measured and noted. Application of this system to the separation of a hydrolysed nucleic acid sample demonstrates the feasibility of the technique to the analysis of volume-limited samples in low concentration with notable sensitivity.

The benefits of a miniaturized liquid chromatographic system under pressure-driven flow is studied and the improved permeability of micropacked capillary columns is exploited in the manufacture of several 25 to 30cm columns which achieve high efficiencies with relatively low pressure drops. Van Deemter plots illustrate the performance characteristics of the columns.

Finally, electroosmotic flow is studied as the motive force for liquid chromatographic separations. This combination of two techniques, packed capillary liquid chromatography and capillary electrophoresis, results in a system which achieves good resolving power through maximization of selectivity and efficiency.

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CHAPTER I

INTRODUCTION

1.1 Research Objectives.

The ultimate goal of most separations is the achievement of high resolving power in a minimum of analysis time. For some applications, this goal can be satisfied through the use of Capillary Gas Chromatography (GC) since properly designed columns can generate up to one million theoretical plates. An overwhelming majority of compounds, however, are unsuitable for GC analysis for reasons of thermolability or limited volatility. This leaves the separations chemist with the problem of achieving high resolving power in the liquid phase. This is no trivial task when it is remembered that mass transfer in liquids is not nearly so facile as that in gases. The theory of High Performance Liquid Chromatography (HPLC or LC) should be consulted to find those parameters in system design which should be adjusted to provide the optimum resolving power achievable in an LC separation. This describes (in general) the scope of the research presented in this document: the achievement of maximum resolving power in a liquid phase separations system through the judicious application of chromatographic and electrophoretic theory.

The objective of this dissertation is to convey the potential benefits of: 1) downsizing Liquid Chromatographic instrumentation and; 2) applying an alternative method of moving mobile phase through the column such that maximum efficiency can be achieved. Experimental results are presented which suggest that, when carefully and properly applied, micro-LC is capable

of delivering important benefits over conventional analytical-scale HPLC systems. That a need for micro-LC exists is a moot point: an increasingly large audience of chemists is finding a purpose for LC in the laboratory, while the operating costs surrounding the technique rise at an alarming rate. Solvents of the purity required for HPLC are extremely expensive, and disposal of some solvents is not only a cost concern, but also a concern because of the environmental hazard posed. For these reasons, the low solvent consumption of micro-LC alone justifies research into miniaturized systems. It is fortunate for those interested in separations research that miniaturization of LC systems yields potential performance benefits as well. In fact, the performance enhancement which can be achieved in packed capillary columns for LC can be surprising when these columns are operated in an electrochromatographic mode, as will be discussed later in this text.

1.2 Miniaturization of Liquid Chromatographic Systems.

Previous research efforts into microscale HPLC have taken a variety of forms[1]. In order to establish a common nomenclature, Jinno[2] mentions that microscale LC columns should be classified according to their flow resistance parameter, ϕ:

$$\phi = d^2/K_o = \Delta P d^2/\eta\mu L \qquad \{1\}$$

where d is the particle diameter of the packing material used in a packed column or the tube bore of an open tubular column, K_o is is the specific column permeability, ΔP is the pressure drop across the column, η is the viscosity of the mobile phase, μ is the linear velocity of the mobile phase, and L is the length of the column. The flow resistance parameter is simply a means of assessing the ease with which mobile phase can pass through the column. Using ϕ, microcolumns can be divided into four basic classes: open-tubular, packed microcapillary, micropacked capillary, and micro- or semi-micro columns. The properties characteristic of these columns are listed in Table 1, and each is described in detail below.

Open-Tubular capillary columns for HPLC represent the ultimate in miniaturization[3]. These columns have inside diameters in the range of three to twenty microns, and operate at flow rates of one to ten nanolitres/minute. Columns are typically manufactured of fused silica capillary material, although much good work has been performed in drawn quartz tubing. True capillary columns, however, are at present only a research novelty, and require highly specialized instrumentation.

Packed microcapillary columns[4,5] are manufactured in quartz and Pyrex glass tubing by first packing tubing of one to two millimeter inside diameter with sorbent material, then drawing the column to the desired inside diameter in a glass drawing machine. Diameters range from forty to eighty microns, and the columns are typified by their loose packing structure, stabilized by the impression of some of the packing material into the walls of the tubing as it is being heated and drawn. The application of high temperatures in the drawing process precludes the use of derivatized packing

Table I. Properties of microcolumns in HPLC.[a]

Column Type	ID(mm)	Length(m)	Particles(mm)	K-P ratio*	f
Low permeability	250-1000	0.1-0.3	5-30	50-200	500-1000
High permeability	50-200	0.1-20	10-100	2-8	100-300
Open tubular	10-60	20-40	n / a	n / a	32

[a] Adapted from reference 30.

* Knox-Parcher ratio, as discussed in text.

f = Flow resistance parameter (dimensionless)

materials, though it is possible to derivatize in situ after the capillary has been drawn. Like their open-tubular counterparts, these capillaries exhibit very high permeabilities (low resistance to mobile phase flow), making very long, efficient columns possible. Flow rates for optimal efficiency are in the vicinity of twenty to fifty nanolitres/minute.

Micropacked capillary columns[6] (also called slurry-packed capillary columns) represent the research area covered in this dissertation. They generally range in inside diameter from fifty to 530 microns, and are tightly packed with sorbent materials as are traditional analytical scale columns. The material of choice for the manufacture of these columns is fused silica. The flow rates generally applied in these systems range between seventy nanolitres/minute (50 micron ID) and eight microlitres/minute (530 micron ID). Depending on the diameter of the packing material used (relative to column inside diameter) these columns may also achieve higher permeabilities than are seen in analytical scale LC[7]. This in turn can allow for columns of greater length to be used, such that total efficiency is greater than might be expected.

Microbore and Semi-micro columns complete the picture of downsized LC systems. These columns are packed in the more traditional tubing materials using procedures common to conventional commercially available columns. Microbore columns generally have a 1mm inside diameter, and semi-micro columns a 2mm inside diameter. These formats offer the benefits of decreased solvent consumption while maintaining their usefulness on traditional instrumentation.

In the case of the micropacked capillary columns in this work, and indeed for all of these column types, careful system design an construction is imperative is system performance is to be optimal. Bowermaster[8] addressed extracolumn volume effects in micro-LC, and suggested that special attention be paid to the minimization of connective tubing and detection cell volumes.

If a micro-LC system is properly designed and built, it is possible according to several researchers[9,10] to gain the following advantages as a recourse of miniaturization:

1. Increased column permeability (less resistance to flow)
2. Higher efficiencies through the use of longer columns
3. Improved mass sensitivity in concentration dependent detectors
4. Decreased consumption of both mobile & stationary phase materials
5. Improvement in resolution (R_s) due to improvement in efficiency.

All are important advantages, but in order to realize them, one must produce a good-quality column. (In this authors' opinion, the manufacture of a good column is not a trivial task.) A discussion of each of these potential benefits follows.

An improvement in column permeability must inherently mean that a less obstructed flow path is achieved. In order to achieve a less obstructed flow path, though, a more "open" packing structure must be achieved without yielding an unstable bed structure. This is possible if the structure imposed on the packed bed by the column wall is propagated across the entire diameter of the column, as illustrated in figure 1. If this is indeed the case, it stands to reason that columns can be made to greater lengths without exceeding an acceptable level of backpressure. These longer columns in turn can provide

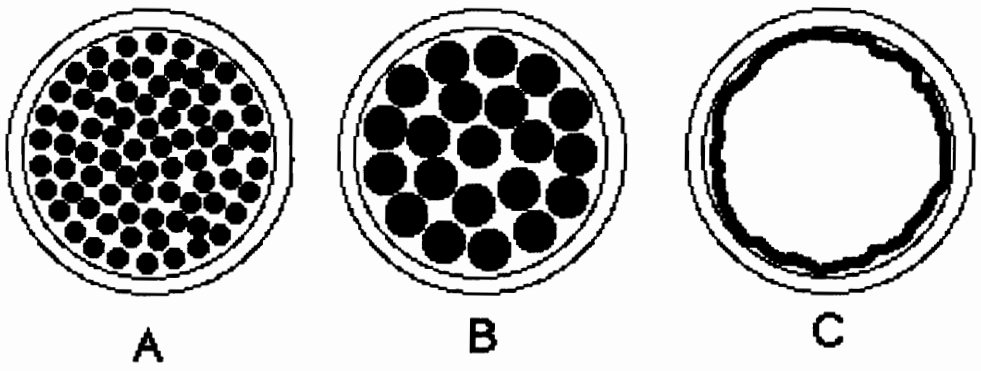


Figure 1. Column formats commonly employed in microscale HPLC, classified according to column permeability.

A: Conventional packed column (low permeability). B: Highly permeable column (d_c / d_p <6). C: Wall coated open tubular column (highest achievable permeability).

greater total efficiencies, N, and yield separations of higher resolution, since N is a component of resolution (R_S).

The phenomenon of improved efficiency in miniaturized systems, measured as reduced plate height ($h = H / d_p$), has been previously demonstrated. Knox and Parcher[11], in 1969, explored the interrelationship between column inside diameter, particle size, and plate height. This work was carried over into the realm of packed capillaries by Wilson and McNair[12], who again substantiated that when the Knox-Parcher ratio, $\rho = d_c / d_p$, falls between 4 and 6, an inflection point occurs in the plot of h vs ρ as seen in figure 2. Apparently, when the column I.D. is 4 to 6 times larger than the particle diameter, a uniform packing structure exists through the cross-section of the capillary. The resulting similarity in all possible flow paths gives rise to less transcolumn inhomogeneity of flow velocities, and improves "h."

On the matter of mass sensitivity, once again the cross sectional area of the column comes in to consideration. If two columns of different inside diameter are taken to have the similar efficiencies per unit length, it becomes apparent that the concentration of an analyte eluting from the smaller diameter column, on the same volume of injection, will be greater. This important characteristic is related as a squared function of the column radius, visible in the formula for the circular cross sectional area of the column.

If columns of smaller volume are employed, it also becomes evident that smaller volumes of packing material and mobile phase solvent will be required. This is important in that waste disposal problems can be avoided, and also in that more exotic or precious materials may be used without incurring to great a cost in effecting the required separation. Table 2 illustrates the

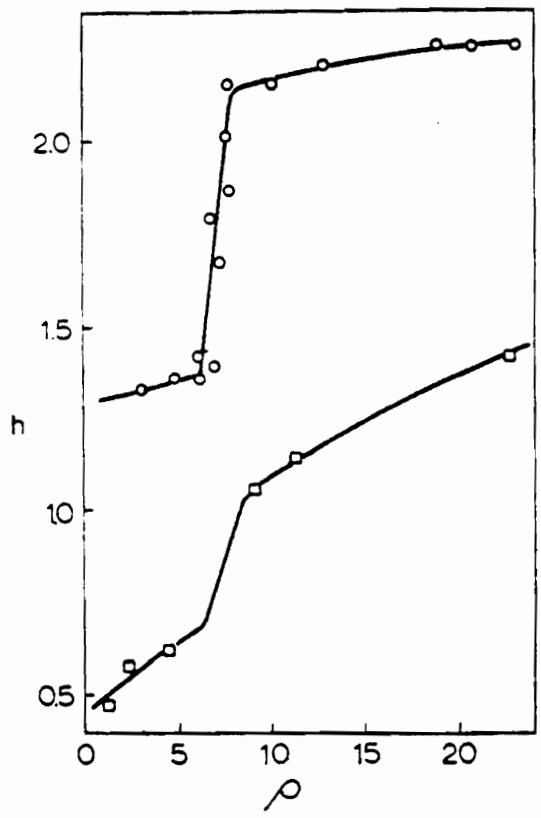


Figure 2. A Knox-Parcher plot illustrating the effect of column diameter to particle diameter ratio on h.

Knox-Parcher plot of reduced plate height (h) as a function of ρ (Knox-Parcher ratio, d_c / d_p). From reference 31.

Table II. Flow rate as a function of column ID.

Column Type	ID (mm)	Rel. cross-sectional area	Flow rate (µl/min)
Dense-packed	4.6	16	1000
Dense-packed	2.0	4	250
Dense-packed	1.0	1	75
Packed Capillary	0.53	0.25	20
Packed Capillary	0.25	0.05	5

(Using a 1.0mm ID as a reference point, and assuming that all linear velocities are equal.)

dependence of volumetric flow rate (for identical flow velocity) on column inside diameter.

Research in micro-LC has met with some rather substantial obstacles. As previously mentioned, a decrease in column size must be accompanied by a decrease in extracolumn volume, lest resolution be lost due to factors outside the column. This means that detector cell volumes must be extremely small, and that connective tubing must be essentially eliminated. Even the small volume contributed by a column end frit can be large enough to substantially degrade the separation. It cannot be stressed enough that careful system design and fabrication is critical to success in micro-LC applications.

1.3 High-Efficiency Electrophoretic Separations.

Electrophoretic separation, as will be discussed, has been a longstanding tool in the biochemistry laboratory. The miniaturization of electrophoretic instrumentation, however, has been a relatively recent interest in the instrumentation community[13]. It seems that downsizing of electrophoretic instrumentation can yield improvements in performance much as it can in chromatographic separations. Indeed, the use of very small separation tubes allows for electrophoresis to be conducted in free solution, rather than on solid matrices. This in turn allows for improved heat dissipation and therefore higher efficiencies through higher applied potential fields and less band defocussing through variations in mobile phase flow velocity.

Capillary Electrophoresis (CE) is typically conducted in open tubes of twenty to one hundred micron inside diameter. These capillaries are usually fifty to one hundred centimeters in length, and are most often crafted from fused silica tubing, though polymeric tubing and glass tubing have also been used. Each end of this tubing is immersed in a separate reservoir containing an operating buffer, and the capillary is filled with the buffer as well. If the capillary contains no material other than operating buffer identical to that in the reservoirs, the technique is called "free zone" electrophoresis, more commonly known as Capillary Zone Electrophoresis (CZE). This is the most popular of the CE techniques, though many others do exist and offer different selectivities. By immersing an electrode into each of the two reservoirs, one of which serves as earth ground and the other of which is used to apply a high potential field (5 - 50 kV) across the capillary, a separation of analytes can be affected based on their hydrodynamic volume and their charge in solution. Finally, a small section (2 to 10 mm) of the polyimide coating on the outside of the fused silica capillary is removed so that a UV detector can be placed in line between the reservoirs for in situ detection. A schematic of the instrumentation for CE is shown in figure 3.

Victor Pretorius[14] once called sample introduction in GC the "Achilles Heel" of the technique. In CE, this characterization applies not only to sample introduction, but equally to detection. Concentration limits-of-detection are in the tens of parts-per-million for CE with UV detection. The reasons for this insensitivity are twofold. First, as an open tubular technique, CE provides low sample loading capabilities. In other words, only very small volumes (1 to 20 nanolitres) of sample can be injected before the capacity of the system is

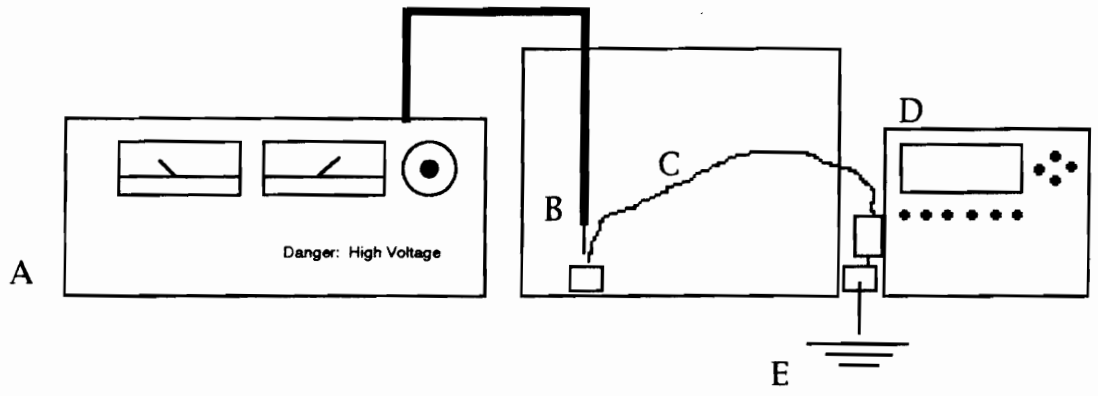


Figure 3. A schematic of the instrumentation used in Capillary Electrophoresis.

A: High voltage power supply. B: Platinum wire electrode. C: Capillary. D: UV Detector. E: Earth ground. Operator is protected from electrical shock by enclosing the high voltage electrode in a plexiglass box.

exceeded and efficiency suffers. For this reason, the amount of sample present for detection is necessarily small. Secondly, when CE is used in conjunction with UV detection, the detection cell is actually a part of the fused silica capillary separation tube from which the polyimide coating has been removed in order to allow for in situ UV detection. As CE is conducted in very small inside diameter tubes (again, 20 to 100 microns), the path length for detection is likewise very small. As indicated by Beers' law, the path length is directly proportional to absorbance intensity, meaning that the detector signal achieved in the case of a short path length as in CE will be miniscule. The end result of the two factors described is poor concentration sensitivity due to small sample size and geometric constraints.

As mentioned earlier, only a limited degree of selectivity (relative to HPLC) is available in CE methods. In classical CZE analytes can be separated only if there is a difference in their electrophoretic mobility, i.e. in their charge to volume ratio. Alternative capillary electrophoretic methods of separation are available for use. Capillary Gel Electrophoresis (CGE)[15], Capillary Isoelectric Focussing (CIEF)[16], and Micellar Electrokinetic Capillary Chromatography (MECC)[17] provide mechanisms which separate analytes based on molecular size, isoelectric point, and partition coefficient between a micellar phase and the mobile phase, respectively. The only method which applies to the separation of neutral analytes is MECC. However, in order for micelle formation to occur in MECC, the surfactant used as the pseudostationary phase must be soluble in an aqueous solution at a concentration above the critical micelle concentration (CMC). In point of fact, most neutral analytes are not readily soluble in aqueous solution to the extent

that they are detectable, given the poor concentration sensitivity of CE mentioned before. For this reason, MECC may not necessarily be a viable technique for the separation of many mixtures containing neutral analytes. Figure 4 schematically represents the major classes of CE and indicates their respective separation mechanisms.

In short, Capillary Electrophoresis can offer the benefit of high efficiency, but cannot offer the benefits of high capacity, good detection sensitivity and selectivity.

1.4 Capillary Electrokinetic Chromatography.

There is a perceived need for a technique which can provide higher sample loadings than CE (for improved limits of detection), and the flexibility of working in organic operating solutions or mobile phases. The natural choice of a competitive method falls to liquid chromatography. LC offers excellent selectivities through solvent optimization and careful stationary phase selection, high mass loading due to a high total stationary phase surface area, and the ease of working in organic mobile phases. What LC *cannot* offer is extremely high efficiency, as columns are limited in total length due to the high pressure drop which exists along the length of the tightly packed bed of small particles. CE *can* offer extremely high efficiency, because of (in part) the plug flow profile which electroosmosis presents. In contrast, pressure-driven LC yields a laminar flow profile, which widens analyte bands thus

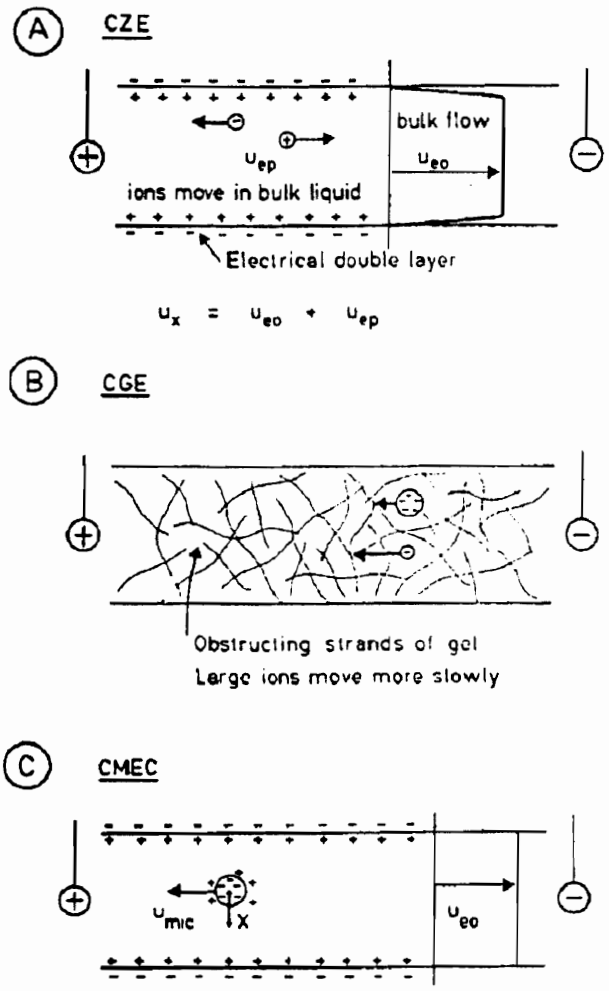


Figure 4. **Several modes of separation commonly employed in CE. (From ref. 18)**

reducing efficiency. (Plug flow and laminar flow are addressed in depth in the Theory section of this dissertation.)

A marriage of the two techniques, CE and packed capillary LC, could potentially fill the niche of a separations method yielding high efficiency, good selectivity, high mass loading, and good detectivity. This type of system has as yet only been touched upon in the literature, being first described in theory by Knox[18] in 1988 and titled Capillary Electrokinetic Chromatography (CEC). Much of the work described in this dissertation is an outgrowth of the tenets of Knoxs' work, and his important contributions are chronicled elsewhere in this manuscript.

In order for CEC to be deemed worthy of study, a careful assessment of its potential benefits must first be conducted. For CEC, a chromatographic technique, chromatographic theory should first be consulted. According to general chromatographic theory, resolving power is described by the master resolution equation:

$$R_S = 1/4 \; (k' \; / \; (1+k')) \; ((\alpha \; -1) \; / \; \alpha) \; (N)^{1/2} \qquad \{2\}$$

Here, it is seen that resolution arises as a function of three factors: k', the capacity factor for an analyte; α, the selectivity of a column for one analyte relative to another; and N, the efficiency of the separation system. Each of these is described separately.

The capacity factor is a thermodynamic parameter which is directly related to the partition coefficient of the analyte between mobile and stationary phases:

$$K = \beta\, k' \qquad \{3\}$$

where: **K = thermodynamic partition coefficient** β **= phase ratio**

In this equation, the phase ratio is the volume of mobile phase in the column divided by the volume of stationary phase. The partition coefficient, K, is a measure of the affinity of an analyte for the stationary phase relative to its affinity for the stationary phase. Simply arrived at, K is a measure of the concentration of analyte in the stationary phase divided by the concentration of analyte in the mobile phase at equilibrium[19]. Since CEC is performed in packed capillaries identical to those used in micropacked capillary HPLC, and since the mobile phases employed in the two techniques are essentially the same, no great change in capacity factor for a particular analyte is expected between the two techniques. CEC will not offer improved resolving power in the capacity factor term of the master resolution equation.

The α term of the master resolution equation conveys the importance of selectivity in the resolution of complex mixtures. Selectivity is a measure of analyte affinity for the stationary phase relative to that of another analyte. Alpha is in fact the ratio of partition coefficients for the two analytes. It is in this term that HPLC derives its power as a separations technique, since the selectivity of HPLC systems is readily tunable by way of changing either mobile phase or stationary phase composition. Once again, the similarities of CEC and micropacked capillary HPLC seem to indicate that neither will have the edge in providing more resolving power than the other due to changes in

selectivity. The two techniques should in fact offer almost identical selectivities. In Capillary Electrokinetic Chromatography as in HPLC, selectivity is a major contributor to total resolving power, since both the mobile and stationary phases can be changed in order to tune α.

Finally, system efficiency is listed as a contributor to resolution. Efficiency (N) is measured as a total number of achievable theoretical plates; the greater the value of N, the better the system functions in terms of resolving power. Efficiency is calculated from chromatographic data using the following equation[20]:

$$N = (t_r / \sigma)^2 \qquad \{4\}$$

where: t_r = **retention time of an eluting analyte**

σ = **standard deviation of the analyte distribution**

Efficiency is not the strong suit of HPLC, where columns generate about 12,000 theoretical plates; but it is indeed the strong suit of CE which easily generates efficiencies of 100,000 theoretical plates. Likewise for other electrokinetic methods, including CEC, total measured efficiencies are higher than is typical for packed capillary HPLC. As in Capillary Electrophoresis, the plug (or bulk) flow profile of CEC tends to keep band spreading, measured as σ, to a minimum. According to the equation for N, above, this in turn yields higher efficiency. Put simply, the efficiency term of the master resolution equation will be greater in CEC than it is in micropacked capillary HPLC. For this reason, the

total resolving power of CEC should be superior to that of micropacked capillary HPLC.

The benefits which could foreseeably be offered by CEC, then, include:

1. The high efficiencies afforded by a bulk flow profile,
2. The high selectivities typifying liquid chromatographic sorbents,
3. Increased sensitivity over that in CE due to higher mass loading,
4. Ease of interfacing to existing CE detectors, and
5. High resolving power relative to both CE and LC.

1.5 Summary.

The chief goal of the research enumerated in this text has been to provide a separation technique which yields high resolving power in the liquid phase. In effect, the goal has been to find a liquid phase counterpart to capillary GC: an efficient, rapid, flexible, and practical separation technique. A significant portion of this research has therefore been dedicated to generating improved efficiency in LC systems by decreasing analyte band broadening through: 1) changing the column geometry, or; 2) providing a different means of analyte transport such that the rate of band broadening is decreased.

CHAPTER II

HISTORICAL

2.1 Chromatographic Separations.

In 1906, a paper was published by Michael Tswett, a Russian botanist, which described a process by which plant pigment extracts could be fractionated into their various components[21]. This publication, which utilized calcium carbonate as the stationary phase and a liquid mobile phase in an adsorption chromatographic mechanism, marked the beginnings of a new analytical technique (chromatography) which would revolutionize chemical and biochemical measurements. Unfortunately, widespread acceptance of chromatographic separations was forced to wait until the early 1930's, when Richard Kuhn and coworkers[22] again used adsorption chromatography in separations of carotenes from egg yolk samples. This research effort in liquid chromatography was published in 1931. It was gas chromatography, though, that marked the beginning of rapid growth in chromatographic separations research, with liquid chromatography lagging behind insofar as high performance applications are concerned.

The publication in 1941 of a paper describing the theory of partition chromatography [23] ushered in a new era of separations research. Martin and Synges' Nobel Prize winning publication outlined a separation process of partitioning analytes between two liquid phases, one of which was held stationary. In the same treatise, the foundations of chromatographic theory were laid, as Martin compared the partitioning process to distillation via the

"plate theory ". Interestingly, this same paper pointed out the possible benefits of gas-liquid chromatography, which soon began to develop at a faster pace than liquid chromatography. Later, in 1959, Craig[24] also published on the plate theory and described another liquid-liquid separation technique which he called Countercurrent Distribution.

The plate theory, though, did not mark the end of the theoretical description of chromatographic processes. Probably the second most influential contribution in the history of chromatography (second, that is, to Martin and Synges' work) is the classical van Deemter equation introduced by the Dutch chemists van Deemter, Zuiderweg, and Klinkenberg[25]. Their efforts introduced the chemical community to the "rate theory," and described the rate of band broadening in terms of thermodynamic and kinetic parameters. It was at this point that the advantage of downsizing chromatographic systems first became interesting to researchers. According to the original van Deemter equation, higher efficiencies would be achievable with small support particles and thin stationary phase films:

$$H = \{ A \} + \{ B / \mu \} + \{ C * \mu \} \qquad \{5a\}$$

where "A" represents a "multipath effect," "B" represents molecular diffusion, and "C" indicates mass transfer. More completely, the equation is expressed as:

$$H = \{2\lambda d_p\} + \{2\gamma D_m/\mu\} + \{1/30 \ (k' d_p^2 \mu/(1+k')^2 D_{sz})\} \qquad \{5b\}$$

Here, "H" is defined as the height equivalent to a theoretical plate, and:

λ = **packing factor** d_p = **particle diameter**

γ = **obstruction factor** k' = **capacity factor**

μ = **average linear velocity of the mobile phase**

D_m = **diffusion coefficient of solute in the mobile phase**

D_{sz} = **diffusion coefficient of solute within the particles**

The juxtaposition of the μ term in the equation (most easily visualized in equation 5a) suggests that a certain flow velocity exists at which H is minimized. This point is represented as a minimum in the familiar curve bearing van Deemters' name: a plot of H as a function of μ. Theory and experiment seemed to agree, and therefore the van Deemter equation became useful in determining which column parameters could be changed in order to affect a given experimental result.

In 1957, Marcel Golay introduced his idea of ultimate efficiency through miniaturization: Capillary Gas Chromatography[26]. In open tubular GC, the need for a coated support particle was alleviated by reducing the column inside diameter to capillary dimensions and coating the wall of the tube with a thin layer of the stationary phase. Golays' experimental results supported the previously proposed rate theory, lending even more credibility to the Dutch equation. In 1958, Golay made some important modifications to the rate equation[27] in order that it might better describe the physical events occurring in an open tubular column. The multipath ("A") term was dropped, since in an open tube only one path through the column exists.

GC led the way to rapid, reliable qualitative and quantitative chemical analysis through automation. The fact that GC is limited to the separation of volatile analytes, though, opened the door for research into liquid phase separations. The usefulness and accuracy of the rate theory in GC suggested that it might be applicable to other forms of chromatography as well, and research into the modernization of liquid chromatography began.

2.2 Liquid Chromatography.

The marked success of Gas Chromatography, and the amazing accuracy of the theoretical description of its' processes, led many researchers to believe that Liquid Chromatography (LC) could be similarly described. A good theoretical description could then be utilized to guide efforts in updating and streamlining LC to the point that it might be as useful and practical as GC had proven to be. Spackman, Stein, and Moore[28], in 1958, automated the process of amino acid analysis and started the push toward research in updated LC techniques. In 1959 Giddings[29] published some work on the use of LC under "high performance" conditions. With these efforts and others, the process of modernization of LC began.

Improvements in the theoretical description of chromatographic processes came in the early 1960's. Of particular note is the text presented in 1965 by J. Calvin Giddings entitled Dynamics of Chromatography[30], in which several modifications to the rate equation are explained.

Chief among the improvements specified by Giddings is the proper placement of the eddy diffusion or multipath term (formerly the "A" term in van Deemters' equation) within the mobile phase mass transfer term. In this modification, Giddings recognizes that diffusion in the mobile phase occurs both axially and radially. Diffusion axially leads to band dispersion and an efficiency reduction, which the van Deemter equation recognized. Radial diffusion, however, aids in maintaining band integrity by effectively time averaging the flow velocities experienced by different analyte molecules. The original rate equation did not account for this dependence of the multipath term on mobile phase mass transfer, and Giddings' improvement on the equation allowed the prevalent theory on efficiency to better parallel experimental results. This modification is commonly referred to as the "coupled equation," as it points out the coupling of the multipath and mass transfer effects.

Another important message of the equation, in the context of this dissertation, is that miniaturized chromatographic parameters can potentially yield improved separations. Particle diameters should be made as small as is practically possible. Unfortunately, the equation cannot convey the difficulties which plague the process of downsizing. Smaller particles are more difficult to pack well, and once packed into a column yield a much higher pressure drop per unit length. This makes small plate heights possible, but requires that column length be kept shorter and therefore total efficiencies are not as high as might be desirable.

John Knox, at the University of Edinburgh, was in agreement with Giddings on the idea of the coupled rate equation. His means of demonstrating

the coupling effect, however, was somewhat different from the approach Giddings took. While Giddings chose to focus on the effects of decreasing particle size, Knox had an interest in the effect of decreasing column inside diameter. In 1965 Knox presented data which seemed to indicate that the coupling of eddy diffusion and mass transfer was real[31]. His experiment was conducted by packing glass beads into tubing of diameters between 3 and 6mm, injecting a potassium permanganate solution, and eluting it with 10% aqueous potassium nitrate. He found that measured values of plate height more closely corresponded to the predictions of the coupled rate theory than those of the original van Deemter equation, especially at high mobile phase linear velocity where the two equations vary most widely in their predictions. Knox also indicated that the ratio of column diameter to particle diameter seemed to be of some importance in determining plate height.

Horvath, Preis, and Lipsky[32], in 1967, made specific use of microscale LC for perhaps the first time. Drawing on the tenets of the coupled rate equation and the work of Knox, a separation of nucleotides was achieved in a column of 1mm inside diameter. As noted in their text, the choice of small inside diameter was made in order to alleviate transcolumn flow inhomogeneities which degrade column performance. Their implication was that decreasing column inside diameter while holding particle diameter constant could possibly result in an improvement in system performance, based on the ideas presented by Giddings in his description of the rate theory. Later, in the Theory section of this dissertation, the topic of flow inhomogeneity is discussed in great detail, and it is proposed that alleviating flow inhomogeneitites can lead directly to improved efficiency by decreasing the

mobile phase mass transfer term contribution to plate height. As Horvaths' and Knoxs' studies suggested, decreasing the column inside diameter relative to the particle diameter may result in a more uniform arrangement of particles within the packed bed. This in turn could make differences between mobile phase flow velocity near the wall of the column and that near the center of the column less pronounced, and could increase column efficiency. Microscale HPLC had become a possibility in the quest to improve LC performance.

2.3 Microscale High-Performance Liquid Chromatography.

Snyder and Kirkland[33] attribute a special section on LC at the Fifth International Symposium on Advances in Chromatography with bringing LC to the forefront in separations research. In their estimation, the 1969 symposium marked the beginnings of the practice of "Modern LC". Indeed, several research endeavors important to microscale HPLC, and therefore to the work described in this manuscript, were publicized in 1969. In particular, Jack Kirkland studied column inside diameter effects on efficiency[34] and found that increased plate counts were achievable when column inside diameter was reduced from 3.2mm to 2.1mm. When a further reduction of inside diameter to 1.6mm was studied, a loss in efficiency was noted. Kirkland suspected that extracolumn effects were responsible, though he had gone to great lengths to minimize extracolumn volume. This note did not bode well for microscale LC, but it is important to recognize that Kirkland did not implicate the smaller 1.6mm ID column itself in assigning a cause for the decrease in efficiency.

Also in 1969, Knox[35] returned to the study of column inside diameter which he had embarked on in 1965. This work studied the interrelationship between column inside diameter and particle diameter which had seemed important in determining efficiency in the earlier experiments. The columns used in this study were not microscale columns, but the results are important in the study of micro-LC. In these experiments, column inside diameters ranged from 11.1 to 3.3mm, and column length was 150cm. 480 micron diameter glass beads were chiefly employed as a packing material, and kinetic

effects which would complicate interpretation of the results were avoided by making measurements on the elution of an unretained solute.

Knox and Parcher in part interpreted their data by constructing plots of the reduced plate height as a function of ρ, the Knox-Parcher ratio. (The Knox-Parcher ratio is defined as the column internal diameter divided by the packing material particle diameter.) A typical Knox-Parcher plot was shown previously in figure 2. According to the rate theory, these plots should result in an unbroken line with positive slope, since the plate height should increase with increasing particle diameter. Interestingly, the results obtained in these experiments showed a break in the line at Knox-Parcher ratios between 4 and 6. The interpretation of the data indicates that lower reduced plate heights (higher efficiencies) are achieved when a more ordered packing structure exists, as is the case when particle diameter is about 1/5th of the column ID.

It took until 1974, though, for serious investigation into micro-LC for reasons based solely on the benefits of miniaturization to begin. At this point in time, Ishii[36], and Scott & Kucera[37], began their studies in micro-LC. The reasoning for these efforts lay partly in the rate theory mentioned above.

In 1974, Daido Ishii[36], in a Jasco technical report, presented some of the first data on miniaturized LC systems. Ishii realized that for micro-LC to be successful, careful attention would have to be given to each phase of the separations process. He therefore addressed column packing and chromatographic system design. Several facets of system design were described in detail by Ishii and coworkers between 1974 and 1977[38-40].

Column packing had been studied extensively by the mid 1970's, with some of the best columns being manufactured in the usual stainless steel

tubing by the balanced density slurry packing method[41]. In this method, the density of the slurry solvent was closely matched to that of the packing material to be suspended, requiring the use of solvents such as carbon tetrachloride. These slurries had the property of staying well suspended such that particle "sizing" did not occur, the result being a more homogeneous packed bed. High pressures aided in the formation of a well-packed, even chromatographic bed, with typical displacement pressures falling in the range of 10 to 15 thousand psi. For Ishii's work, these pressures were excessive, as his column material was PTFE, which could not withstand pressure this high. He therefore developed an alternative packing method in which a slurry of the desired packing material was made, and the slurry pulled into the column blank under syringe vacuum. As the bed would not then be very tightly packed, a compression step was then required. The column was fritted at one end with a glass wool plug, and pressure was applied with a syringe pump in order to consolidate the bed. Ishii's columns generated 50 to 80% of theoretical efficiency as compared to the 70 to 90% typical of balanced-density slurry packed steel columns.

Extracolumn volume, in all types of column chromatography, degrades efficiency. Dead volume tolerances in micro-LC are extremely low, being on the order of 1 microlitre for Ishii's 200 to 500 micron ID columns. This required that detector cell design be modified, which Ishii accomplished by directly coupling a quartz flow cell to the end of the micropacked PTFE column, and detecting as close to the end of the packed bed as possible using a UV detector. Sensitivity was sacrificed because the path length of the detector cell

was short, being about equivalent to the column inside diameter, but extracolumn volume was minimal.

The invention of fused silica capillary tubing provided a new medium for constructing micro-LC columns. These capillaries were developed at Hewlett-Packard's Avondale Division by Dandeneau and Zerenner[42] in 1979. The first investigator to study LC in fused silica capillaries was Frank Yang[43] of Varian Associates, who in 1980 prepared a series of open tubular columns. These columns were derivatized with octadecylsiloxane in several inside diameters, the best results being obtained with 30 micron ID capillaries. In this publication, Yang proposed in situ or on-column detection, possible easily if the protective polyimide coating is removed from a small section (2-10mm) of the capillary. This design outperformed even Ishii's in that it effectively eliminated any extracolumn volume contribution from a detection cell.

One year later, in 1981, Yang again used on-column detection but this time in packed fused silica capillaries[44]. Figure 5 shows the general design of Yangs' detection scheme. By using a packed capillary format, Yang drastically increased the loading capacity of the column, and was able to demonstrate marked improvements in sensitivity over the open tubular design. A 20x improvement in signal intensity was demonstrated over conventional 4.6 mm ID columns, though the operating conditions used did slightly skew the results in favor of the capillary system.

Yang's work sparked interest in packed fused silica capillary columns for LC, though only Yang was capable of producing columns which performed at close to theoretical maximum efficiency. In order to offset their poorer plate heights, Ishii and Takeuchi[45], in 1982, decided to increase their total

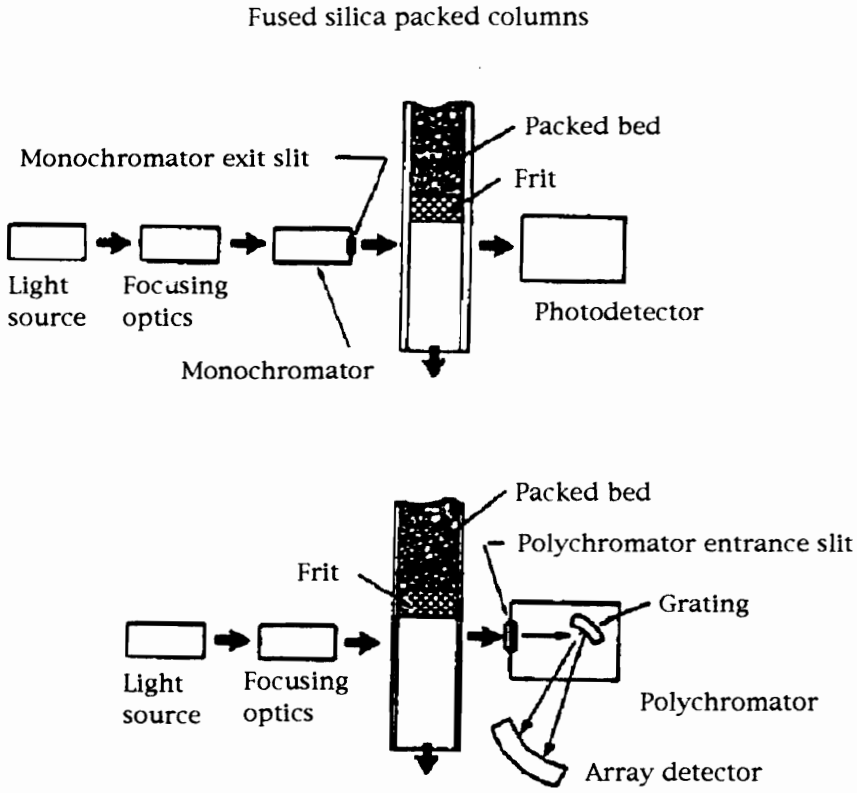


Figure 5. On-column detection in capillary microseparations. (From ref. 43)

efficiency by coupling several columns in series through the use of extremely low dead volume unions. Their efforts provided good results, and generated plate counts which were close to the sum of the efficiencies of the joined columns. But as mentioned earlier, manufacturing a good packed capillary column is a difficult task. Manufacturing several was not practical, and was time consuming.

In 1983, Milos Novotny[46] at Indiana University undertook a research effort which would attempt to elucidate the proper conditions for the production of high-quality packed capillary columns in fused silica. The study found that denser slurries (ca. 3.8 : 1), when used with a less viscous solvent (acetonitrile) as both slurry and displacement fluid, yielded columns with better performance characteristics. The use of acetonitrile had the pleasant side-effect of decreasing the time required to pack and depressurize the columns as well.

The advent of capillary electrophoresis in this time frame (the early 1980's) diverted much interest. Many of the dedicated microseparations researchers began research programs in CE, which also made use of the fused silica capillaries and on-column detection mentioned earlier.

2.4 Zone Electrophoresis.

The early development of electrophoresis began as a natural consequence of Faradays' 1791 presentation of the laws of electrolysis. First mentions of the theory that was to spawn the technique of electrophoresis

came in the early 1800's from Wiedemann[47]. After these early efforts, Helmholtz[48], in 1877, performed experiments in the application of an electric field across a glass capillary filled with a salt solution. He was able to determine that the wall of the capillary acquired a negative charge on application of the potential field, and that particles of opposite charge loosely associated themselves with the charged wall. As would be normally expected, the cationic species associated with the wall were noted to move toward the cathodic end of the capillary, and the process of electroosmosis, also called electroendosmosis, was discovered. Even at this early stage, Helmholtz was able to describe in mathematical form the electroosmotic phenomenon.

Electroosmosis was put to early use with the experiments of Hittorf[49] (1853), Nernst[50] (1897), and Kohlrausch[51] (1897), who used a system similar to Helmholtz' to determine the ionic radii and specific conductances of small ions in solution. In fact, all three experimenters tabulated transport numbers for a variety of ions.

Kohlrausch made another important contribution to the study of electrophoresis in deriving the Kohlrausch regulating function[52] in 1897. Using this function, the order of migration and the concentration of a series of ions could be determined. Further research by Kohlrausch yielded equations which describe the interaction of an electric field at a boundary within the system. Of greater importance at the time, though, was the application of Kohlrausches' discoveries to the boundaries between moving zones in an electrophoretic separations system. In particular, the high resolution separations of proteins, which required very sharp and highly

concentrated bands, would have been much more difficult to realize without the work of Kohlrausch.

Separations of mixtures became an area of major importance and interest in the early 20th century. Tiselius, in 1937, began his work in electrophoresis[53] and demonstrated the utility of the technique in the characterization of proteins. Also interested in protein purification was Svedberg[54], who received the Nobel Prize for his invention of the ultracentrifuge. It is important to note that neither technique (electrophoresis or ultracentrifugation) alone was sufficient for the resolution of all of the components of a complex mixture of proteins.

Tiselius' work in 1937 was some of the first performed in U-shaped electrophoresis chambers. He also introduced the use of photography as a detection technique, photographing in the UV region of the electromagnetic spectrum: perhaps the first documented use of UV detection in electrophoresis. At this point, one of the major limitations of electrophoresis was both recognized and treated (in part) by Tiselius[55]. Resistive heating of the operating solution in the electrophoretic U-tube led to band dispersion via thermal convection, decreasing the resolving power of the system. In response to this, Tiselius designed a system in which a rectangular U-tube was cooled to 4° C. This invention was paramount to one of the most important discoveries of the 20th century: the isolation of γ-globulin. From serum albumin, Tiselius isolated α-, β-, γ-, and δ-globulin. Deservedly, Tiselius was presented a Nobel Prize in 1948 for adsorption chromatography and moving boundary electrophoresis, two separations techniques which are still today in regular use.

Like the moving-boundary electrophoresis discussed above, more modern techniques of electrophoresis, such as isotachophoresis and zone electrophoresis, are described readily by Kohlrausches' theoretical relations. One of these newer techniques, isotachophoresis, sprang from the separation of compounds in solution based on their isoelectric points. This discovery by Hardy[56] in 1905 allowed for the 1909 work of Michaelis[57] who was able to characterize enzymes based on their isoelectric point (pI) as determined via electrophoresis. The necessary pI data is collected simply by running a series of electrophoretic experiments at a variety of pH conditions. This important work led to the 1923 discovery of an electrophoretic technique based on a principle other than moving boundary electrophoresis. The "Ion Migration Method" of Kendall and Crittenden[58] was useful for the separation of rare earth metals. Kendall and Crittenden discovered that ions in solution can obey Kohlrausches' regulating function by both separation and concentration adaptation to the concentration of the first zone.

A difficulty of the Ion Migration Method was the close spacing of the separated bands of materials in solution, making visualization of the separation troublesome. Also, real-time detection techniques were not readily available. To this end, Kendall[59] suggested in 1928 the addition of species to the sample which had migration values intermediate to the analytes of interest. These were later called "spacers," and were selected such that they were coloured in solution. They provided a convenient means of detection. In the same 1928 publication in which he suggested the use of coloured spacers, Kendall mentioned that other methods of detection (namely spectrometry, thermometry, and conductivity) should be explored. "Displacement

Electrophoresis" was another moving zone technique explored in the mid 1900's. Martin, in 1942, used the technique to affect a separation of glutamate, aspartate, chloride, and acetate, which he later referenced in a work published with Everaerts[60].

Although much good work in the field of electrophoresis took place in the 1940's and 1950's, it took until about 1960 for another major breakthrough to be made. In the early 60's, the practice of electrophoresis in tubes of small inside diameter (<500microns) became popular. With a technique developed in 1967 by Hjerten[61], electroosmosis could be eliminated via a surface-coating process. Hjerten developed a system in which all of the previously determined factors affecting on separation quality were optimized. Electroosmosis was eliminated by surface treatment, thermal convection was minimized by cooling, and sensitive, real-time detection (UV at 280nm) was employed. This system generated some of the most alluring data of the time, providing separations of proteins at concentrations in the 0.1% range, requiring extremely small amounts of sample, and yielding high resolution.

Isotachophoresis, as an individual technique, was championed by LKB, which manufactured and sold a displacement electrophoresis instrument advertised specifically as an isotachophoresis instrument. This instrument operated on the principle that once zones of analytes had been established within the capillary, as in Tiselius' early experiments, these zones would be moved through the capillary (all at the same velocity) such that all resolution would be maintained. Isotachophoresis to a large degree has served as the forerunner of Capillary Electrophoresis (CE), in that it is frequently performed under conditions very similar to those employed in CE. The use of

on-line UV (Arlinger and Routs[62], in 1970) and conductivity (Verheggen[63], in 1972) detectors, and high potential fields (100 to 300 V/cm) have provided high resolution separations with excellent elution time precision and good sensitivity.

2.5 Capillary Electrophoresis.

The practice of electrophoresis in very small diameter glass and fused silica capillaries has been termed capillary electrophoresis. Several separation mechanisms have been employed, including two-phase partitioning (Micellar Electrokinetic Capillary Chromatography or MECC) invented by Shigeru Terabe[64] in 1984, free zone electrophoresis (Capillary Zone Electrophoresis or CZE) studied in depth by Jorgenson[65], and capillary isotachophoresis. Typical capillary inside diameters are in the 50 to 200 micron range, and the potentials applied across the tubes are in the vicinity of 200 V/cm. Many types of detection have been described, with UV being the most widely used to date.

The introduction of CE has been credited to Mikkers, Everaerts, and Verheggen[66], who in 1979 published a work in which they resolved a complex, sixteen component mixture in under 10 minutes. It is notable that their system, which made use of a 200μm inside diameter PTFE capillary, maximized the resolving power of CE by operating in the absence of electroosmotic flow, as Hjerten had previously done in surface coated glass tubing.

Little work was performed in the arena of CE until the 1981 demonstration by Jorgenson and Lukacs[67] of electrophoresis in fused silica capillaries. CZE rapidly gained popularity as a high resolution technique for the separation of charged species in solution. Note, however, that it was only useful (in the free-zone format) as a method for the resolution of charged species. This limitation led to much effort in the extension of the technique to neutral species.

Terabe and coworkers[68] (1986) finally arrived at a solution to the problem of separating neutral analytes by developing MECC (which is sometimes also referred to as MEKC). In this technique, an anionic surfactant is added to the operating buffer of the CE system at a concentration in excess of the critical micelle concentration (CMC) of the surfactant. This allows for the transient formation of micelles, which have been referred to as a "pseudostationary phase." The process of electroosmosis carries the micelles toward the cathode, despite the electrophoretic vector of the surfactant towards the anode, as the electroosmotic velocity here is greater than the electrophoretic velocity. The movement of the micelles is at the velocity which represents the vector sum of electrophoresis and electroosmosis. A neutral analyte which is associated with a micelle moves also at that velocity, though the same molecule in free solution moves at the electroosmotic velocity. Molecules which have a greater affinity for the pseudostationary phase, then, are selectively retained for a longer period, allowing for a separation to occur based on the partition coefficients of the analytes similarly to reversed phase HPLC.

The invention of MECC did not, however, lead CE to maturity. At the time, many problems still faced (and to this day surround) the young science. As close observation indicates, the sample capacity of a 50 to 200 micron I. D. capillary is woefully inadequate to allow for reasonable concentration sensitivity. An increase in sample load, after a point, begins to markedly hamper the resolving power of the technique by degrading efficiency. Clearly, the injection of larger sample volumes is not a feasible solution to poor sensitivity. Remembering the early electrophoretic experiments in large tubes, and the inherent loss of efficiency due to excessive heating in the large volumes of these tubes, the use of bigger capillaries to increase capacity was also rejected. To increase sensitivity, then, new detection schemes have been explored. Two of the most sensitive techniques employed include amperometric detection[69], demonstrated by Andrew Ewing in 1987, and laser induced fluorescence detection[70], studied by James Jorgenson in 1986.

Although the invention of MECC by Terabe broadened the applicability of capillary electrophoresis to include some neutral species, it did not broaden the technique to the point of universal utility. That is, any compound which is to be resolved from a mixture by MECC must be soluble in the operating buffer which is being used in the separations system. In order to allow for the formation of micelles critical to the operation of the technique, only small amounts of organic modifier can be added to the aqueous buffer. Many mixtures capable of being separated by a partitioning mechanism are only sparingly soluble in aqueous phases. These thoughts lead to a paradox: a mixture of relatively nonpolar compounds can be resolved by partitioning (as

in MECC), but may be inherently insoluble in the aqueous phase used within the separation capillary.

2.6 Capillary Electrokinetic Chromatography.

In order to make use of CE's ultra-high efficiencies for a wealth of neutral compounds, a technique which allows the use of high concentrations of organic solvents, provides a means of partitioning, allows higher sample capacity than CZE or MECC for improved sensitivity, and still uses an applied potential as the motive force for affecting a separation is desired.

Victor Pretorius[71], in 1974, described electroosmosis in packed glass tubes under the influence of a high potential field. His work described only measurements made using unretained solutes, but was encouraging in that high efficiencies were measured. Pretorius also found that, as theory would indicate, irregularities in the packed bed did not affect chromatographic performance. Pretorius also provided an equation for electroosmotic flow velocity:

$$v_{eo} = [\, \varepsilon \zeta / 4 \pi \nu \,] E \qquad \{6\}$$

where: **e** = **solvent dielectric constant** **n** = **solvent viscosity**
E = **applied potential** **z** = **zeta potential.**

The article pointed out the lack of any geometric constraint on electroosmosis, which is paramount to the operation of electrochromatography in packed capillaries.

A similar attempt at the use of electroosmosis as the motive force for the mobile phase in HPLC was made by Jorgenson and Lukacs[72] in 1981. The UNC effort made use of a pyrex glass tube of 170 micron ID 68cm in length. For the tube to be packed, and end frit was constructed at one end of the column by filling about 5mm of the tube with a common silica-based HPLC packing material of 30 micron diameter. Brief application of heat to the packed zone sintered the material into a porous frit. The column was then packed with a 10 micron reversed-phase packing slurried in acetonitrile. After packing, the column was dried by applying gas pressure, and the other end fritted as the first was. The apparatus used was the same as that commonly employed in capillary electrophoresis, shown earlier in figure 3. Using acetonitrile as a mobile phase and applying 30kV resulted in a separation of the test sample (9-methyl anthracene & perylene) in about 45 minutes. Although the method seemed slow, it generated over 20,000 theoretical plates for both analytes, corresponding to reduced plate heights of 1.9 and 2.5. Given that the practical optimum for packed columns is accepted to be 2.5, these values should have generated much interest and excitement. The main thrust of the publication, though, was free zone electrophoresis, which was gaining proponents quickly, and the packed capillary work went virtually unnoticed. Jorgenson also mentioned directly in the article that the technique was "a bit difficult and inconvenient to work with" and indicated that the performance enhancement provided "may not justify the increased difficulty in working

with electroosmotic flow." Apparently, the research community accepted his advice.

It should be reiterated that the work Jorgenson presented was performed in glass capillaries, which certainly added to the difficulty of column production and use. After this 1981 effort, when the much more rugged fused silica capillary tubing was more readily available, the outlook may have been much brighter.

John Knox[73] described the theory of operation of CEC in 1988. His paper, entitled "Thermal Effects and Band Spreading in Capillary Electro-Separation," presented an overview of the theory of CEC, CZE, MECC, and CGE. In it, he also referenced the work he and I.W. Grant presented at the 16th International Symposium on Column Liquid Chromatography in June of 1988. Unfortunately, CEC still was not widely researched, again presumably because of the popularity of the other electrokinetic techniques.

The most recent research effort in CEC was published in October of 1991 by John Knox[74]. Here, Knox used both slurry packed capillaries and drawn-packed capillaries in a comprehensive study of the effect of particle size on electroosmotic flow velocity. He found that particle size had essentially no effect on velocity, and that electrolyte concentration in the mobile phase also had only a small effect on flow velocity. By constructing a plot of plate height and flow velocity versus electrolyte concentration, Knox was able to ascertain that an electrolyte concentration of about 0.002 M resulted in a relatively fast analysis with high efficiency. He applied the system in measurements of aromatic hydrocarbons by reversed phase electrochromatography. In this

first concentrated effort into capillary electrokinetic chromatography, reduced plate heights as low as 1.3 were measured.

CHAPTER III

THEORY

3.1 Efficiency.

As previously discussed, efficiency in liquid chromatographic methods is described by the van Deemter equation. Efficiency is a measure of the quality of the chromatographic column, a means of assessing the potential the column has for delivering good separations based on its physical characteristics and the operating parameters employed. The chemical characteristics of the column are not implicated here, as they are covered by selectivity and capacity factor descriptors. In a true sense, efficiency should not be thought of as a characteristic of a column, but rather as a characteristic of the entire system of which the column is the major part[75]. For this reason, it is important to carefully design a separations system such that each part of it: injection valve, connective tubing, column, and detector cell, contributes minimally to loss of efficiency.

In order to establish a better understanding of the concept of efficiency, the concepts of plate height and reduced plate height are introduced. Until the advent of chromatography, one of the principle methods of separating mixtures was distillation. In the process of distillation, components of the mixture are separated based on their boiling points, by vaporizing them and condensing them onto stationary plates within a cooled column. The larger the number of plates in a column, the more capable it was of fractionating complex mixtures containing compounds with similar boiling points, to a reasonable approximation. In other words, the greater the number

of plates, the more efficient the column. Another way of viewing this was that, in a certain length of column, the largest number of plates could be included and the highest efficiency achieved if the distance between the plates (the height of a plate, H) was kept small. The smaller H is, the more efficient the column is. This concept was widely used by petroleum chemists, so the use of theoretical plates as a measure of column efficiency in chromatographic separations was natural. The length of chromatographic column traversed in the process of partitioning between the mobile phase and the stationary phase is viewed as one plate of height H. Dividing the plate height H by the particle diameter d_p results in a reduced (dimensionless) parameter, h, the reduced plate height. This term can be used to compare column efficiency between columns of different length and different particle diameter without bias. Unfortunately, the plate theory was not as comprehensive and descriptive as it should have been, and so the rate theory was proposed.

The rate theory was proposed by van Deemter et al, as described earlier. The rate equation has undergone a number of important revisions, one of the most comprehensive treatments being that presented by Giddings[76]:

$$H = \left\{(2\psi D_m/\mu) + (2\psi_S D_S (1-R_R)/\mu R_R)\right\} + \left\{q (k'/(1 + k')^2 (d_f^2\mu/D_S)\right\} + \left\{\omega d_p^2\mu/D_m\right\} + 1/\left\{(1/2\lambda d_p) + (1/H_{mtm})\right\} \quad \{7\}$$

where: R_R = retention ratio (= t_m/t_r);

H_{mtm} = $\{\omega\ d_p^2\ \mu\ /\ D_m\}$;

t_r = retention time of a solute;

q = configuration factor;

t_m = dead time; and

ω = packing factor.

The motivating factors for the research contained in this text lie in this equation, and for that reason each term of the equation will be discussed in depth.

The first contributor to H is molecular diffusion:

$$H\ \alpha\ \left\{(2\psi D_m/\mu)\ +\ (2\psi_S\ D_S\ (1-R_R)/\mu\ R_R)\right\} \qquad \{8\}$$

Molecular diffusion is described by Ficks laws[77], the first of which was described in 1855, making this term one of the best defined and best understood of the contributors to plate height. If a band of analyte is injected using an injection valve, at the time of injection the analyte appears as a concentrated plug of sample. As a function of time, this plug will become increasingly ragged at its edges and will become broader (see figure 6), as Fick's laws describe: "the flux of matter is proportional to the concentration gradient in the solution."[78] The process of diffusion arises because of the random motion of molecules in solution, and the fact that regions of high concentration diffuse is simply a result of statistics and probability. That random motion gives rise to diffusion, though, is important. In random

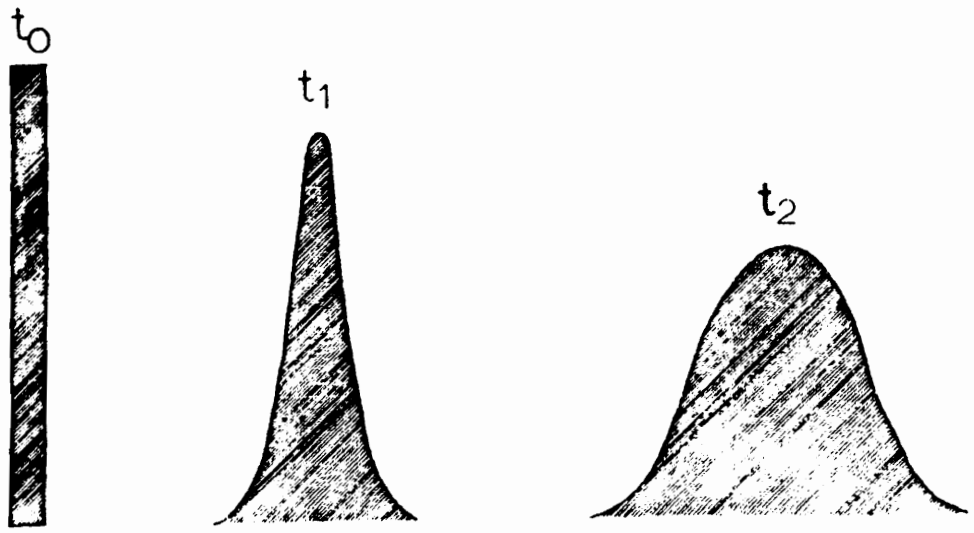


Figure 6. **Band broadening, partly arising from diffusion in the mobile phase as a function of time. (From ref. 121)**

motion, or in a random walk, it is simple to define x as a distance covered by a molecule in a time interval Δt. The mean square distance moved in that time interval is directly proportional to the time period over which the movement occurs. Einstein[79] determined that the proportionality constant was twice the diffusion coefficient of the analyte:

$$x^2_{avg} = 2D\ \Delta t \qquad \{9\}$$

where: x^2_{avg} = **mean square distance;**

D = diffusion coefficient; and

Δt = **time.**

That distance is related to time here as a squared function is fortuitous, since this means that diffusion over long distances is a slow process. Also, in HPLC, this term is a minor contributor to plate height since diffusion coefficients of analytes in liquid media are quite small compared to diffusion in a gas. Note that the diffusion term of the van Deemter equation is inversely proportional to mobile phase flow velocity: the faster an analyte is removed from the column, the less time it has to diffuse and band broaden. Optimization of the molecular diffusion term through the use of faster flow velocities is not a realistic possibility for improving on system efficiency in HPLC, because of the larger negative effects which high mobile phase velocities have on the other terms of the rate equation. The "B" term contribution to band spreading is illustrated in figure 6, which in fact points out the benefits of working at

high mobile phase velocity in order to minimize plate height contributions of molecular diffusion.

The most important modifications to the rate theory appear in the mass transfer ("C") term which is expanded, describing mass transfer in both the mobile and stationary phases independently. Stationary phase mass transfer is found to be a function of the capacity factor for the analyte, a configuration factor (constant), and two other important factors; the square of the thickness of the stationary phase film and the diffusion coefficient of the analyte in the stationary phase:

$$H \alpha \{q\ (k' / (1 + k')^2\ (d_f^2\ \mu / D_S)\} \qquad \{10\}$$

where: q = configuration factor;

d_f^2 = film thickness; and

D_S = diffusion coefficient.

The equation suggests that film thicknesses should be exceedingly small for this term to contribute minimally to plate height (to maximize efficiency). In the case of bonded phase HPLC packings, the stationary phase is a chemically bonded monolayer of a small organic moiety, making the "film" thickness extremely small. This term also indicates the important role temperature control plays in HPLC separations. Changes in temperature affect diffusion coefficients in liquids markedly, and therefore can alter efficiency and (more importantly) selectivity.

The most important and effective changes in the rate theory are seen in the mobile phase mass transfer term. Here, Giddings noted the dependence of eddy diffusion (the "multipath effect") on flow by moving the "A" term, formerly $A = 2\ \lambda\ d_p$, which appeared at the beginning of the van Deemter equation, into the "C" term. He termed the process which made this change in the theory necessary the "coupling effect."

The change was motivated by the fact that diffusion of an analyte in the mobile phase is equally facile and equally likely in all directions, both in the direction of flow and radially. Radial diffusion would have the effect of placing an analyte into different "streampaths" at different points in time. As

figure 7 indicates, the time spent in each of the different streampaths can be viewed as a series of individual steps. A highly segmented path (a path with many steps) would result in a *larger total number* of steps of *shorter* length, while a less fragmented path would have *few steps*, but each with a *greater length*. Giddings' proposal certainly had a more realistic basis than that presented in the original rate equation, and offered a pleasant surprise: plate height, which should be decreased for best efficiency, is *directly* proportional to the *number* of steps, but increases as the *square* of the step *length*. This meant that the more realistic, multistep path resulted in lower plate heights (better efficiency) than was previously thought possible according to theory for higher linear velocities. This is illustrated in figure 8, which shows the C term contribution to plate height for both the coupled and the non-coupled terms.

The term which deserves closest attention in terms of improving system performance is the mobile phase mass transfer (C_m) term just described. According to Giddings, "zone spreading itself originates in the velocity inequalities of the flow pattern, but the extent of spreading is governed largely by diffusion between fast and slow stream paths."[80] He continues on to note that five particular velocity inequalities which result in zone spreading include:

1. *transchannel* flow velocity inhomogeneity, velocity differences within a single flow path;
2. *transparticle* flow velocity inhomogeneity, exchange of analyte between stagnant mobile phase within pores of a porous packing material and the moving mobile phase;

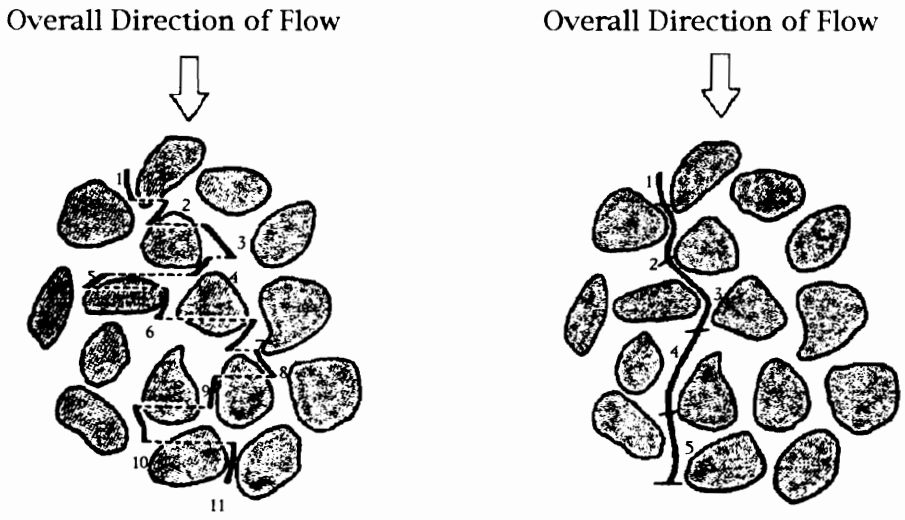


Figure 7. A schematic representation of the coupling effect in chromatographic separations. (From ref. 20)

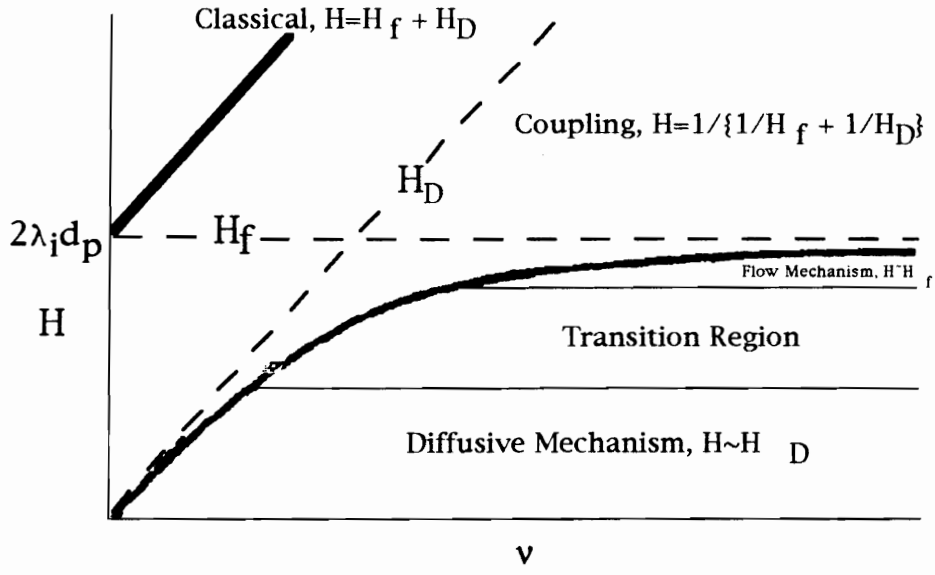


Figure 8. **Eddy diffusion curves showing the theoretical plate height achievable at a given velocity for the coupled equation and the original rate equation. (From ref. 20)**

3. *short range interchannel* flow velocity inhomogeneity, velocity differences between regions of tightly packed and nearby more loosely packed material within the column;
4. *long range interchannel* flow velocity inhomogeneity, velocity differences between regions of different packing structure throughout the column; and,
5. *transcolumn* flow velocity inhomogeneity, differences in velocity of the mobile phase across the diameter of the column.

In order to minimize band broadening due to mobile phase velocity effects in HPLC, then, one must pack HPLC columns which are as homogeneous in packing structure, particle size distribution, and packing density as possible[81]. Yet, even if the "perfect" column were produced, flow velocity differences would exist which would result in zone spreading and efficiency loss because of the transparticle effect and the transcolumn effect. Stagnant mobile phase would still occupy the pores of a porous sorbent, and laminar flow would still exist in the pressure-driven HPLC system between particles (transchannel) and transcolumn. In the case that column diameter is less than 25 times the particle diameter, the transcolumn effect can be minimized, though it will still exist[82]. To minimize the transcolumn effect as Giddings suggested, the column inside diameters listed in Table 3 must be employed. Notice, though, that some of the suggested particle diameters are exceedingly large compared to those typically used in HPLC. This is due to the fact that in the final mathematical expression for mobile phase mass transfer, it is found that particle diameter should be minimized for best efficiency. One clear means of coming to agreement between the desire for small particle diameter and that for a

Table III. Minimization of the transcolumn effect.

Column Type	Particle Diameter (μm)	ID (mm)
Packed Tubular (Prep)	400	10.0
Packed Tubular (Analytical)	185	4.6
Packed Tubular (Semi-micro)	85	2.1
Packed Tubular (Micro)	40	1.0
Packed Capillary	25	0.53
Packed Capillary	10	0.25
Packed Capillary	4	0.10
Packed Capillary	2	0.05

column diameter of only 25 times the particle diameter is to utilize columns of very small inside diameter. Packed capillary columns, according to HPLC theory, will provide improved efficiency if they can be packed as well as larger columns and if they are packed with the particle sizes suggested in Table 3.

3.2 Improving Efficiency in Liquid Chromatography.

The earlier stages of the research enumerated in this dissertation center on producing high-efficiency micropacked capillary columns for micro-LC through minimization of the transcolumn effect. Small diameter capillary columns were employed, and particle size was selected based on the recommendations listed in Table 3. These measures were taken as a means of decreasing the derogatory effect which transcolumn inhomogeneity has on plate height. The desire was to provide similar mobile phase flow streampaths throughout the cross-section of the column.

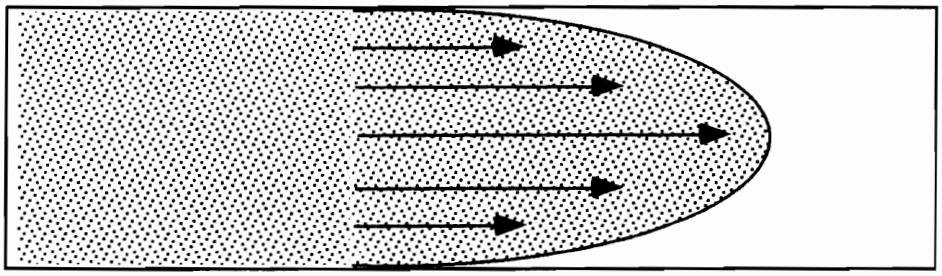
There does exist, however, another even more effective means of reducing flow velocity differences which exist in packed liquid chromatographic columns. In pressure-driven systems, drag at the interface of the moving phase and that which is stationary causes the flow profile to be laminar. Given this condition, it stands to reason that when a molecule diffuses from a region of relatively high velocity (such as the center of a channel) to one of lower velocity nearer the edge of a channel (close to the surface of a packing particle), a velocity difference will exist between that

molecule and one identical to it which is in the faster moving center of the channel. These two molecules will move different distances in a unit of time, causing the distribution of molecules within the band of like molecules to widen. Band broadening, as mentioned earlier, arises in part due simply to *flow velocity profile*. Figure 9 represents laminar and bulk flow profiles. If the flow profile can be changed from laminar to bulk in nature, analytes will for the most part experience only one flow velocity of the mobile phase, and velocity induced band broadening will be at its minimum. An increase in achievable efficiency will result simply because the transchannel and transcolumn effects have been minimized. A means of producing a bulk flow profile exists, and is a common feature of capillary electrophoretic systems.

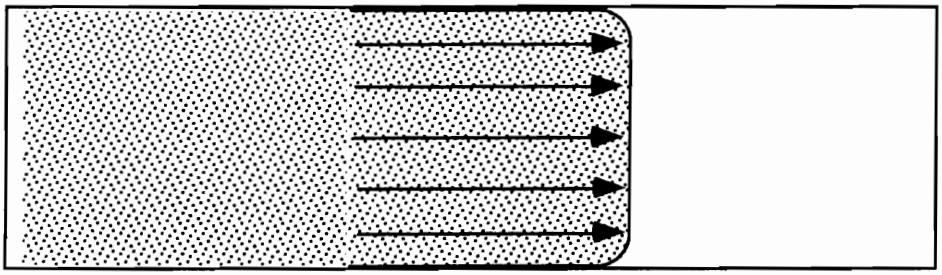
3.2.1 Electroosmosis and the Bulk Flow Profile.

In capillary electrophoresis there are two forces which act on a molecule to cause it to migrate. The two processes are electrophoresis and electroosmosis, and will be addressed separately.

In the case of electrophoresis, analytes are moved within a separation tube by virtue of their charge in solution. Unionized species, then, are not moved through the tube through an electrophoretic process. Ionized solutes, because of their charge in solution, are drawn toward the pole of opposite charge through the bulk solution at velocities which are dictated by the charge and hydrodynamic volume of the solute. The fact that this solute is ionized in solution results in the formation of an electrochemical double layer



Laminar Flow arising from a Pressure-driven system.



Bulk Flow arising from an Electroosmotically-driven system.

Figure 9. A comparison of laminar & bulk flow velocity profiles.

around the solute, the first layer of which is tightly associated with the solute and which is termed the Stern layer[83]. The second or outer layer is called the Gouy layer, and is more loosely associated with the solute, such that species in the Gouy layer can freely diffuse into the bulk solution and exchange with ions residing there. When this electrochemical double layer system is placed in an electric field, a shear force is exerted between the Gouy and Stern layers as the ions in the system are attracted to their opposite pole. Viscous drag on large solutes results in lower velocities, while increases in net charge result in higher velocities. This process of movement describes electrophoresis. Equation {11} mathematically relates the electrophoretic velocity of a solute as:

$$u_{ep} = (0.67)\ [\varepsilon_o\ \varepsilon_r\ \zeta\ E\ /\ \eta] \qquad \{11\}$$

where: ζ= **the zeta potential;**

E= field strength;

η= **solution viscosity;**

ε_o= **permittivity of a vacuum; and**

ε_r= **dielectric constant of the separation medium.**

An interesting feature of capillary electrophoresis in open fused silica tubes, though, is the fact that neutral species in solution, as well as charged species, are capable of being moved through the capillary electrokinetically. Remember that no geometric constraint is placed on the the formation of an electrochemical double layer. This means that an electrochemical double layer exists at the interface of the wall of a capillary tube and the solution

filling the tube. When an potential field is applied axially to the tube, ions associated with the wall of the tube in the Gouy layer will migrate towards their opposite pole, as described earlier. On the surface of a fused silica capillary, by virtue of the fact that it is comprised in part of silanol moieties, the surface carries a net negative charge (pH > 2), such that cationic species are associated with it[84] (figure 10). These cationic species will be drawn towards the cathode, and will by process of momentum transfer drag along with them surrounding bulk solution. This phenomenon is termed electroosmosis, and results in the transport of neutral species in the direction of the cathode in fused silica capillaries when a potential field is applied.

The flow profile in electroosmosis is dependent on the size of the tube in which it is occurring, and indirectly on the temperature profile across the diameter of the tube. Rice and Whitehead[85] described electroosmosis in capillaries with circular cross section, and carefully addressed tube diameter effects on flow profile. Their equation for electroosmotic flow velocity is:

$$v_{eo}(r) = -\varepsilon\zeta E/4\pi\eta * (1-I_o(\kappa_d a)/I_o(\kappa_d r)) \qquad \{12\}$$

where: ε = **the dielectric constant of the mobile phase;**

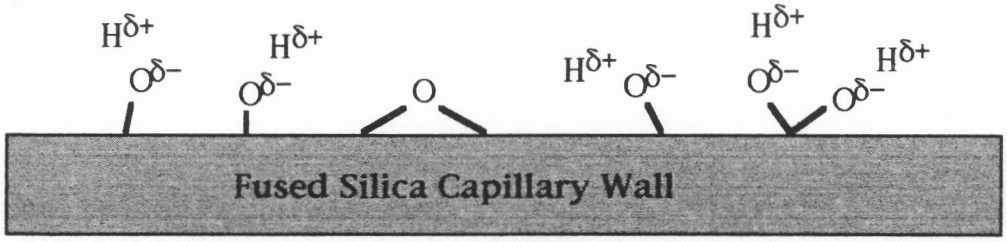
ζ = **the potential across the solution/surface interface;**

κ_d = **the reciprocal of the thickness of the double layer;**

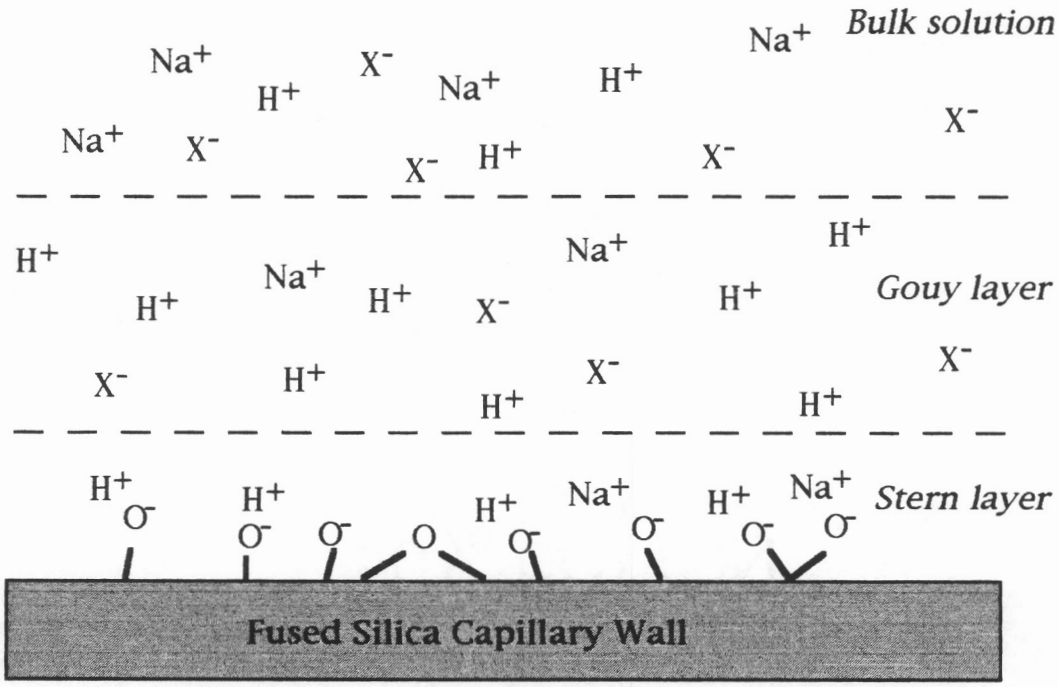
a = distance from the center of the capillary;

r = the radius of the capillary; and

I_o = **a zero order Bessel function of the first kind.**



A representation of the surface of fused silica tubing.



Hydrated silica surface illustrating the electrochemical double layer.

Figure 10. A representation of the surface of a fused silica capillary when filled with an aqueous solution.

The double layer thickness is typically quite small (on the order of 1nm), and so Pretorius' equation {6} mentioned earlier falls directly out of the Rice and Whitehead equation:

$$v_{eo} = [\ \varepsilon\ \zeta / 4\ \pi\ \eta\ ]\ E \qquad \{13\}$$

where: **e = solvent dielectric constant;**

η = **solvent viscosity;**

E = applied potential; and

z = zeta potential.

In very large open tubes, sheath flow may result in which the outer layers of solution in close proximity to the tube wall move towards the appropriate pole, while momentum transfer is insufficient to convey the more distant molecules nearer the tube center as easily. In addition to these forces, if high potentials are applied, further distortion of the bulk flow profile can occur due to resistive heating of the solution within the tube. In this case, the surface area of the tube is insufficient to dissipate the heat generated by applying a high voltage, and solution nearer the center of the tube remains hotter than that near the wall. A corresponding difference in viscosity results which allows flow in the less viscous solution at the tubes' center to be more facile. The end product of this heating phenomenon (commonly called Joule heating) is a parabolic flow profile. In short, large open tubes are not indicated for high resolution electroseparations.

In very small tubes, where the tube diameter is less than 20 times the double layer thickness, double layer overlap can occur[86]. The results of double layer overlap can in effect completely obliterate any electroosmosis, so that no mechanical transport of mobile phase or analyte occurs. Figure 11, taken from reference 85, indicates flow velocity profile in the region where double layer overlap occurs. The importance of electrochemical double layer overlap in this research becomes evident when electroosmosis in packed capillaries is considered.

When a capillary is tightly packed with a typical HPLC sorbent, about 5 microns in outside diameter, the interstitial spaces between the particles of packing material are sufficiently large that electrochemical double layer overlap will not impede the bulk flow nature of electroosmosis. Most modern HPLC sorbents are microporous, though, and it is this highly porous nature which gives the packing a high loadability. The question to be answered is: will electroosmosis arise inside the pores of the packing material and mechanically transport analyte to these areas. According to the equations proposed by Rice and Whitehead, a pore size ("tube" size) this small will typically result in some double layer overlap. This hypothesis is supported by John Knox[87]. No measurable amount of electroosmosis will occur within the pores of the packing material.

The promise of Capillary Electrokinetic Chromatography lies in the flat-faced (bulk) flow profile generated when a high potential is applied across a glass tube filled with a silica-based packing material. Just as with electroosmosis in open tubes, a zeta potential is measurable at the interface of the glass-like packing material and the filling solution. This means that the

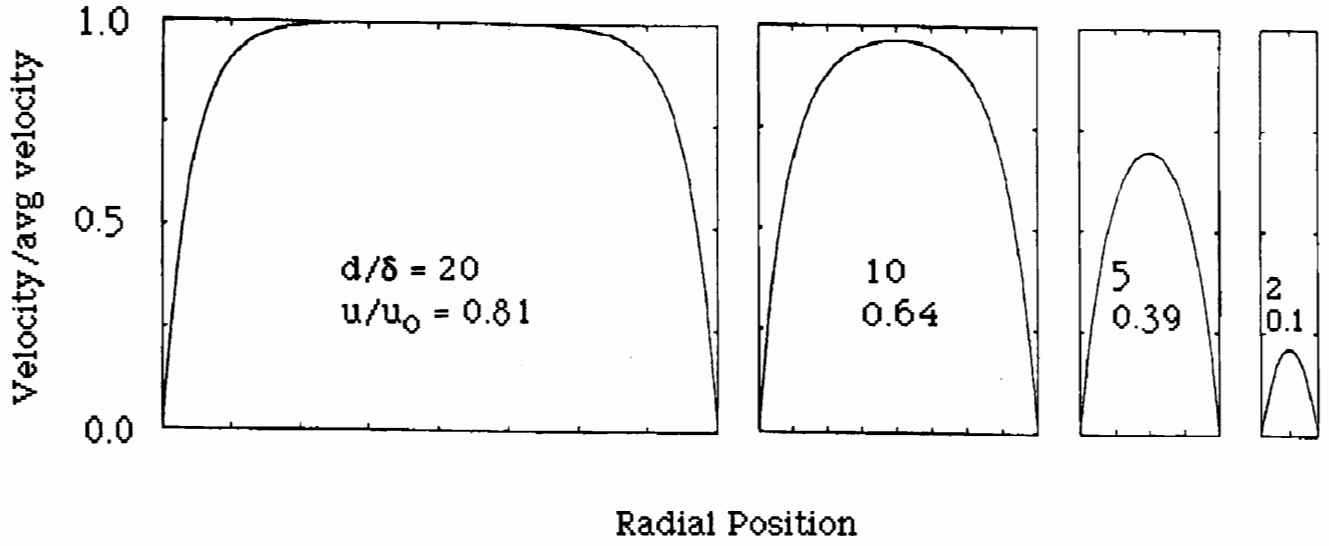


Figure 11. Electrochemical double layer overlap, which occurs when the inside diameter of a liquid filled tube is less than ten times the thickness of the double layer. (From ref. 85.)

motive force for flow in the packed-tube system comes from the packing material as well as from the walls of the container in which the sorbent is packed. In a system of chromatographic nature, the utility of a bulk flow profile rather than a laminar one, as is generated in pressure-driven systems, is a decrease in longitudinal diffusion which in turn yields increased efficiencies. According to equation {12}, above, electroosmotic flow is geometry independent so long as conditions preclude the formation of a thermal gradient due to resistive heating as discussed previously. A diagrammatic representation of the bulk flow phenomenon arising in packed capillaries when a high potential field is applied is shown in figure 12, taken from reference 73.

3.2.2 Mass Transfer Under Conditions of Bulk Flow.

The premise of the mass transfer term in the rate equation is that movement of solute between the stationary phase and the mobile phase is not instantaneous. It stands to reason that the speed with which the transfer can occur is limited by the diffusion coefficients of the solute in the mobile phase and in the stationary phase. Knox and Scott addressed mass transfer theory in 1984[88], and implicated the diffusion coefficient of the solute in the stationary phase particles (D_{sz}) as the "key parameter" in mass transfer. D_{sz} can be represented as:

$$D_{sz} = D_m \{\gamma_{sm}\,\phi + [k'(1-\phi)-\phi]\,\gamma_{ss}\,(D_s/D_m)\} \,/\, \{k'(1-\phi)\} \qquad \{14\}$$

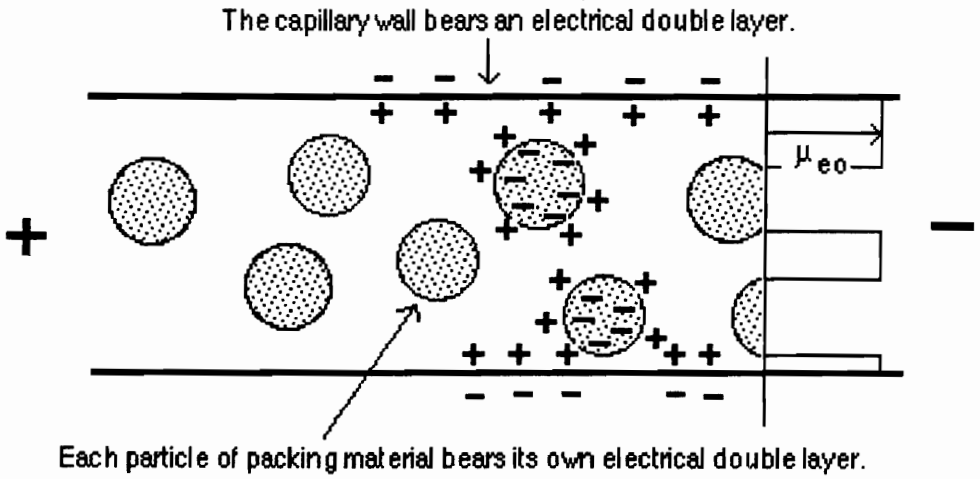


Figure 12. The bulk flow profile which arises in packed capillaries when a electroosmosis is used as the motive force for LC separations.

where γ_{sm} and γ_{ss} are factors representing obstruction of diffusion in the stagnant mobile phase and the stationary phase and ϕ is the proportion of mobile phase within the particles. When diffusion of solute in the stationary phase is very slow, the D_s/D_m term becomes negligible[89] and an abbreviated form of the D_{sz} equation holds:

$$D_{sz} = D_m \{\gamma_{sm} \phi\} / \{k'(1-\phi)\} \quad \{15\}$$

In order to make an approximation on the C term contribution to plate height, a simpler version of the van Deemter equation is used, as presented in equation {5b} earlier:

$$C = \left\{1/30 \quad (k' d_p^2 \mu / (1+k')^2 D_{sz})\right\} \quad \{16\}$$

Now, taking ϕ to be 0.5, this term can be represented as:

$$C = \left\{1/30 \quad (k' d_p^2 \mu / (1+k')^2 D_{sm})\right\} \quad \{17\}$$

where D_{sm} represents the diffusion coefficient of the solute in the stagnant mobile phase within the particles, with $D_{sm} = \gamma_{sm} D_m$ [90]. The largest possible C contribution to plate height (remember, plate height is desired to be quite small) comes with k' values which are large. The worst-case scenario is represented by a k'/1+k' of 1. Assuming this, and assuming that μ is 1mm/sec and $D_{sm} = 0.6 \times 10^{-9}$ m^2/sec, C becomes:

$$C = 55 \times (d_p/mm)^2 = 55 \times 10^{-6}(d_p/mm)^2 \quad \{18\}$$

so that with d_p = 5 microns, C = 1.3 microns. This calculation indicates that CEC can approach other electrokinetic techniques in terms of efficiency, especially if submicron particles can be used, since as d_p decreases, C (a contributor to plate height) will likewise be reduced. The limiting factor in terms of efficiency then becomes axial diffusion, as it is in CZE and MECC.

Knox[91] has suggested that for all capillary electrokinetic methods, including now CEC, the ultimate performance characteristics are:

Ultimate Plate Height	$H = 2 D_m\eta / \varepsilon_o\varepsilon_r\zeta E$	so that	$H \alpha 1/E$;
Ultimate Plate Count	$N = (L/H)$	so that	$N \alpha E$;
Ultimate Dead Time	$t_o = (L/\mu) = L^2\eta / \varepsilon_o\varepsilon_r\zeta E$	so that	$t_o \alpha L^2$;
		and	$t_o \alpha 1/E$.

For maximum efficiency and minimum run time, high voltages should be applied. Also, short columns should be used for fast analysis. Not mentioned here is the need to use low volume (small cross-sectional area) capillaries in order to dissipate heat.

3.3 The Capacity Factor & Selectivity.

As mentioned earlier, CZE is incapable of resolving mixtures of neutral species because they do not differ in their charge to hydrodynamic volume

ratio. The invention of MECC offset this limitation of CE methods, but only in a limited way. One of the major limitations of MECC is that it cannot provide the variety of capacity factor values which HPLC can, and therefore it cannot yield the wealth of selectivities which are possible in HPLC. This is because selectivity is simply a ratio of capacity factors:

$$\alpha = k'_2 / k'_1 \qquad \{19\}$$

where alpha is selectivity, and the k's are the capacity factors of two different analytes. Since selectivity arises out of the capacity factor, k' will serve best as a term for the comparison of two techniques. In order to compare methodologies fairly, MECC is discussed (rather than CZE) and contrasted to HPLC and CEC.

A comparison of MECC to conventional reversed-phase HPLC is natural, since both techniques presumably operate on a partitioning mechanism. The chief difference between the two is that in HPLC, one phase is held stationary, while in MECC the second phase is micellar and is dynamic. Because the micellar phase is not static, it must be treated differently in order for theory to justly represent chemical phenomena.

Terabe[92] defines the capacity factor of an analyte in MECC as:

$$k' = n_{mc} / n_{aq} \qquad \{20\}$$

where n_{mc} is a measure of the number of moles of solute in the micellar phase and n_{aq} is the number of moles of solute in the aqueous phase. In order to quickly calculate the k' of an analyte from chromatographic data, the formula:

$$k' = t_r - t_o \; / \; t_o \; (1-t_r \; / \; t_{mc}) \qquad \{21\}$$

can be used, where t_r is retention time of the analyte, t_o is the elution time of an "unretained" solute (one which spends no time in association with the micellar phase), and t_{mc} is the elution time of an analyte which spends all of its time in the micellar phase. Here, t_o and t_{mc} define the "elution window" of MECC. Already, theory indicates that an envelope of useful capacity factors for MECC exists, rather than the continuum which HPLC provides. Fortunately, the envelope covers a useful range of k' values.

Terabe[93], realizing that these changes in the description of the capacity factor would affect the proper presentation of the master resolution equation, suggested that it be represented as:

$$R_S = 1/4 \; (N)^{1/2} \; [(\alpha - 1)/\alpha] \; [k'_2/(1+k'_2)] \; [(1+(t_o/t_{mc})/1+(t_o/t_{mc})k'_1)] \qquad \{22\}$$

If t_{mc} is assumed to be infinite, as when one phase is held stationary (like in HPLC), this equation becomes the original master resolution equation. To compare MECC and HPLC in resolving power, the terms in this equation which include k' should be approached. Figure 13 from reference 94 illustrates the situation in which the two k' terms in the equation are assumed to be equal[94].

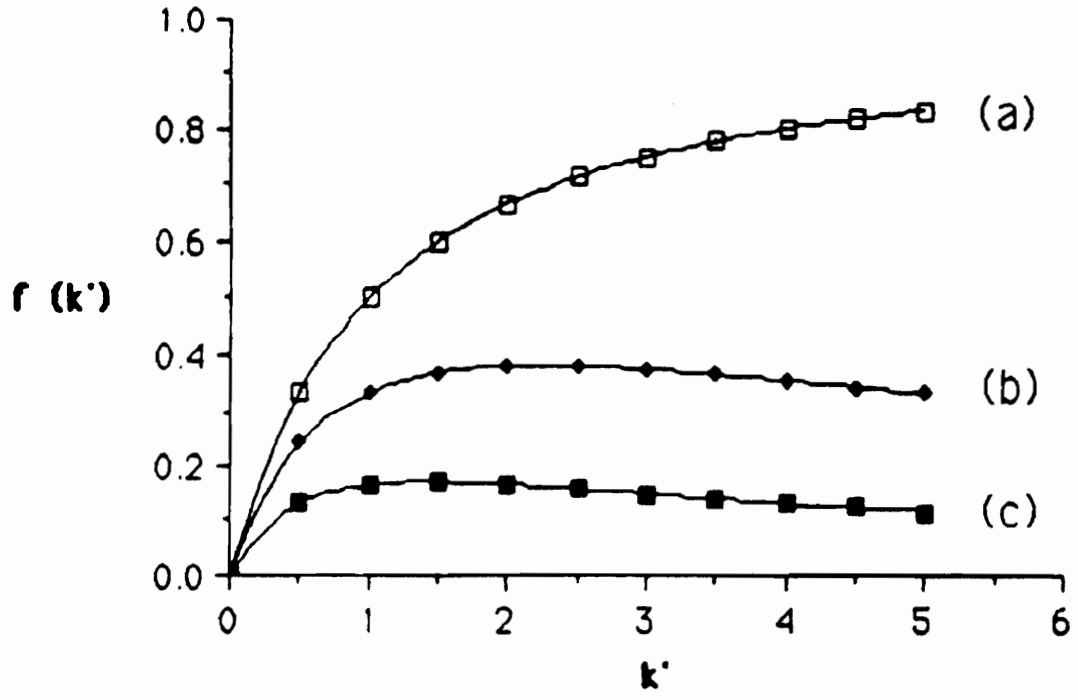


Figure 13. The capacity factor function in HPLC and MECC, which in part dictates resolution. Here, "b" and "c" represent two different surfactant concentrations in MECC, and "a" represents the stationary phase in HPLC. (After ref. 94)

This plot is made based on Terabe's equation which relates the capacity factors' contribution to resolution. In the case that t_o / t_{mc} = 0, traditional HPLC is represented. The relation plotted is:

$$f(k') = [k'/(1+k')] \quad [(1+(t_o/t_{mc})/1+(t_o/t_{mc})k')] \qquad \{23\}$$

with f(k') plotted as a function of k'. Although resolution can be optimized in MECC by adjusting t_o / t_{mc} , it will never approach what is achievable in HPLC. This does not sound a death knell for MECC, but it does indicate a limitation which HPLC does not impose. A wider range of possible capacity factors for MECC would in turn result in a better selectivity, further improving resolving power. Capacity factor (and therefore selectivity) tuning is possible in a limited way by altering surfactant concentration or type, though this still does not allow MECC to match HPLC in terms of selectivity. Additionally, selectivity tuning through the addition of organic modifiers in high concentration is not possible because these solvents are not compatible with the micellar phase.

3.4 Improving Selectivity in Electroseparations: CEC.

At this point it has been established that HPLC can offer the ultimate resolving power in terms of selectivity, but suffers from mobile phase flow velocity inhomogeneity which limits efficiency. Also, it has been ascertained that electrokinetic techniques such as CZE and MECC offer the ultimate in resolving power in that they provide extremely high efficiency, though they

cannot compare to HPLC in terms of selectivity. For the absolute highest resolving power, a method is required which provides the best of both worlds: the efficiency of and electrokinetic method along with the selectivity of HPLC. CEC is capable of filling this niche. Since HPLC packing materials and mobile phases are employed in performing CEC, it can be expected that CEC will deliver the selectivity which HPLC delivers. As an electrokinetic technique, CEC is not encumbered with the flow inhomogeneities which limit HPLC. Capacity factors of solutes in CEC are as flexible as in HPLC, since any proportion of organic solvent can be employed without affecting the integrity of the stationary phase, as opposed to MECC where disruption of micelle formation will occur in the presence of high concentrations of organic solvent.

CEC seems to address each of the three terms of the master resolution equation. High efficiency, variability in capacity factor, and tunable selectivity together yield a technique which can in theory surpass both MECC and HPLC in resolving power.

3.5 Extracolumn Effects.

Each component of a liquid chromatographic system is responsible for contributing a finite volume to the total volume of the system. These volumes adversely affect the quality of a separation if they exceed certain minimums, by allowing analyte bands to disperse. In micro-LC, the importance of regulating extracolumn volume is magnified because of the extremely low volumetric flow rates employed and the small peak volumes encountered.

If injection valves were ideal, an injection of sample would result in the introduction of a plug of liquid to the system. As a function of diffusion in the mobile phase and interaction with the packed bed inside an HPLC column, this plug of sample would begin to broaden at the onset of the analysis, and would continue to broaden as the separation progressed. The broadening process, if no chemical phenomena are involved, results in a Gaussian distribution (peak) of analyte molecules. The variance of the peak is a sum of the variance contributions of each of the components of the chromatograph[95]:

$$\sigma^2_{tot} = \sigma^2_{inj} + \sigma^2_{tub} + \sigma^2_{col} + \sigma^2_{det} \qquad \{24\}$$

where: σ^2_{tot} = **the total measured peak variance;**

σ^2_{inj} = **the variance caused by the injector;**

σ^2_{tub} = **the variance contribution of connective tubing;**

σ^2_{col} = **the columns' contribution to variance; and**

σ^2_{det} = **the variance due to the detector cell.**

The variance contribution of the column is in part dictated by how well the column is packed, and cannot be changed. For the system to perform at its best, the column variance should be the largest contributor to total variance, and all others should be minimized.

If the performance of a column is to be judged, it is important to designate which portion of the band broadening process is occurring as a function of the column, and to assess the column based only on this. The simplest means of determining column variance contribution is to measure

the variance contribution of each of the other parts of the system, and later to subtract this extracolumn variance from the total peak variance.

There are several methods of assessing extracolumn variance. Reese and Scott[96] quantitated extracolumn variance by adding an extra volume of connective tubing to the chromatographic system. By starting with a given length of tubing, making a measurement, and moving to progressively shorter tubing lengths, a plot of peak variance as a function of tubing length could be constructed. The measured variance would be the equivalent of the original variance plus the variance contribution of the added tubing, since variance is additive:

$$\sigma^2_{observed} = \sigma^2_{original} + \sigma^2_{added} \qquad \{25\}$$

Using this method, they were able to assess the variance contributions of each of the system components and the total variance contribution of the system hardware.

An alternative to actually measuring the variance is to calculate it based on a theoretical description of the process of band spreading. Lochmuller[97] demonstrated the calculation of extracolumn variance by recognizing two different sources of band broadening: static volume contributions of the system components (injector, detector, tubing) and the axial dispersion of analyte due to the process of laminar flow in open connective tubes and detection regions. The laminar flow profile discussed previously indicates that analytes near the center of the column are moving at a velocity which is about twice the mean velocity of the band. Analyte in the radial positions near the

tube wall are moving at velocities approaching zero. In time, this process results in a widened analyte band, as illustrated in figure 14. This process is not as damaging to efficiency in capillary GC as in LC because of the speed of diffusion in gases, which time averages the radial concentration profiles. In LC, the averaging process is not as facile, and at high flow velocities this results in an exaggeration of the laminar flow effect on band dispersion. Bakalyar[98] derived an equation which is useful for calculating the connective tubing contribution to extracolumn variance:

$$\sigma^2_t = 2000\ d_t^4 L_t F \qquad \{26\}$$

where: d_t = **the inside diameter of the tubing in mm;**

L_t = **the tubing length in mm; and**

F = **the flow rate of the mobile phase in ml/minute.**

In order to minimize tubing-caused band broadening the connective tubing should be as short as possible. Most important though is the tubing inside diameter, related to variance as diameter to the fourth power. The use of extremely small inside diameter tubing minimizes band spreading, but also increases the likelihood of plugging the tubing. In micropacked capillary HPLC, eliminating connective tubing altogether is the best option. Connecting the column directly to the injector, and using Yangs' on-column UV detection scheme results in essentially no connective tubing.

Also important in determining the extent of tubing induced band broadening is the flow rate. In the context of this dissertation, this term may

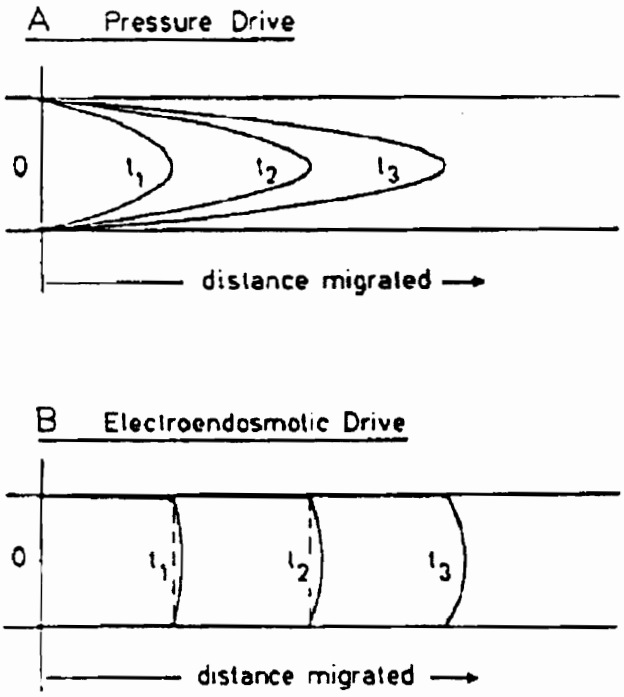


Figure 14. Radial positional differences in flow velocity in open tubes, and the band broadening which results. (From ref. 74)

in fact be the single most important contributor to efficiency loss, in that it relates the flow profile to band spreading. As flow rate (and therefore flow velocity) is increased, the laminar flow profile is exaggerated: with the mobile phase at the tube center still travelling at about twice the average velocity, and that near the wall still near zero, the *range of velocities* is wider. A wider range of velocities translates directly to a wider analyte band when sample is introduced to the system. This observation holds in the case that flow is laminar, as in pressure-driven liquid flow. In the event that flow is not laminar, as in CZE, the equation does not hold because of the bulk flow nature of the system. In other words, if connective tubing is used in CEC, and if that tubing is the same size as that used to manufacture the CEC column, no connective tubing contribution to band spreading is expected other than that due to axial diffusion of the analyte. This could be important in interfacing the technique of CEC to complex, information-rich detection systems such as mass spectrometers. Volumetric flow rate differences between open and packed tubes make application of this phenomenon more difficult than the theory indicates, since the open tubular portion of the separation capillary will have a greater "appetite" for mobile phase than the lower volume packed portion of the column.

Column-induced band broadening is discussed here to serve as a benchmark for determining how much extracolumn volume can be allowed in a given system. Column-induced band broadening in liquid chromatographic methods should be the determining factor in band spreading if the separations instrument is properly designed. The width of a chromatographic band is dependent on its' residence time in the column, such that the efficiency of the

column (N) and the capacity factor of the peak of interest (k') must be used in the calculation of σ^2_{column}:

$$\sigma^2\mathbf{column} = \{4V_m\ (1 + k')\ /\ (N)^{1/2}\}^2 \qquad \{27\}$$

where V_m is the void volume (or "dead volume") of the column. For 4.6mm ID columns, V_m is estimated as 100µl/cm of length. Likewise for 2mm ID columns it is estimated as 19µl/cm, and for other column dimensions it is related approximately as the square of the radius of the column. To minimize column volume contributions to band broadening, the void volume must be minimized. This is done by packing the column properly, so that the bed is tight and close packed, and by using small diameter packing materials such that the interstitial volume is minimized, as well as using small volume (small inside diameter) tubing for manufacturing columns. Additionally, using high-efficiency systems aids in decreasing band spreading. In order to improve efficiency, flow dynamics, as discussed earlier, must be recognized as an important determiner of plate height. Minimization of the transcolumn effect through careful choice of particle diameter relative to column diameter[99] decreases flow inhomogeneity and therefore decreases plate height. The ultimate in efficiency enhancement through flow inhomogeneity reduction is offered by electrokinetic techniques, which yield bulk flow. In CEC, transcolumn and transchannel effects are both minimized, and the contribution of the column to band broadening is therefore minimal.

According to John Dolan[100], the contribution of the injector to total bandwidth is is about twice the injection volume. In an ideal situation, the

contribution would be much smaller (on the order of 1.15 times the volume of injection), but the typical valve design adds to total extracolumn volume because of the "flushing characteristics of the valve."[101] Here Dolan is alluding to two factors: the passage volume of the valve, and the flow dynamics associated with sample introduction.

Any injection valve will present a small additional volume of passageway which serves to connect the sample loop to the column head, even when the valve is specifically designed for use in microseparations. Sternberg[102] determined that the minimum variance contribution of an injector was determined by:

$$\sigma^2_{inj} = V^2_{inj} / 12 \qquad \{28\}$$

where V^2_{inj} represents the square of the volume of the injector. This equation pertains to an ideal situation in which flow dynamics do not affect zone width.

The more realistic situation was mentioned by Coq[103], who states that flow dynamics do dramatically affect delivery of an injected plug of sample to the column. Elimination of laminar flow through the use of a sample loop packed with beads results in the variance predicted by the Sternberg equation.

Proper selection of a sample solvent can correct for the non-plug format with which injection valves introduce sample. Dynamically, regardless of sample solvent, the delivery of sample to the head of the column is the same. Chemically, though, the flow problems are corrected by using a sample solvent which is weaker than the elution solvent. This has the effect of focussing the sample at the head of the column. Consolidation of the injected zone has been

studied by Slais[105] for microbore HPLC, and has been found effective in virtually eliminating injection valve contribution to peak variance.

In CEC, injection valve effects on band broadening are eliminated because the injection format does not make use of a valve. In CEC (as presented in this text), electrokinetic injection is employed. Electrokinetic injection is accomplished by placing the head of the packed capillary column into a reservoir containing the sample solution. A low (1-5kV) potential is then applied axially, allowing electroosmosis to draw analyte into the column for several seconds. If only neutral analytes are present in the sample, each is introduced in representative concentration to that in the sample. Some bias is introduced in the case where ionized species are analyzed, as their velocity will be the vector sum of their electrophoretic velocity and the electroosmotic velocity. Regardless of sample type, though, zone broadening induced by laminar flow is eliminated, such that the variance contribution of injection in CEC to band width is negligible when compared to that in a pressure-driven micro-LC situation in which solvent focussing is not employed.

Finally, detector cell volume contributions to peak variance can degrade system efficiency. Again, as in the case of injection valves, band dispersion arises in part due to laminar flow effects. Mathematically, zone broadening in the detector cell can be expressed as:

$$\sigma^2_{det} = (V^2_{det} / 12) + (F\pi r^4 L / 24 D_m) \qquad \{29\}$$

where: **F = the volumetric flow rate;**

r = detector cell radius;

L = the cell length; and

D_m = diffusion coefficient of the analyte in the mobile phase.

Small cell volumes obviously will reduce the zone broadening effect, but also reduce sensitivity by reducing path length. In Yangs' on-column detection design path length is short (equal to the ID of the column), limiting sensitivity, but the detector cell contribution to band spreading is minimal. In CEC, elimination of laminar flow allows the elimination of the ($F\pi r^4L$ / $24D_m$) term, and results in the ideal detection format in terms of extracolumn effects.

CHAPTER IV

EXPERIMENTAL

4.1 Semi-micro Ion Chromatography.

4.1.1 Column Production Procedure.

Column blanks for the production of semi-micro Ion Chromatography (IC) columns consisted of 2 mm x 230 mm DuPont Tef-zel^{TM} (Wilmington, DE., USA) tubing. The tubing ends were heat flared to decrease the likelihood of deforming under pressure. Column end fittings were PEEK^{TM} (poly ether ether ketone) minimum dead volume unions obtained from Dionex (Sunnyvale, CA., USA), and were designed for use with 2mm plastic tubing. A 2 micron fritted teflon^{TM} disc, also from Dionex, was placed at the outlet of each column blank inside the low dead volume union in order to terminate the column. A schematic diagram of the column end fitting design is shown in figure 15.

Dionex AS4A anion exchange resin was utilized as the stationary phase. This packing material consists of a poly(styrene-divinylbenzene) particle of 15 micron diameter. The polymer is highly crosslinked, making it essentially non-porous on the scale of the analytes. The surface of this bead is sulfonated, giving it a net negative charge and making it useful for cation separations. In order to produce an anion exchanger, the surface is modified with an aminated latex. The strong coulombic interaction of the amine functionalities of the latex with the sulfonated PS-DVB particles makes the newly applied surface quite stable[106]. The end result is a strong anion exchanger of low capacity (low surface area). The capacity of these resins is

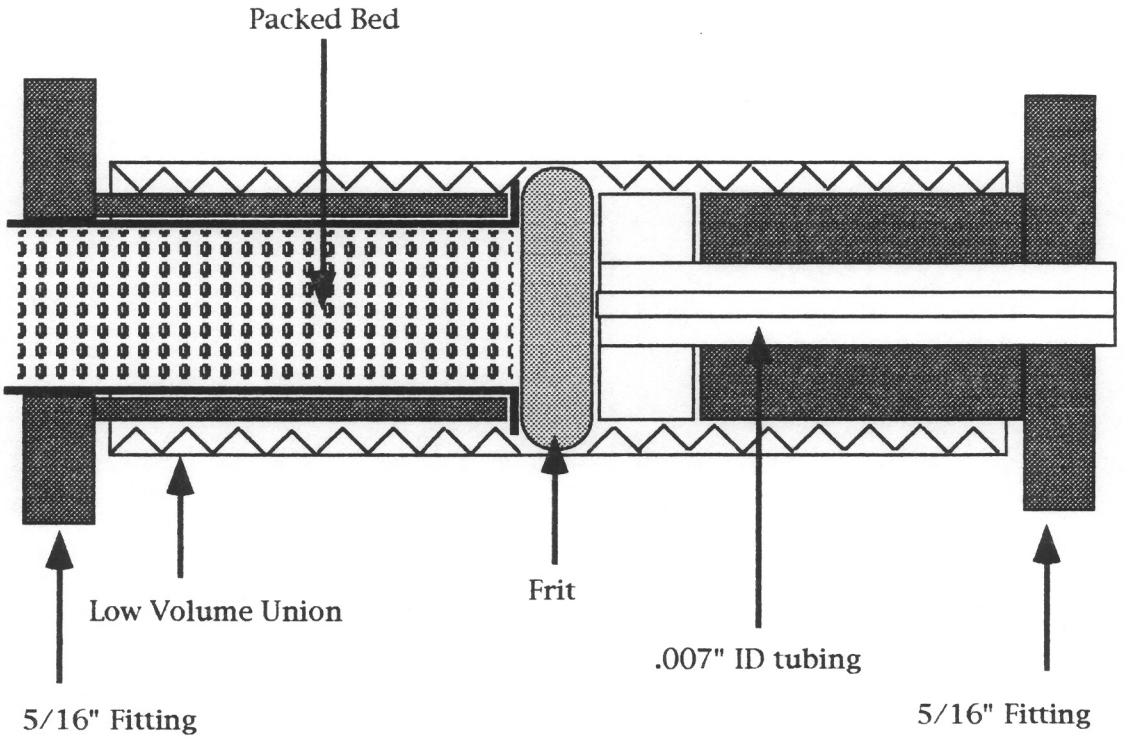


Figure 15. A cutaway view of the end fitting used in semi-micro Ion Chromatography.

typically in the vicinity of 0.02 meq/g [107]. The packing material was washed by soaking it in a solution of 150mM NaOH overnight, then sonicating it in fresh 150mM NaOH for 10 minutes. After rinsing the material in fresh distilled, deionized water several times, a slurry of the material was prepared. A slurry of 1g resin to 15ml distilled, deionized water was prepared in a 50ml erlenmeyer flask, which was then sonicated for 10 minutes prior to transfer in to a slurry reservoir, a Micromeritics (Norcross, GA., USA) model 705. Given the large diameter (high sedimentation coefficient) of the packing particles, stirring was necessary in order to ensure that the packing particle size distribution remained the same throughout the length of the column. Without stirring, it is likely that the largest particles would have begun to settle out of the slurry rapidly, thus changing the composition of the slurry both in terms of slurry density and in terms of particle size distribution. These changes would have been reflected in the composition of the bed of the column, resulting in a non-homogeneous bed structure.

The column blank, with one fritted end fitting attached, was connected to the slurry reservoir and held vertically using a ringstand and clamp. A Hitachi (Mito, Japan) Model 655-15 isocratic HPLC pump was used to deliver the slurry displacement solution of distilled, deionized water to the stirred slurry reservoir containing the column packing material. In line between the pump and reservoir were a liquid-filled pressure gauge and a needle valve, which allowed for pressure to be developed without affecting the slurry, then rapidly applied in an effort to instantaneously pressurize the packing system. The pump was used in the constant pressure mode at 150 kg/cm^2 (~2100psi). Figure

16 illustrates the packing apparatus used to manufacture 2mm ID Tef-zel^{TM} Ion Chromatography columns.

After dispensing the slurry into the Micromeritics reservoir and stirring for 10 minutes, the packing pump was started and pressure was allowed to build to 150 kg/cm^2. Once the desired pressure was reached, the needle valve was opened and pressure applied to the slurry reservoir. The column was maintained in a pressurized state for 20 minutes, after which the pump was turned off. Pressure was allowed to bleed off through the column for 10 minutes, although the pressure had dropped to zero after approximately four minutes. Removal of the column from the packing apparatus prior to bleed-down might have resulted in bed deconsolidation and would have ruined the column. The column was then removed form the packing apparatus and upper end fitting and frit assembly was attached. After flushing the column with 150mN hydroxide for approximately 12 hours at a flow rate of 150µl/min, the column was ready for use.

Dionex CS3 cation exchange resin consisting of 15 µm surface sulfonated PS-DVB particles was utilized in the suppressor column. This material was obtained by removing the end fitting of a commercially-available Dionex CS3 column and forcing the packing material from it using a 50cc disposable plastic syringe and hand pressure. The packing material thus obtained was soaked in 500mN H_2SO_4 overnight, sonicated in fresh 500 mN H_2SO_4 for 10 minutes, then triply rinsed with distilled, deionized water before packing. The CS3 column was packed using the same methodology as described for the AS4A columns, but was flushed after packing with 500 mN sulfuric acid at 150 µl/min for about 12 hours. A head frit was not used so that the

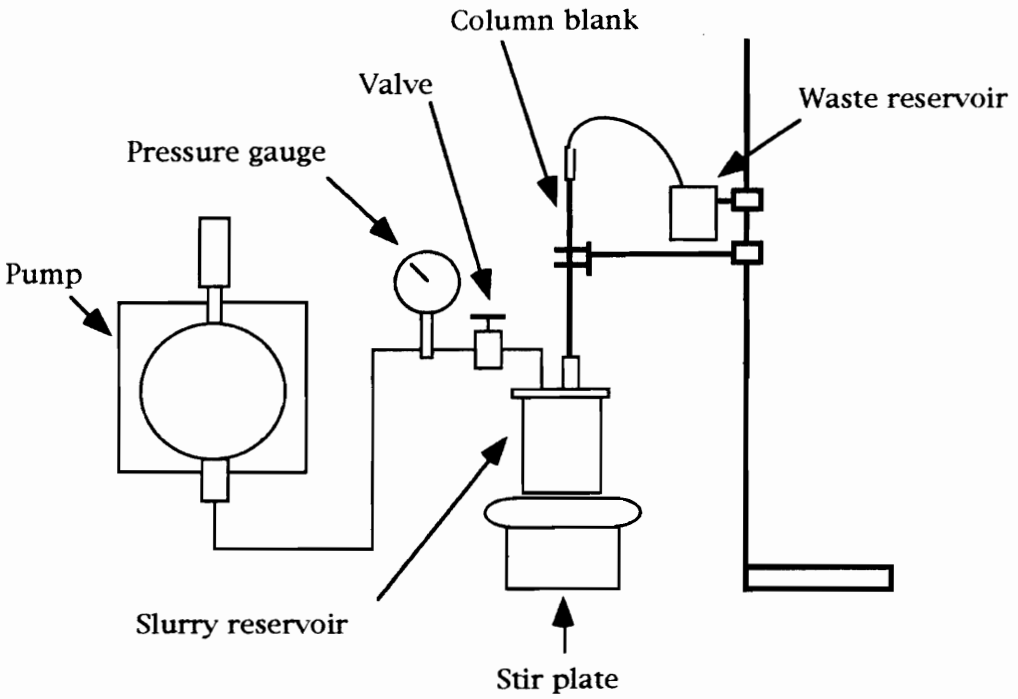


Figure 16. Schematic showing the packing apparatus used in preparing semi-micro IC columns.

suppressor column could be attached directly to the end fitting of the analytical column, minimizing extracolumn volume.

The small surface area of the resin dictated that the column be regenerated (returned to the fully hydrogenated form) frequently. This was accomplished by removing the column from the system and flushing with 500 mN sulfuric acid for at least 3 hours at 150 µl/min. For use as a suppressor column, a microporous cation exchanger would have been preferred because of its larger exchange capacity. This would have resulted in less frequent system shutdown for regeneration. Unfortunately, a microporous cation exchanger was not available in our laboratory.

4.1.2 Instrumentation Design.

A Dionex Model 4000i gradient pump was used with minor modifications to reduce volumetric flow rate without introducing air leakage and reproducibility problems. The metal-free system consisted of glass one liter mobile phase reservoirs, teflon mobile phase frit filters, and teflon transfer lines for delivery of mobile phase components to the pump. The pump was a dual-piston design which also was metal-free.

The features of Ion Chromatography (IC) which set it apart from Ion Exchange Chromatography (IEX) were pioneered by Hamish Small at Dow Chemical in 1975[108]. A primary advantage of IC over IEX is the ability to elute strongly retained ions with relatively weak mobile phases. This is possible through the use of low-capacity packing materials. The use of ion exchange

interactions was thus extended to the polyvalent species which had previously been more difficult to analyze by chromatographic methods. The principle advantage of IC over IEX, though, comes in the possibility of using gradient elution, which previously was not possible with conductivity detection.

In separations of ions, a common feature amongst all analytes is that they all increase solution conductance. This common feature suggests that measuring solution conductance in the flowing stream of chromatographic eluent is the most simple and universal form of detection for separations of ions. For this reason, conductivity detection has been quite popular in IEX methods. If the mixture of ions to be resolved is diverse, containing mono-, di- and polyvalent species, several isocratic chromatographic "runs" are required, such that the weakly retained monovalent ions are resolved in one run, then the highly retained species are separated in a second run. In order to analyze all of the species in one run, gradient elution was necessary. However, increasing eluent strength (ionic strength and/or pH) results in increased solution conductance and therefore causes severe baseline drift. Consequently, gradient elution was not feasible in classical IEX.

IC solves the problem of gradient elution by incorporating a suppressor column between the analytical column and the detector cell. This suppressor column, in the case of anion exchange chromatography, exchanges protons from the suppressors' stationary phase for sodium ions in the aqueous sodium hydroxide mobile phase. The highly conductive sodium hydroxide solution is converted to much less conductive water. This results in a marked decrease in mobile phase conductance, and improves sensitivity in addition to allowing the use of gradient elution in IC.

In order to convert a Dionex model 4000i IC system for microseparations, flow was split prior to the sample valve between two similar columns. In order to accomplish this, a polymeric tee union was attached to the pump outlet with 0.007 in ID teflon tubing. Similar tubing connected one leg of the tee to a Dionex AS4A anion exchange column of 4.6mm ID and 25cm length. The other leg of the tee led to the sample injection valve and the semi-micro IC column and suppressor assembly. This arrangement resulted in an approximate flow split of 5.3:1. Here, the approximate split ratio is obtained by dividing the cross-sectional areas of the two columns, based on the assumption that they are similarly packed. Flow to the semi-micro column was determined volumetrically by collecting the effluent from the split column such that any variability due to suppression would be eliminated. The difference between flow at the pump and flow from the split column then represented flow to the semi-micro column. Flow was adjusted at the pump to yield a flow rate of approximately 250 µl/min. Following the split, flow entered a standard Dionex inert high pressure sample valve (part #038598) fitted with a home-made 10 µl sample loop. The entire apparatus is represented in figure 17.

The injection valve was coupled to the analytical column using a 4.5cm length of 0.007" ID teflon tubing, the minimum length possible considering the depth of the fittings used in attachment of the column to the valve. As indicated in the instrument diagram, the analytical and suppressor columns were directly coupled to one another so as to reduce extracolumn volume. In order to minimize any further system volume contributions, the detector cell was located as close to the suppressor column outlet as possible, again by using a 4.5cm long piece of 0.007" ID teflon tubing. Two detector cell

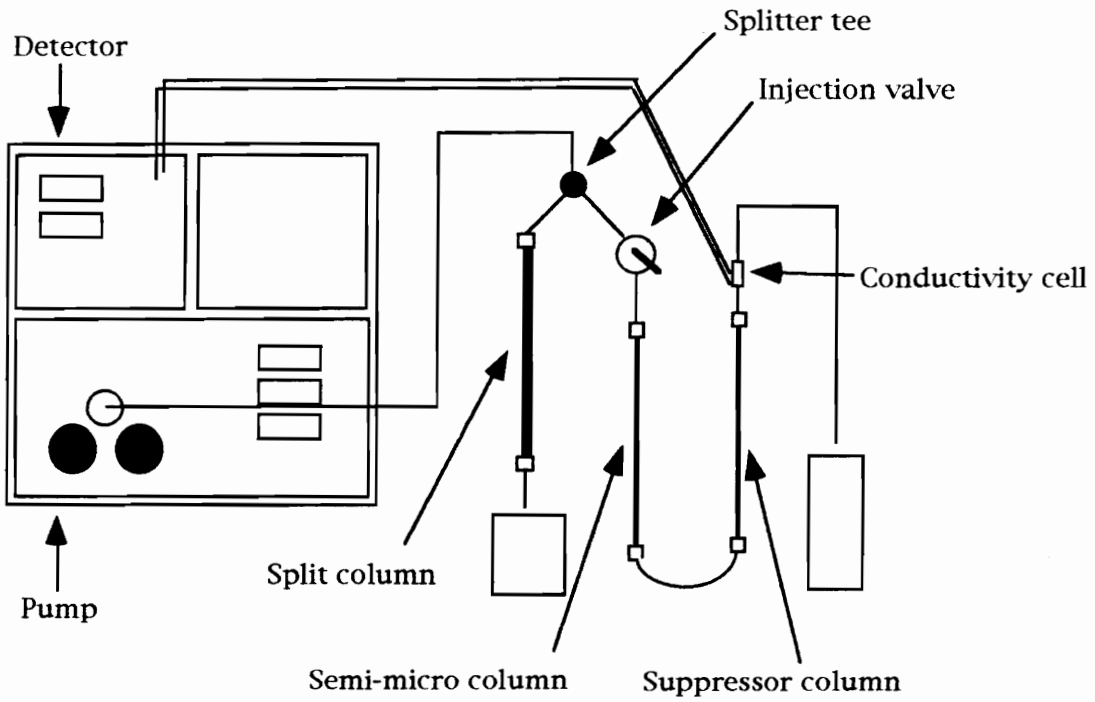


Figure 17. The home-made flow split design used to adapt a conventional IC system for use in microscale IC.

designs were employed: the standard Dionex conductivity detector cell and a home-made low volume "micro" conductivity cell.

The micro conductivity cell was modeled after that suggested by Dasgupta et al[109]. Teflon tubing of 0.007" ID was used to build the detector cell and inlet. Tuberculin needles served as electrodes. The cell was assembled as shown in figure 18 by cutting a one centimeter length of teflon tubing, reaming both ends of the tubing to a conical shape with a set of micro burr reamers (Supelco, Bellefonte, PA., USA), and sliding a needle into each end of the tube to fit snugly. Under a microscope, the ends of the needles could be positioned such that their beveled tips were parallel to one another and about 0.5mm apart. Another piece of teflon tubing was then cut, for use as a detector cell inlet. This tube was 3.0cm in length, and was reamed at one end to slide snugly over one of the needles used in the detector cell. The other end was attached directly to the suppressor column outlet using a commercial fitting.

The standard Dionex conductivity detector electronics were used to both drive the cell and process the analytical signal. Cell drive and return cables were salvaged from an older Dionex conductivity cell, and attached to the electrodes (the two tuberculin needles) of the microconductivity cell. All measurements were made at a sensitivity setting of 30 micro-Siemens full scale as set on the detector. The processed signal was sent to a Hewlett-Packard (Avondale, PA., USA) model 3394A integrator.

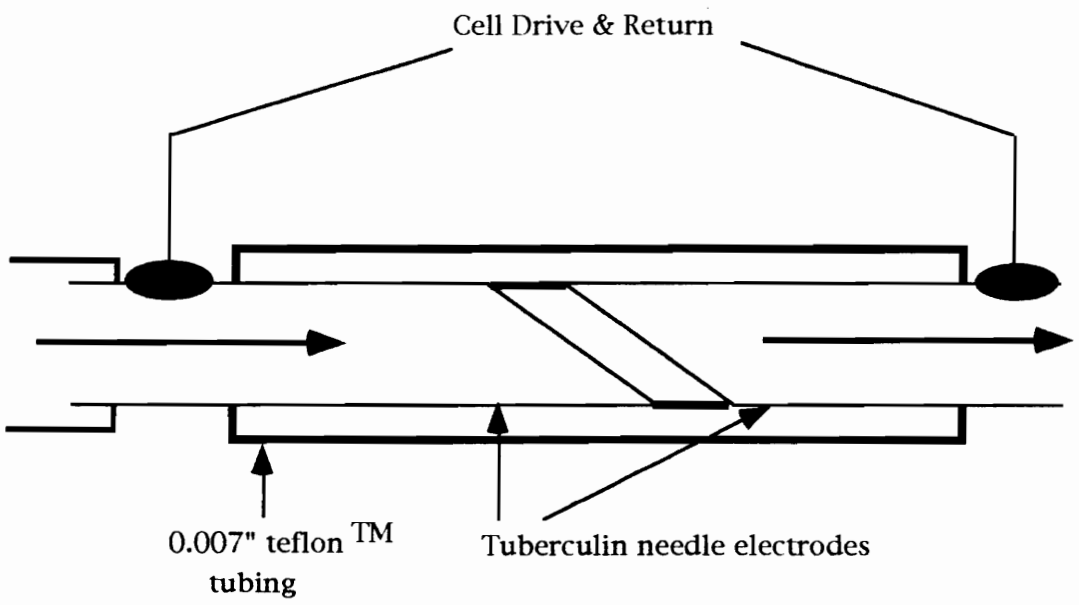


Figure 18. The home-made micro conductivity cell used in the semi-micro IC studies.

4.1.3 Reagent Selection & Preparation.

Semi-micro IC columns were evaluated by running samples of weakly retained (low k') inorganic anion test probes. Solutions of fluoride and chloride were prepared from master standards of high purity obtained from E.M. Science (Cherry Hill, NJ, USA) in concentrations of 1.000 +/- 0.002 g/l. These standards had been prepared as sodium salts. Samples for IC were made by diluting aliquots of each of the master standards to 10ppm concentration in doubly distilled, deionized (18 megohm) water. The mobile phase was prepared from 30% w/w sodium hydroxide solution obtained from Fisher Scientific (Fair Lawn, NJ, USA). One liter solutions of 25mN hydroxide were prepared in helium sparged 18 megohm water from the freshly opened bottle of sodium hydroxide, and the fresh solution was immediately dispensed into a sealed vessel and placed under a helium atmosphere. These precautions were intended to decrease the formation of carbonate by absorbing CO_2 from the atmosphere which would affect mobile phase strength. Regenerant for the manually regenerated suppressor column was made with ACS reagent grade concentrated sulfuric acid (Fisher Scientific, Fair Lawn, NJ, USA) and 18 megohm water. A 500mN solution was prepared.

4.2 Micropacked Capillary Ion Exchange Chromatography.

4.2.1 Column Production Procedure.

The packing of flexible fused-silica capillaries has been well addressed in the literature in the past five years[110-113]. Several researchers have made concentrated efforts to further the technology of micropacked capillary HPLC. The focus, however, has been on packing capillaries with both bonded phase type materials and silica gel. One mode of separation has been overlooked in this research, namely, ion exchange. By using ion exchange media in the micropacked format, very small samples of ionized species can be separated and detected at low limits. In the particular application of separating nucleotides illustrated here, a micropacked anion exchange resin is essential.

Dionex (Sunnyvale, CA, USA) AS4A anion exchange resin was used in the fabrication of micropacked capillary ion exchange columns. The packing material was slurried in doubly-distilled, deionized water at 1g packing per 25ml water. The slurry was then sonicated for 15 minutes to thoroughly disperse the packing material, and immediately poured into a home-made stirred slurry packer with a 15 ml reservoir. The cap of this reservoir is commercially available as part of a stirred slurry packer sold by Micromeritics Instrument Corp. (Norcross, GA, USA). Two fittings on the reservoir cap allow for attachment of a pneumatic amplifier packing pump (Haskel, Inc., Burbank, CA, USA) and a column blank. A pressure gauge and needle valve were placed between the pump and the slurry reservoir, as before, so that the necessary high pressure for packing could be developed and instantaneously applied to the slurry.

Column blanks consisted of 530 μm ID fused silica tubing (Polymicro Technologies, Inc., Phoenix, AZ, USA). Each column blank was fitted with an end frit at the upper end (outlet) utilizing Kasil^R #1 (PQ Corp, Valley Forge, PA, USA). Kasil^R #1 is a 2.5:1 solution of SiO_2 and K_2O. This potassium silicate solution, when mixed with formamide (Sigma Chemical Co., St Louis, MO, USA) in a volume ratio of 5:1, can be drawn into the column blank by placing the capillary into a vessel of the mixture. A 4mm length of solution was drawn into several 20cm long column blanks by capillary action, and the blanks were placed into a 250ml Erlenmeyer flask frit end down for drying. The flask containing the blanks was placed into a GC oven at 120° C, and the frits were left to cure for one hour. Upon drying, a porous ceramic frit is formed with a 3000 to 5000 angstrom pore size[114]. These end frits were stable up to 5000 psi, the normal packing pressure, when made to a length of 4mm. Once the columns had been packed, the frits were cleaved to 2mm for use. Frits shorter than 2mm, though contributing less to extra-column volume, were easily displaced at pressures over 800 psi and could not be used.

After the end frits had been produced, and before packing, the column blanks were rinsed with acetonitrile by pressurizing the column blanks with acetonitrile at 2000psi, using an ISCO μLC-500 syringe pump (ISCO, Lincoln, Nebraska, USA). A final rinse with distilled, deionized water at 500psi left the blanks ready for packing. The inlet to the stirred slurry reservoir was connected directly to the pneumatic amplifier pump, which was driven by compressed air. The pump was adjusted such that a liquid pressure of 5000psi was exerted on the slurry, forcing it into the column blank. Distilled, deionized water was used as a displacement solvent. Once packed, the pressure

on the column was maintained for 15 minutes to ensure a tightly packed, well settled bed. Pressure was allowed to bleed off through the column. The column was then removed from the apparatus and washed with 150mN sodium hydroxide at 15 µl/min for 2 hours to ensure its cleanliness. A total of five columns were made.

4.2.2 Instrumentation Design.

The apparatus used consisted of an ISCO µLC-500 syringe pump, a Rheodyne (Cotati, CA, USA) 7520 micro injection valve with a 500nl loop, the home-made anion exchange column, and a Linear (Reno, NV, USA) model 206 PDA detector.

Fused silica tubing of 75um I.D. provided the transfer line and flow cell assembly for the experiments. This tubing was joined to the micropacked capillary column by a home-made slip-tight zero dead volume union. The union was fabricated by cutting a 1cm length of 0.010in ID teflon tubing and reaming the ends of the tubing such that they would accept the pieces of fused silica capillary tubing, yielding a snug fit which was leak-tight at low pressure. Since the outside diameter of the 530 micron ID column was fairly large (~720µm), a pin vise drill (Supelco, Bellefonte, PA., USA) was used to machine one end of the zero dead volume union prior to reaming for a tight seal.

The polyimide cladding of the transfer line fused silica tubing, at a point about 2cm from the end of the capillary column, was burned off using a

butane lighter and the soot removed with isopropanol to provide a 6 to 8mm cell window for UV/Vis detection. Figure 19 shows the instrument as assembled.

Use of the photodiode array detector provided for UV/Vis detection at any wavelength from 190 to 800nm, and for more information about an eluting analyte in the form of a UV spectrum taken instantaneously at any given time during the elution of a peak. This UV spectrum, though not confirmatory, provided some means for identifying a given peak. In addition, peak purity could be assessed by comparing spectra taken at different points during the elution of a chromatographic peak. "Pure" peaks will exhibit essentially identical spectra across the peak, while the coelution of two analytes will result in dissimilar spectra at different points during the peaks' elution[115].

4.2.3 Reagent Selection & Preparation.

Nucleobases and nucleosides are weak bases, and as such carry no charge at neutral or slightly acidic conditions. However, nucleotides contain phosphate groups which dissociate under neutral or weakly acidic conditions. This made anion exchange chromatography the separation mode of choice. As the ionization of the phosphate groups is pH dependent, adjustment of the pH of the eluent modified the retention behaviour of the analytes. This was a useful tool in method development, as simple adjustments in pH affected the interaction of the analyte with the stationary phase surface to a large degree.

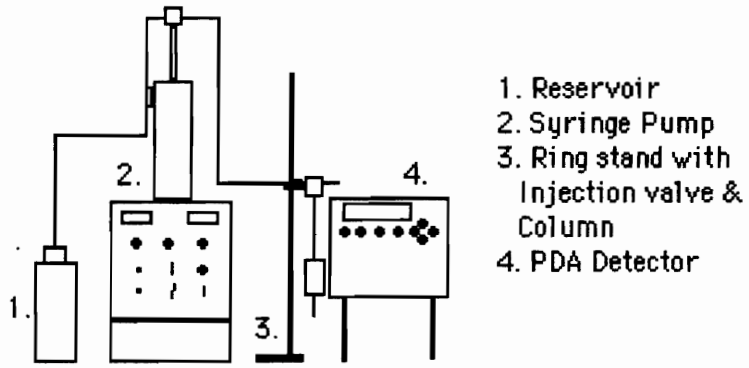


Figure 19. Instrumenatation used in micropacked capillary ion exchange chromatography.

The choice of eluent composition was predicated by the fragility of the sample[116]. An eluent must not only meet chromatographic needs, but must provide an environment in which the sample maintains its integrity. Ammonium phosphate buffers served well in both of these roles. The selection of pH and buffer concentration was made by adjusting the amounts of monobasic and dibasic ammonium phosphate added. A mobile phase strength of 150mM at pH 5.4 was found to yield satisfactory results. The ammonium phosphate salts were obtained from Fisher Scientific (Fair Lawn, NJ, USA), and the mobile phase was prepared in 18 megohm, double-distilled, deionized water.

Samples utilized in method development were obtained from Sigma Chemical Co. (St. Louis, MO., USA). These samples consist of nucleoside 5'-mono-phosphates, specifically: adenosine monophosphate, uridine monophosphate, guanosine monophosphate, and cytidine monophosphate. Thymidine monophosphate was not available. The samples were prepared as individual solutions 10ppm in concentration, and one solution of all 4 components at 10ppm each was also prepared. The solvent used in all cases was ammonium phosphate buffer, also used as the mobile phase described previously.

The sample used in method verification was a rattlesnake venom phosphodiesterase digest of a mixture of synthetic single stranded RNA's produced via the polymerase chain reaction technique purchased from United States Biochemical (Cleveland, Ohio, USA) . This type of sample was used to verify the utility of the method in the analysis of nucleotides prepared by

digesting a "real-world" strand of RNA. The sample was provided in 100mM ammonium phosphate buffer at pH 7.5.

4.3 Pressure-Driven Separations in Packed Capillaries.

4.3.1 Column Production Procedure.

The manufacture of micropacked capillary HPLC columns of 200μm to 320μm inside diameter is not exceedingly difficult, although the success rate in fully packing the desired length of capillary is only 50 to 60%. For the capillaries that fully pack performance characteristics tend to be quite good, on the order of 80 to 85% of theoretical efficiency. These statements allude to the mechanical difficulty of manufacturing micropacked capillary columns. When attempts are made to produce even smaller inside diameter columns, the mechanical forces which impede packing are even further enhanced, and for this reason only a 10 to 20% success rate in manufacturing 50 or 100 micron ID columns was achieved.

Fused silica capillary tubing from Polymicro Technologies (Phoenix, AZ., USA) was used exclusively in producing micropacked capillary columns of 320, 250, 100, and 50 micron inside diameter. All of the 220 and 200 micron ID columns were made with fused silica tubing also, though it was obtained through Scientific Glass Engineering (Victoria, Australia). The tubing was generally cleaved to a length of 15 to 25 cm, and was then fitted with a ceramic end frit at one end using Kasil #1, a potassium silicate solution (PQ Corp., West Lafayette, PA., USA), using a method first described by Cortes[117].

A 10:1 (v/v) mixture of the Kasil^{R} solution from PQ Corp. (Valley Forge, PA., USA) and formamide from Sigma Chemical Co. (St. Louis, MO., USA) was prepared in a 2ml disposable vial, and one end of each column blank was in turn placed in the vial. By holding the vial up to a bright light, a meniscus of liquid could be observed to move slowly up the capillary. In the 50µm column blanks some maneuvering was required in order to be able to see the meniscus, and in some cases a 10x optical comparitor from Supelco, Inc. (Bellefonte, PA., USA) was needed in order to see the liquid movement. Vision correction in the form of prescription glasses was also required, and was necessary in increasing strength over the course of the work. The solution was allowed to fill one end of the capillary to a length of one centimeter before the capillary was removed from the vessel and placed in a flask with the "frit" end down. A laboratory wipe was stuffed into the top of the flask in order to secure the column blanks while they were cured in a GC oven at 120° C for one hour. After removal from the oven, the end frits could be observed as white segments at the end of each capillary length. The frits could then be cleaved using a whetstone or similar cleaving tool for capillary GC columns to any desired length, with 1mm being appropriate for columns larger than 200 microns in ID, and 0.5mm being chosen for the smaller ID capillaries. A number of "batches" of column blanks were prepared by this method.

Borra[118] and others have recommended the use of Triton X-100 as a slurry solvent additive for the packing of capillary columns, but in this work it was not used. The use of another dispersing agent, Brij 35 puchased through Fisher Scientific (Fair Lawn, NJ, USA) at the 1.5% concentration recommended by Borra for Triton was tried, but no significant improvement in packing

resulted. For this reason, 100% acetonitrile was used as both slurry and displacement solvent for all ensuing column production. Slurries were prepared as 1g:5ml ratios using Lichrosorb RP18 packing materials from EM Laboratories, Inc. (Elmsford, NY, USA) of several different mean particle diameters. These particles are irregular in shape, and therefore required special care in handling such that "fines" would not be produced in preparing the slurries. Prolonged sonication of slurries containing these irregular particles resulted in the production of small (submicron) fragments of packing material which were not immobilized in the packed bed structure of the column. These fines would, while the column was in use, eventually migrate to the end frit of the column where they would impede flow. This resulted in increased backpressure, eventually rendering the column useless. A small quantity of Spherisorb (Phase Separations, Ltd., Queensferry, Clwyd, UK) S10 ODS 10 micron spherical packing material was also available, and was slurried using the same methods as described for the Lichrosorb material.

With the exception of liquid pressure source, all of the reversed phase micropacked capillaries were packed using the same apparatus. A Haskel (Haskel, Inc., Burbank, CA, USA) pneumatic amplifier pump was used to pressurize a home-made 4.0mm ID x 50mm length cylindrical slurry reservoir containing the slurried packing material. A liquid-filled pressure gauge and needle valve were, as before, used to monitor pressure and isolate the slurry reservoir from the pump until the system had reached the desired packing pressure. Columns were packed at 2500psi using acetonitrile as the displacement solvent by connecting the column directly to the outlet of the slurry reservoir and allowing the empty capillary to hang loosely from the

immobilized reservoir. Figure 20 is a representation of the apparatus used to pack capillary HPLC columns. In some of the later work in pressure-driven micro-LC presented here, a Hitachi (Mito, Japan) model 655 dual reciprocating piston HPLC pump operated in the constant pressure mode was substituted for the Haskel pump. The aforementioned 10x optical comparitor could be used to observe particles of packing material as they moved through the column and accumulated at the top of the column bed.

The failure rate in manufacturing columns of the desired length was mentioned earlier to have some dependence on column inside diameter. In the 50 micron ID columns, frequently a short segment (5-8cm) of column could be packed successfully followed by a inability to complete the packing process for the remainder of the length of the capillary. Solvent flow through the column would not noticeably decrease during this process. Removal of the column and inspection under a microscope would often reveal a "plug" of packing material wedged into a small portion of capillary near the column inlet. Given the large diameter of the tubing (relative to the packing material particle diameter) it seems most feasible that this occurs due to agglomeration of packing material. The capillaries which met this fate were not useable, and were discarded. The use of slurries at lower ratios did not seem to improve the likelihood of the capillary fully packing, and the original slurry ratios were returned to for all subsequent columns.

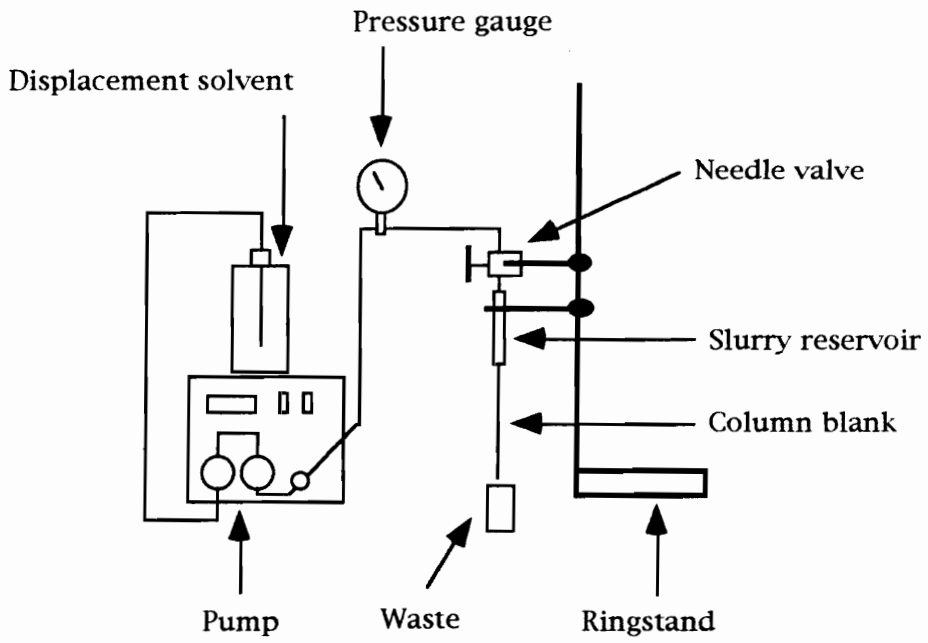


Figure 20. Packing apparatus schematic showing the configuration used in manufacturing micropacked capillary HPLC columns.

4.3.2 Instrumentation Design.

The ISCO μLC-500 syringe pump previously mentioned was used for all of the pressure-driven micro LC experimentation described herein. All of the micropacked capillary HPLC columns were operated at a variety of different flow velocities such that van Deemter curves could be constructed for each. This demanded (for the 50 to 200 micron ID capillary columns) an eluent splitter for the syringe pump. This was a necessity, as the author was concerned about the ability of the pump to reproduce flow rates on the order of one to five μl/min. For all flow rates at or below 5 μl/min, flow was split via a low volume butt-connector designed for splitting capillary SFC effluents for dual detection, sold by Lee Scientific, Inc. (Salt Lake City, UT., USA) shown in figure 21. The author is indebted to Dr. Jack Hensley of the Tennessee Eastman Company for demonstrating the use of this connector in an SFC application. On one arm of this tee a 320 micron ID column was placed as the split column, while the other arm was connected to a Rheodyne 7520 micro injection valve with a 200nl internal loop rotor installed.

The micro valve was a major contributor to extracolumn volume for the 50 and 100 micron ID columns, and was also non-ideal for the 200 micron ID columns produced. A better substitute was unavailable, so the technique of "sample solvent focussing" mentioned earlier in this text was employed for these studies. Packing material inside the capillary was isolated from the injection valve by cutting a small frit from a 0.45 μm HPLC membrane filter obtained from Gelman Sciences (Ann Arbor, MI., USA) using a pair of moustache trimming scissors. This material could be carefully cut to yield a

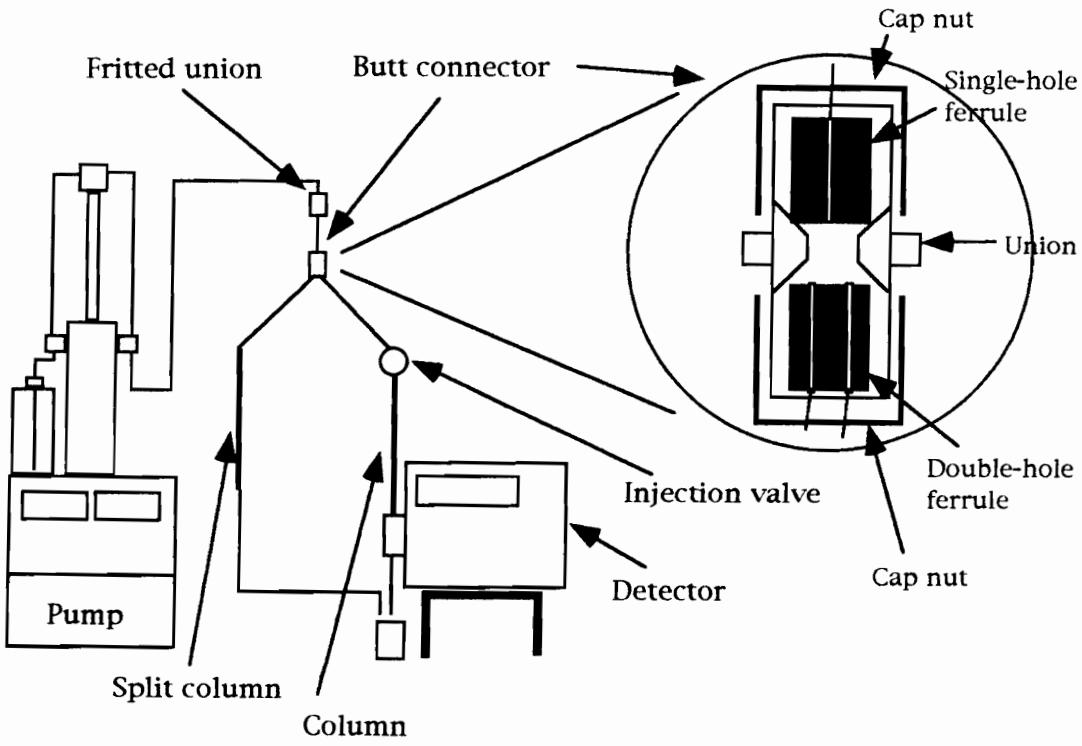


Figure 21. Flow splitting apparatus used in micropacked capillary HPLC, showing the SFC butt-connector employed to split flow.

somewhat circular frit of ca. 1/16th inch diameter. The frit was then placed inside the port on the valve into which the column would be inserted. This process was imperative to the proper functioning of the valve, as it prevented the migration of packing material into the valve interior, which would have resulted in "scoring" of the rotor and led to valve failure. Similar filters, machine pre-cut to 1/16th inch diameter, became available part way through the completion of this work from LC Packings Inc. (Amsterdam, The Netherlands), and were used in the latter stages of the data collection. A cutaway view of the valve & column assembly appears in figure 22.

Only one 50μm and two 100μm columns were made for use in the pressure-driven format due to the difficulties encountered in manufacturing, handling, and operating these ultramicro packed capillary columns. Better success in operating these tiny columns was achieved electroosmotically.

Detection was performed by butt-connecting a detection capillary to the column using the previously described home-made teflon zero dead volume unions. A window was burned into the detection capillary, removing the polyimide cladding, using a butane lighter. In all cases except in the 320 and 250 μm columns, the detection capillary ID was identical to the column ID, necessitating a close placement of the capillary outlet to the detector cell window. The cell holder design of the Linear micro detector allowed easy mounting of the joined capillaries as diagrammed in figure 23. In the larger capillaries, a 75 micron ID transfer/detection capillary was butt connected to the column. This yielded a poorer sensitivity, but was not overly derogatory to performing quality separations. Data was collected on a Hewlett Packard 3390A integrator (Avondale, PA, USA).

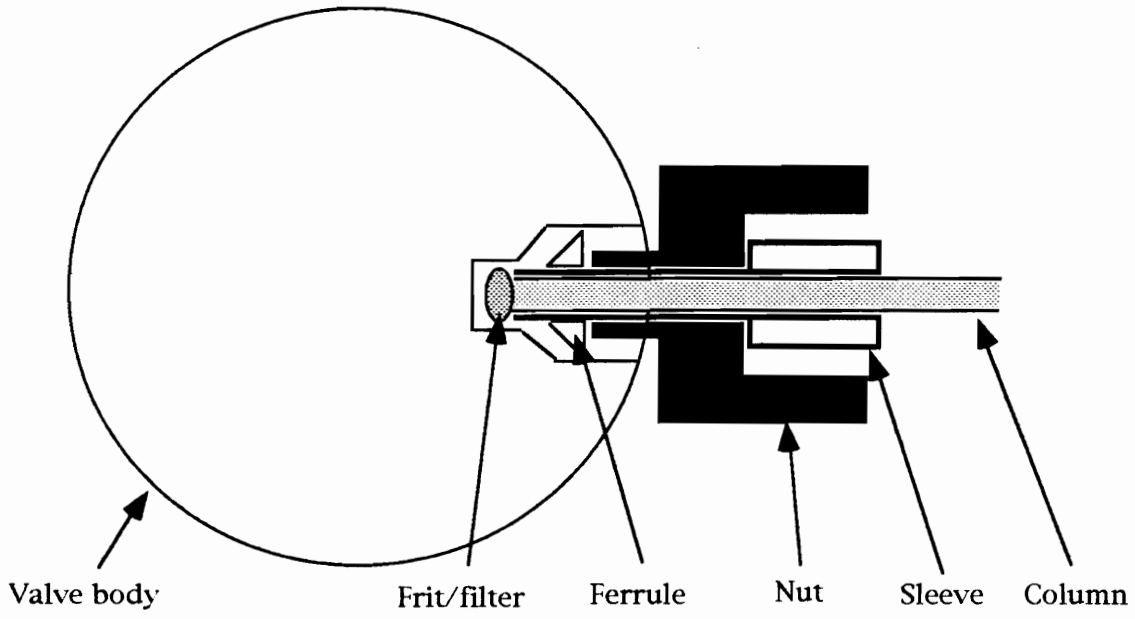


Figure 22. A cutaway view of the injection valve to column connection used in packed capillary HPLC, showing the placement of the removable head frit.

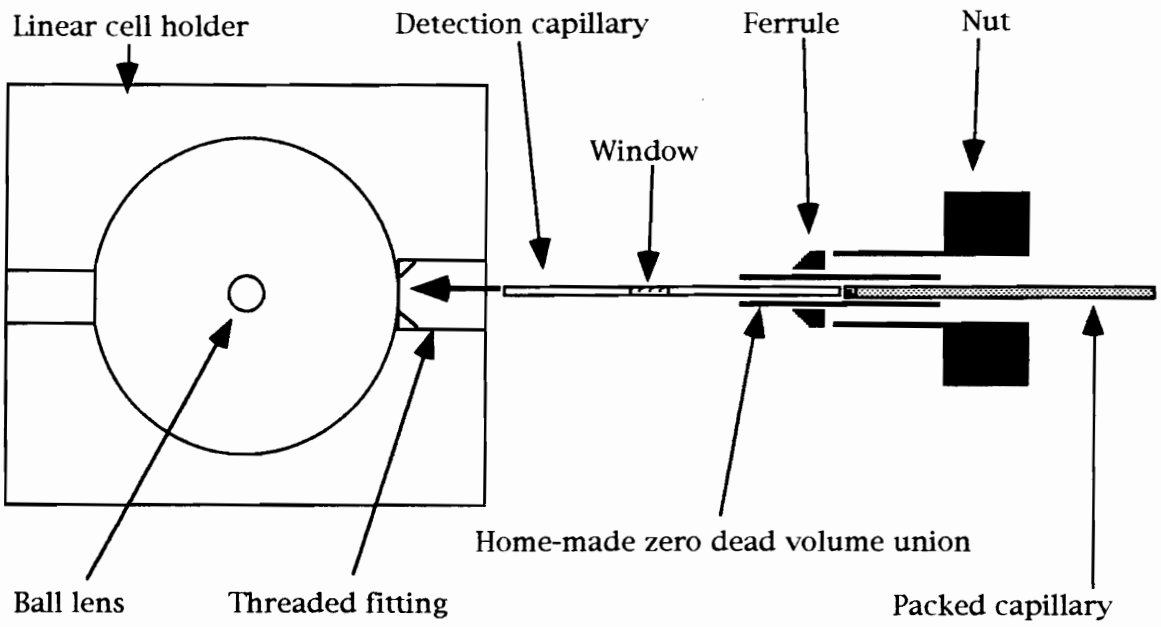


Figure 23. Mounting scheme for the connection of fused silica packed capillary HPLC columns to a Linear model 206 UV detector.

4.3.3 Reagent Selection & Preparation.

A number of different test probe solutions were used throughout this work. All were prepared in HPLC grade solvents from Fisher Scientific (Fair Lawn, NJ, USA), J. T. Baker (Phillipsburg, NJ, USA), or E. M. Science (Cherry Hill, NJ, USA). Mixtures of acetonitrile and water of several different compositions were used exclusively. Diethyl phthalate, acetone, and methyl benzoate test probes were from Fisher Scientific (Fair Lawn, NJ, USA), dibutyl phthalate and biphenyl were from Eastman Kodak (Rochester, NY, USA), benzyl alcohol was from Sigma Chemical Co. (St. Louis, MO, USA), benzene was from Fisher Scientific or Mallinckrodt Inc. (Paris, KY, USA), and 2,6-dimethyl aniline and dimethylterephthalate were from Aldrich Chemical Co. (Milwaukee, WI, USA). Acetone was used as a dead volume marker in all of the reversed-phase packed capillary HPLC presented.

4.4 Electroosmotically-Driven Separations in Packed Capillaries.

4.4.1 Column Production Procedure.

Columns prepared for Capillary Electrokinetic Chromatography were manufactured using the same techniques and apparatus described for the pressure-driven packed capillary columns (section 4.3.1, above). Tubing and packing material choices were the same, with fused silica being purchased from both Scientific Glass Engineering (Victoria, Australia) and Polymicro Technologies (Phoenix, Arizona, USA). The packing materials used were from

the same vials used in the preparation of the pressure-driven micro LC columns. The end fritting procedure was also identical to that described previously in section 4.3.1.

The starting lengths of the capillaries packed were a bit longer than for the earlier study, such that a window for optical detection could be made in the separation capillary rather than in a transfer line capillary as was done in the pressure-driven work. Each capillary was only partially packed, such that bed lengths were between 12 and 20 cm with a long open section of capillary remaining for detection. In the first two capillaries made for electrokinetic chromatography, packing material at the unfritted end of the bed was sintered by heating gently with a blue flame from a hand held microtorch (Archer model B, Radio Shack). This process made the capillaries quite fragile, as the packing material stiffened the capillary, and the protective polyimide cladding was removed. Additionally, this procedure was frightening to the experimenter, in that it could potentially ruin the product of several days work. Careful thought and perusal of the literature led to the realization that no outlet frit was necessary, since the ionized silanols at the surface of the particles of packing material gave each particle of packing material a net negative charge. As the inlet of the system was at the anode, and since the fritted end of the capillary was placed at this electrode, packing material would be held in the capillary when a potential was applied[119]. After two disastrous attempts at sintering frits, the microtorch idea was abandoned and no outlet frit was used. This arrangement worked quite well.

4.4.2 Instrumentation Design.

The instrumentation used in affecting electrochromatographic separations consisted of a Spellman High Voltage Electronics Corp. (Plainview, NY, USA) model UHR100 high voltage power supply, a home-made plexiglass safety box, and the Linear Instruments (Reno, NV., USA) model 206 detector used in the pressure-driven micro-LC work described in section 4.3. The high voltage power supply was modified by removing the one-turn potentiometer it was supplied with and replacing it with a 10-turn potentiometer and a 10-turn metering dial. This allowed for more precise control over applied potential both for running the system and making electrokinetic injections. Another modification consisted of the addition of a microammeter.

Several iterations of plexiglass safety boxes were used, with the last one being the most flexible and convenient. This final design, suggested by Dr. Chris Palmer, was spacious and rugged. It was constructed of 1/4 inch plexiglass with a plywood base and offered access through a hinged door at the front. Several access holes and slots made electrode and capillary placement simple and convenient.

The high voltage cable supplied with the power supply was modified by the addition of a new banana plug to which an alligator clip was attached. The alligator clip offered an efficient means of holding the 0.5 mm outside diameter platinum wire from Aldrich Chemical Co. (Milwaukee, WI., USA) which served as the electrode, and made electrode replacement easy. An earth ground was provided by purchasing a 3/8 inch zinc coated copper rod, one yard in length, from a local hardware store. This rod was driven into the

ground outside of a nearby lab window, and a copper ground wire was attached to it using a 1/2 inch cable clamp. The ground wire could then be connected to the grounding point on the power supply as well as the ground lead for the separations circuit. This wire had an alligator clip attached to hold the platinum wire ground electrode.

The fritted end of a packed capillary was then immersed in the vessel in which the high voltage platinum electrode (anode) was placed. A small window was burned into the packed capillary column using a butane lighter or a capillary window burning tool made from a model airplane glow plug, step-down transformer, and a variac[120]. The window burning tool was particularly effective at producing narrow (2mm) detection windows. Figure 24 shows the burning tool. The section of capillary with the window was then mounted in the cell holder provided with the Linear detector, and the free end of the capillary was placed in a second vial of mobile phase along with the ground electrode. Application of a high potential (5 to 15 kV) gave rise to electroosmosis through the capillary.

Prior to installation, all capillaries were flushed and filled with the mobile phase using the ISCO syringe pump. After one or two electrochromatographic runs (occasionally three) the capillaries required rewashing and refilling. This was necessary because of bubble formation at the junction of the packed bed and the open tubular portion of the capillary, discussed elsewhere. For flushing, the capillary column outlet was connected to the ISCO syringe pump using an Upchurch Scientific (Oak Harbor, WA., USA) low volume fritted union.

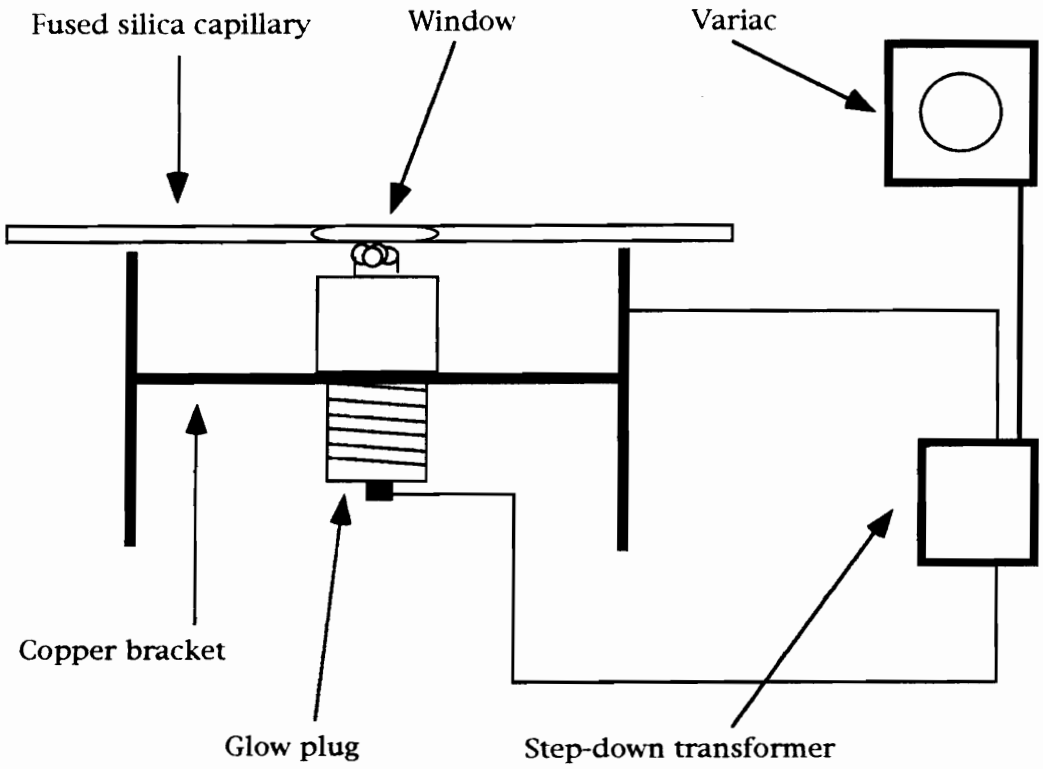


Figure 24. General schematic of a home-built capillary window burning tool made from a model airplane glow plug.

Injections of sample were made electrokinetically, by placing the high voltage electrode and the capillary inlet into a vial of sample solution and applying a low voltage (2 to 5 kV) for a short time period (3 to 10 seconds). This process results in a gentle electroosmotic flow into the capillary which moves a small zone of sample into the column. The electrode and capillary are then replaced in the mobile phase vial, the potentiometer is adjusted to the desired setting, and the separation is started by applying the higher potential. In the latter stages of this work, when transfer line length effects were studied, the usual small vials or test tubes used for mobile phase and sample were replaced by glass tee joints which allowed for shorter capillaries to be used. Data was collected on a Hewlett-Packard 3390A integrator. A diagram of the entire separations system is shown in figure 25, in the conformation used for short columns and transfer lines.

4.4.3 Reagent Selection & Preparation.

All of the Capillary Electrokinetic Chromatography data was collected by running in a 60% acetonitrile/40% aqueous sodium phosphate buffer (5mM, pH ~7) mobile phase. Acetonitrile was from the previously mentioned sources. The phosphate buffer was prepared by making two solutions: one of monobasic sodium phosphate (0.2M) and one of dibasic sodium phosphate (0.2M). These solutions could then be mixed to yield any desired pH and ionic strength in the buffering range of a phosphate buffer by use of the Henderson-Hasselbach equation. Sodium salts of phosphate were from Aldrich

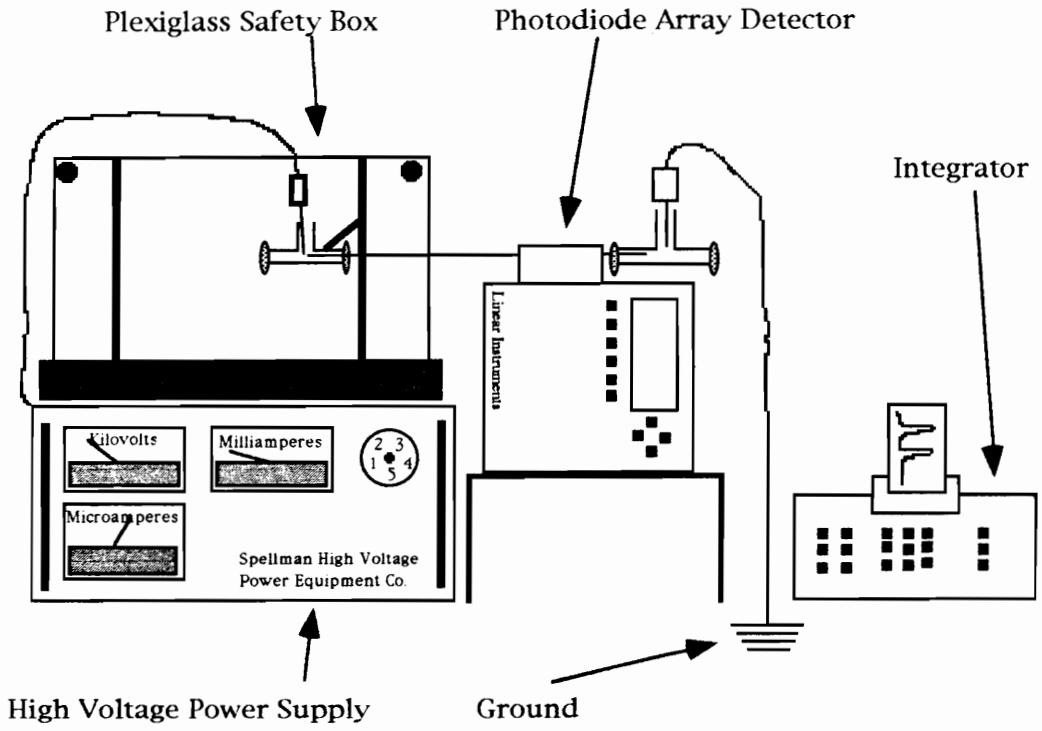


Figure 25. Apparatus used in Capillary Electrokinetic Chromatography.

(Milwaukee, WI, USA) or Sigma (St. Louis, MO., USA) or Fisher (Fair Lawn, NJ, USA). Samples were prepared in 60:40 v/v acetonitrile:water. All solvents used were HPLC grade with the exception of the water in which the buffer was prepared, which was double-distilled, deionized water. The mixed mobile phase was filtered through a 0.45 micron filter prior to use, as were samples.

CHAPTER V

RESULTS & DISCUSSION

5.1 Semi-Micro Ion Chromatography.

5.1.1 System Design & Performance.

The chief advantage of using semi-micro columns over using conventional 4mm ID columns in Ion Chromatography lies in detection. A perceived increase in sensitivity results when column inside diameter is decreased. This phenomenon is only observed when injection volume and detector cell volume are unchanged from those in conventional IC. As mentioned previously, however, decreases in the physical dimensions of HPLC columns must be accompanied by decreases in the volume of all system components if high efficiency is to be maintained. Keeping these facts in mind, experimentation was conducted which would indicate whether semi-micro IC on conventional IC systems was a practical means of increasing sensitivity and lowering detection limits.

In order to provide an unbiased comparison of semi-micro and standard bore IC columns, identical flow velocities must be used in both columns. Flow velocity is related to volumetric flow rate as[122]:

$$v' = (\mu\ d_c^2\ \pi\ \varepsilon_{tot})\ /\ 4 = \mu\ r_c^2\ \pi\ \varepsilon_{tot} \qquad \{30\}$$

where: μ = **linear flow velocity;**

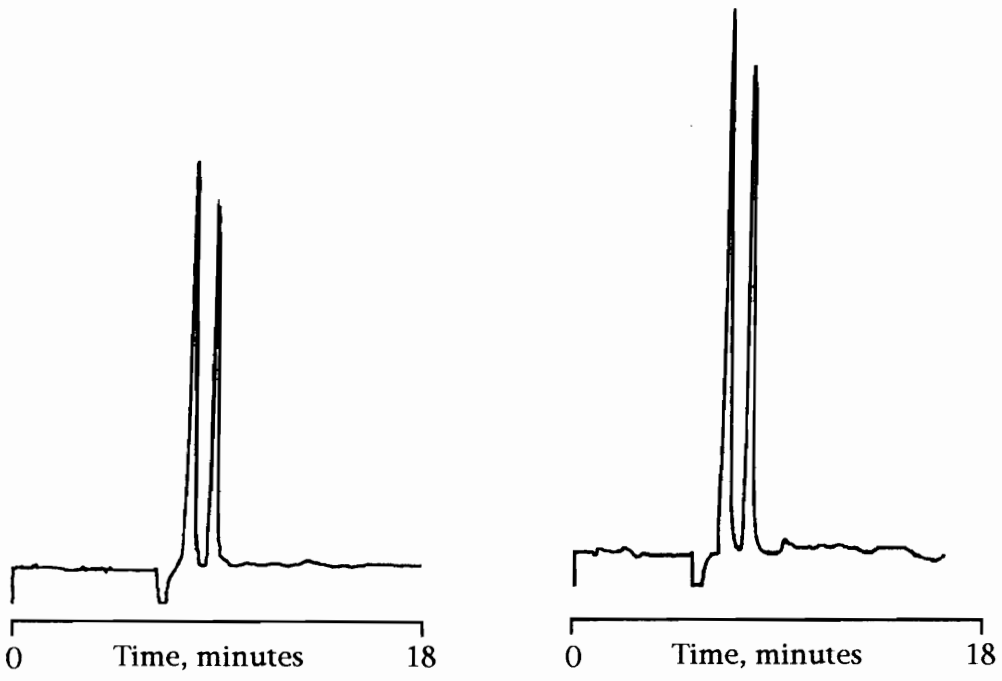
d_c^2 = **square of the column diameter;**

ε_{tot} = **total porosity of the packing material; and**

r_c^2 = **square of the column radius.**

The relation of volumetric flow rate to column size as the square of the column radius indicates that the 2mm ID semi-micro IC column should be operated at 1/4th the flow rate of the conventional bore column. For a volumetric flow rate of 1mL/min in the conventional column, a 250μL/min flow rate was used in the semi-micro column. Under these conditions, flow velocity in both columns is identical. With all other parameters in the system unchanged, the chromatograms in figure 26 were obtained. The results indicate that no sacrifice in separation time is made in either case: semi-micro or conventional IC. The peak heights and areas for both the fluoride and chloride peaks have increased, indicating that better sensitivity has resulted from the use of a semi-micro column.

The increase in peak height and area is easily explained. In both analyses the injection volume was unchanged. The use of a lower flow rate in the semi-micro separation indicates that the volume of sample injected will be diluted into a smaller volume of mobile phase in the same time of analysis as for the conventional system. In other words, the concentration of analyte once it is detected is higher in the miniaturized system than it was in the conventional system: an equal mass of analyte is eluted in one fourth of the volume of mobile phase. Since the conductivity detector used is a concentration dependent detector, the resulting signal is larger for the



A separation of Fluoride and Chloride on a 4mm ID column.

A separation of Fluoride and Chloride on a 2mmID column.

Figure 26. Chromatograms illustrating the sensitivity improvement achieved when a 4.0mm ID column is replaced by a 2.0mm ID column.

Table IV. A numerical comparison of the sensitivity enhancement achieved when a 2.0mm ID column replaces a 4.0mm ID column in Ion Chromatography.

Column ID/Run	Pk. Ht. (F-)	Pk. Ht. (Cl-)	Pk. Area (F-)	Pk. Area (Cl-)
4.0mm / 1	43mm	31mm	619403	598452
4.0mm / 2	47mm	36mm	621856	604519
4.0mm / 3	45mm	35mm	617864	593214
4.0mm / avg	45mm	34mm	619708	598728
2.0mm / 1	118mm	72mm	1146535	984376
2.0mm / 2	104mm	67mm	1157646	993529
2.0mm / 3	121mm	75mm	1095984	981987
2.0mm / avg	114mm	71mm	1133388	986630
Sensitivity Enhancement	2.53:1	2.09:1	1.83:1	1.65:1

microseparation than for the conventional one. This phenomenon would not be observed if the injection volume were scaled down along with column ID, fairly comparing the two different dimensions. Table 4 provides a numerical comparison of the sensitivity enhancement achieved in using a semi-micro IC column.

The use of small scale columns in HPLC should be accompanied by decreases in extracolumn volume so that maximum efficiency is achieved. As discussed in the Theory section of this dissertation, one important contributor to extracolumn volume is the detector cell. In the initial experiments conducted on semi-micro IC, the conventional IC conductivity detector cell was used. Although this cell is not a major contributor to zone broadening in conventional scale IC, it can potentially degrade efficiency in a miniaturized IC system. From theory, remember that efficiency is calculated as:

$$N = t_r^2 \; / \; \sigma^2 \qquad \{31\}$$

where: t_r = **the retention time of an analyte; and**

σ^2 = **the variance of the peak representing that analyte.**

This equation illustrates the effect which an increase in peak variance has on efficiency: the larger σ^2 becomes, the lower efficiency becomes. Clearly, decreasing the variance contribution of the detector cell by decreasing its' volume can reduce total peak variance and thereby increase the measured efficiency of the chromatographic system. The detector cell contribution to this peak variance was given in equation {29} to be:

$$\sigma^2_{det} = (V^2_{det} / 12) + (F\pi r^4 L / 24 D_m) \quad \{32\}$$

where: **F = the volumetric flow rate;**

r = detector cell radius;

L = the cell length; and

D_m = diffusion coefficient of the analyte in the mobile phase.

The inside diameter of the conventional conductivity detector cell was 1.0mm and the length of the cell was 13mm, so that the cell volume was 10.2μL. Diffusion coefficients of analytes in liquid media are typically in the range of 1×10^{-5} cm^2/sec. The flow rate used in the conventional column was 1mL/min and that for the semi-micro column was 250μL/min. Substituting these values into equation 31 yields the σ^2_{det} values:

$$\sigma^2_{det,4mm} = 1808.67 \ \mu L^2$$

for the conventional 4mm ID column with the standard conductivity cell; and

$$\sigma^2_{det,2mm} = 451.82 \ \mu L^2$$

for the semi-micro column used with the same detector cell. Clearly, the volumetric flow rate has a marked effect on peak spreading due to flow velocity profile. The use of a semi-micro column (with a smaller volumetric

flow rate for the same velocity) with a conventional detector is therefore more feasible than might seem evident at first glance. In order to determine whether this theoretical conclusion on peak broadening matched well with practice, an experiment was conducted in which the detector cell contribution to band broadening was measured.

A 20cm length of teflon^{TM} tubing 0.007" in inside diameter was placed between the injection valve and the detector cell. In this configuration, five flow injection analyses were conducted using a 10ppm chloride test solution at a flow rate of 1mL/min. Peak variance data was collected for the chloride band by conversion from width of the peak at half height[123], as in figure 27. Five further flow injection analyses were conducted at 250µL/min, duplicating the flow conditions of a semi-micro IC analysis. At this point, the connective tube was removed and cleaved to 18cm, replaced, and the process of data collection continued as before. Plotting the measured peak variance as a function of tubing length added allows for the determination of peak variance caused by the detector cell itself by extrapolation to zero tubing added. Figure 28 illustrates the extrapolation to peak variance for the conventional conductivity detector under the flow conditions for both conventional IC and semi-micro IC.

Although the variance contribution of the conventional detector cell in both analytical-scale and semi-micro IC is relatively small, this cell causes enough dispersion to affect system performance. In order to determine whether the variance contribution of the detector cell is large enough to significantly degrade the efficiency of the semi-micro IC system, the variance arising from the column itself must be known. The peak variance

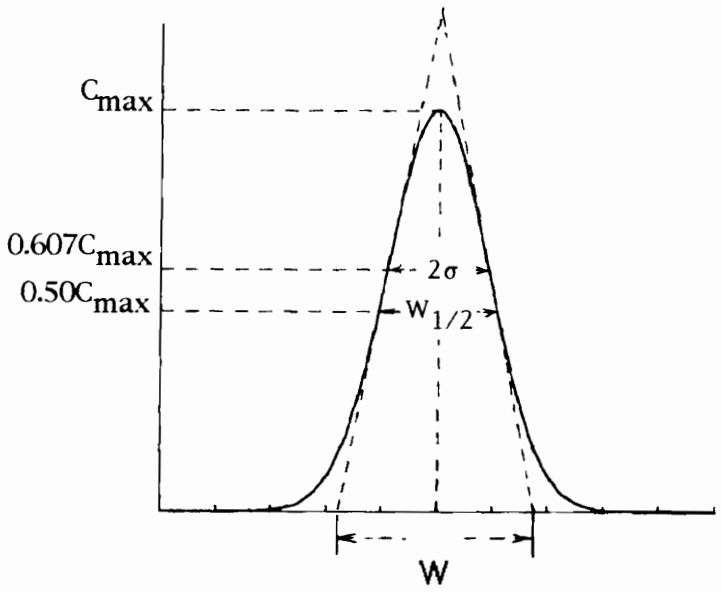


Figure 27. Calculation of peak variance for a gaussian distribution of molecules eluting from a chromatographic column. (From ref. 123)

contribution of the semi-micro IC column can be estimated using equation {33}, first described in the Theory section of this dissertation:

$$\sigma^2_{column} = \{4V_m \ (1 + k') \ / \ (N)^{1/2}\}^2 \qquad \{33\}$$

Using the water front (commonly referred to as a water "dip") as the indicator of t_o, the dead volume (or void volume) for the semi-micro column is determined as:

(elution time of the water dip) x (flow rate) = (dead volume). {34}

For the semi-micro IC column used this study, the dead volume averaged 210µL (n=5). For the chloride peak, the capacity factor (k') mean for 5 determinations was 1.28. Also determined using the chloride peak for 5 analyses was efficiency (N), which averaged 1769 theoretical plates. Substituting these values into equation {32} gives:

$$\sigma^2_{column} = \{4(210\mu L) \ (1 + (1.28)) \ / \ (1769)^{1/2}\}^2$$

$$\sigma^2_{column} = 2073\mu L^2 \quad \text{for the 2mm ID column}$$

Using the guideline that the peak variance contribution of the column should be the largest factor in total variance, it seems that the conventional detector cell does not degrade the semi-micro separation as badly as one might suppose, since:

$$\sigma^2_{col} > \sigma^2_{det}$$
$$2073\mu L^2 > 452\mu L^2$$

However, *any* reduction in extracolumn volume will lead to improved efficiency. In an effort to improve system efficiency, a reduction in detector cell volume was desired.

In order to decrease detector cell volume, a home-made microconductivity cell was constructed. The dimensions of the cell were considerably reduced over those of the conventional conductivity cell. The length of the cell was 11mm, and the ID of the cell was dictated by the ID of the tuberculin needle used as an electrode as 0.4mm. This results in a calculated cell volume of 1.38μL.

The volume of the detector cell was also determined gravimetrically by first weighing the cooled dry cell (dried at 60 C for 12 hours), then subtracting this value from that of the wet cell (filled with distilled, deionized water). Dividing this difference by the density of water yielded a volume of 1.26 μL. Electrode spacing was measured under a microscope at 40 x magnification to yield a value of approximately 0.5 mm.

According to equation {31}, using the larger (calculated) cell volume figure in order to remain objective, the microconductivity cell will contribute $9.76\mu L^2$ to peak variance. This represents a 45-fold decrease in detector cell contribution to peak variance. The connective tubing used in joining this cell to the suppressor column outlet was of the same length and inside diameter as that used with the conventional cell, and the same instrument design was

Table V. Efficiency enhancement through reduction of detector cell induced band spreading.

Column ID	Run No.	H(F-) [microns]	H(Cl-) [microns]
4.0mm	1	68	71
4.0mm	2	65	73
4.0mm	3	66	73
4.0mm	**average**	**66**	**72**
2.0mm	1	56	58
2.0mm	2	53	57
2.0mm	3	52	60
2.0mm	**average**	**54**	**58**

employed. The improvements in efficiency shown in table 5 are therefore due only to the decrease in detector-caused peak variance.

The experimenal determination of peak variance conducted for the conventional detector cell was also conducted for the microconductivity cell. A new piece of 0.007" ID teflon^{TM} tubing was cut to 20cm, and the experiment was carried out exactly as before at the 250µL/min flow rate used in the semi-micro IC determinations. Figure 29 is a composite of two curves used in determination of detector-caused peak variance: variance due to the microconductivity cell at 250µL/min; and variance due to the conventional cell at 250µL/min. Clearly, the use of the low volume micro cell is indicated if maximum system efficiency is desired. Two semi-micro ion chromatographic separations are shown in figure 30, which illustrates the benefits of using a small volume detector for microseparations. It is important to note that the use of a small volume detector does <u>not</u> improve sensitivity, but only improves efficiency. The sensitivity enhancements described are due solely to increasing the mass of analyte eluted in a given volume by holding injection volume and flow velocity constant while decreasing column ID.

5.1.2 Column Performance.

A total of three semi-micro anion exchange columns were produced. All three were evaluated with the same suppressor column in order that an unbiased comparison of the three columns could be made. In each case, a flow rate of 250 µl/min was used. Individual runs were isocratic at an eluent

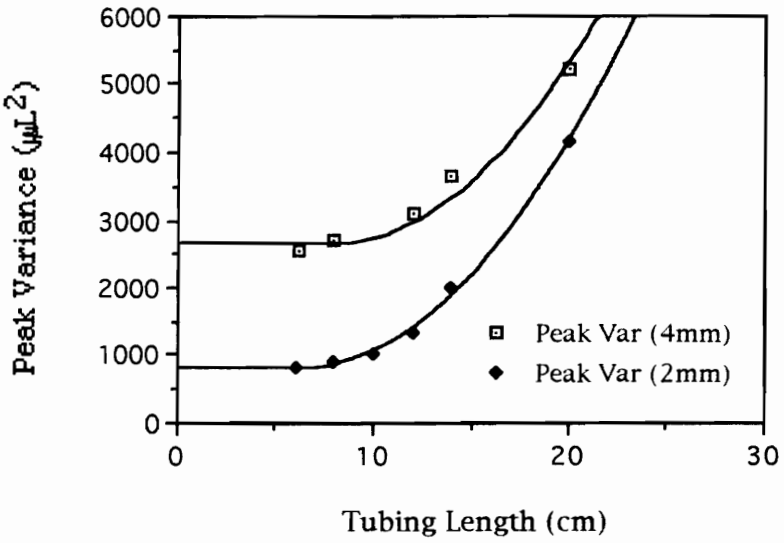


Figure 28. Experimental determination of detector caused peak broadening by extrapolation.

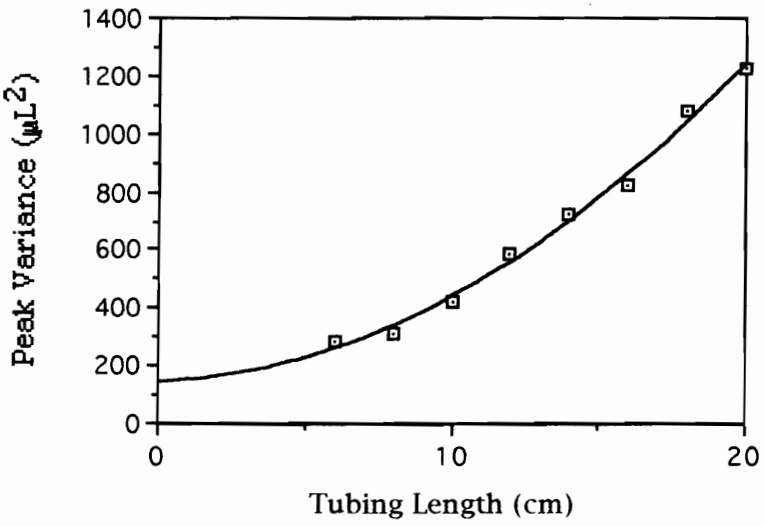


Figure 29. Experimentally deduced reduction in peak variance on reduction in detector cell volume.

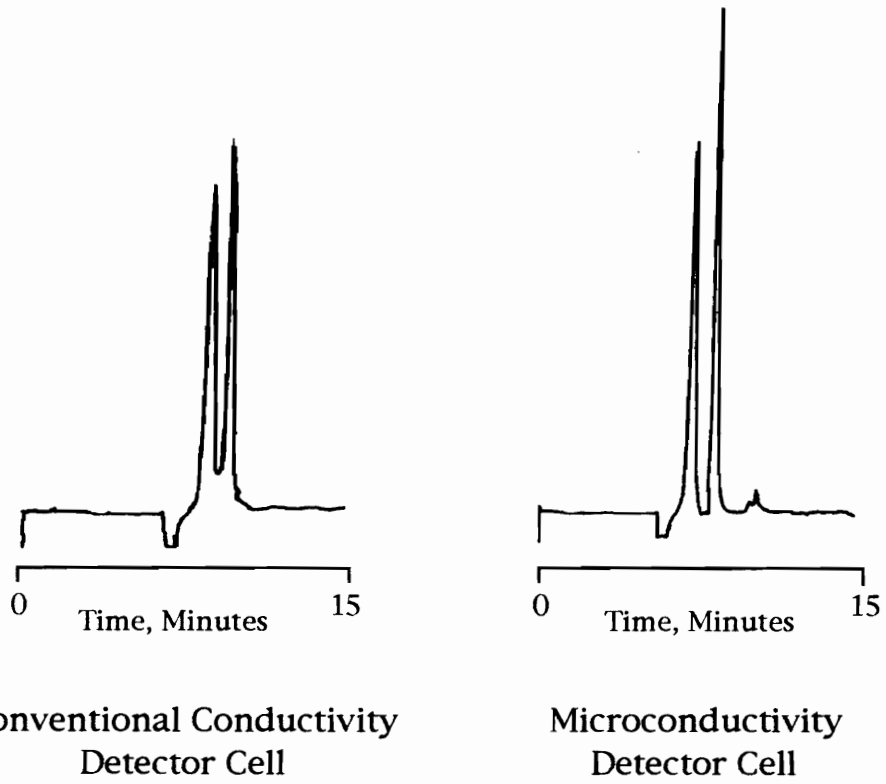


Figure 30. Two separations illustrating the enhancement in efficiency achieved when detector cell volume is decreased.

strength of 25 mN sodium hydroxide. The sample consisted of 10ppm fluoride and 10 ppm chloride in distilled, deionized water, prepared as described in section 4.1.3. Weakly retained analytes were chosen such that several runs would be possible before regeneration of the suppressor column became necessary.

Each analytical column was evaluated by running the sample three times in succession, followed by regeneration of the suppressor column as described previously. A second and third set of runs were then conducted on each column, each set again followed by the regeneration of the suppressor column. The data in table 6 represent the nine runs on each column, conducted in sets of three runs per column, with columns evaluated in random order. A t-test was performed at the 95% confidence interval in order to compare the three columns to one another in terms of height equivalent to a theoretical plate, and this test indicated that the columns were not significantly different from one another.

The plate heights measured for the three columns ranged between 0.13 and 0.19mm for chloride. For a 15mm particle, as was used in these experiments, the arbitrarily selected "theoretical limit" of plate height for maximum efficiency is given by:

$$H_{opt} = h_{opt} \times d_p = (2)\ (15\mu m) = 30\mu m = 0.03mm \quad \{35\}$$

Table VI. Column efficiency (measured as H) for nine runs on each of three columns, and t-test results for comparing the three in terms of H. (All columns 2.0mm ID.)

Run No.	H (Cl-) column A	H (Cl-) column B	H (Cl-) column C
1	58	63	56
2	61	62	59
3	62	59	58
4	57	62	60
5	59	61	61
6	60	58	59
7	61	59	60
8	63	60	59
9	59	58	61
mean	60	60	59
s	1.94	1.86	1.56

t_{calc} A-B = 0.25 t_{tab} A-B = 2.36 A & B not different (95% CI, 7df)

t_{calc} A-C = 0.53 t_{tab} A-C = 2.36 A & C not different (95% CI, 7df)

t_{calc} C-B = 0.72 t_{tab} C-B = 2.36 C & B not different (95% CI, 7df)

where: H_{opt} = best (lowest) achievable plate height;

h_{opt} = the optimum reduced (dimensionless) plate height, accepted[124,125] to be 2; and

d_p = particle diameter of the packing material.

Evidentally, these columns are not perfect according to theory, as they yield plate heights which are several times larger than optimum. It must be remembered, however, that these are ion exchange columns manufactured using a micropellicular support, and are operated in a system with fairly high extracolumn volume due to the use of a suppressor column. The optimum h suggested is pertinent to microporous reversed-phase and normal-phase HPLC columns. In order to assess the performance characteristics of the semi-micro IC columns manufacured in this study in the absence of an appropriate theoretical optimum, a direct comparison to a conventional, commercially-produced IC column was conducted.

Using the conventional detector cell and an identical sample flow path and injection volume, several analyses were conducted on both a semi-micro column and a Dionex (Sunnyvale, CA., USA) HPIC^R AS4A 4mm x 250mm anion exchange column. The runs on each column were conducted using several mobile phase linear velocities, such that van Deemter curves for each column could be constructed. These curves are illustrated in figure 31. That the two curves are practically coincident indicates that the home-made semi-micro columns are packed as well as the commercial columns, and in fact as well as can be expected.

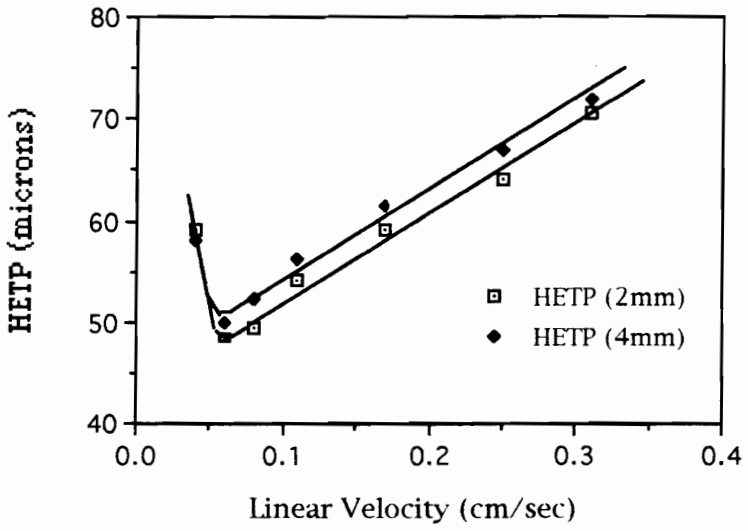


Figure 31. van Deemter curves for a semi-micro IC column and a conventional column packed with the same packing material.

The largest single benefit of the semi-micro IC system employed here is the increase in sensitivity achieved when a small column is employed in a conventional system. Care must be taken in employing conventional injection volumes on semi-micro columns so that overloading of the column is avoided, but in the limit that the columns' capacity is not exceeded, a useful amplification of signal is achieved with little sacrifice in efficiency. The use of a low-volume microconductivity cell maintains the sensitivity "enhancement" while improving on system efficiency through a reduction in extracolumn band dispersion.

5.2 Micropacked Capillary Ion Exchange Chromatography.

Packed capillary columns, though not yet in widespread use, are being researched avidly by several groups[126,127]. The general concensus of these researchers is that packing capillaries, though not a trivial task, is possible. Most experimenters have met with good results in their attempts, obtaining reduced plate heights approaching 2 in many instances. Again, though, these values have been reported for normal-phase and reversed-phase separations on microporous sorbents in packed capillaries. For ion exchange chromatography on large pellicular sorbents, the best achievable reduced plate height may in fact be somewhat higher than 2, based on the semi-micro experimentation previously described.

5.2.1 Column Performance.

In comparison to the 2mm semi-micro IC columns previously described, and to conventional IC columns, the results obtained in these experiments were surprising. As evidenced by Table 7, the measured efficiencies were substantially higher in the 530μm micropacked capillary ion exchange columns. In order to make these efficiency measurements, the home-made microconductivity cell used in the 2mm semi-micro experiments was directly coupled to the micropacked capillary IC column. This was accomplished by cutting the connective tube leading to the cell to a length of 7mm, reaming the end with a burr reamer, and snugly inserting the column. This resulted in a low dead volume union, and allowed for the detection of the 10ppm chloride test probe used in the earlier experiments. All of the columns were evaluated at the same mobile phase linear velocity, corresponding to flow rates of 2.0mL/min, 0.5mL/min, and 32μL/min for the 4mm, 2mm, and 0.53mm columns, respectively.

The data in table 7 indicate that the smaller ID column provides the highest efficiency. This is misleading, though, in that the suppresor column used in the 4mm and 2mm work was eliminated from the 530μm system. The much smaller micropacked capillary column is less tolerant of extracolumn volume, and attempts to use a suppressor column severely degraded efficiency. In all likelihood, the 530μm column is not so much better than the 2mm and 4mm columns as is suggested in table 7.

Table VII. Efficiencies (measured as H) determined for 4.0mm, 2.0mm, and 0.53mm ID Ion Chromatography columns.

Run No.	H (Cl-) 4.0mm ID	H (Cl-) 2.0mm ID	H (Cl-) 0.53mm ID
1	71	58	51
2	73	61	53
3	73	62	50
4	70	57	49
5	69	59	51
average	71	59	51

5.2.2 Practical Utility of Micropacked Capillary IC Columns.

A more realistic assessment of the performance characteristics of micropacked capillary IC columns was desired, as well as a practical application of the technology. For these reasons, the separation of nucleoside 5'-monophosphates was undertaken.

The test probes used to calculate reduced plate height and capacity factor data in these studies were cytidine 5'-monophosphate (CMP) and guanosine 5'-monophosphate (GMP). Reduced plate height (h) was easily calculated from the integrator output of retention time and area/height ratio using the formula mentioned by Bidlingmeyer and Warren[128]:

$$N=2(\pi)\{t_r * \text{height}\}^2 / \{\text{area}\}^2 \qquad \{36\}$$

which rearranges to yield:

$$N=2(\pi)\{t_r / (\text{area/height})\}^2 \qquad \{37\}$$

From this point, h is obtained from H = L / N and h = H / d_p. Capacity factors are calculated using k' = t'_r / t_m and selectivities (between CMP and adenosine 5'-monophosphate (AMP)) from α = k'_2 / k'_1.

As indicated in table 8, reduced plate heights around 3 were obtained. This indicates an appreciable improvement over the reduced plate heights measured for the semi-micro and conventional columns, which again is most

Table VIII. Performance characteristics of 530 micron ID micropacked capillary IC columns using a nucleoside 5'-monophosphate test mixture.

Column No.	h (CMP)	h (GMP)	α	k' (CMP)	k' (GMP)
1	3.3	2.4	1.89	2.47	7.64
2	3.5	2.4	2.00	2.21	7.52
3	3.1	2.3	1.94	2.53	7.89
4	3.3	2.3	2.11	2.42	7.67
5	3.0	2.4	1.92	2.39	7.58

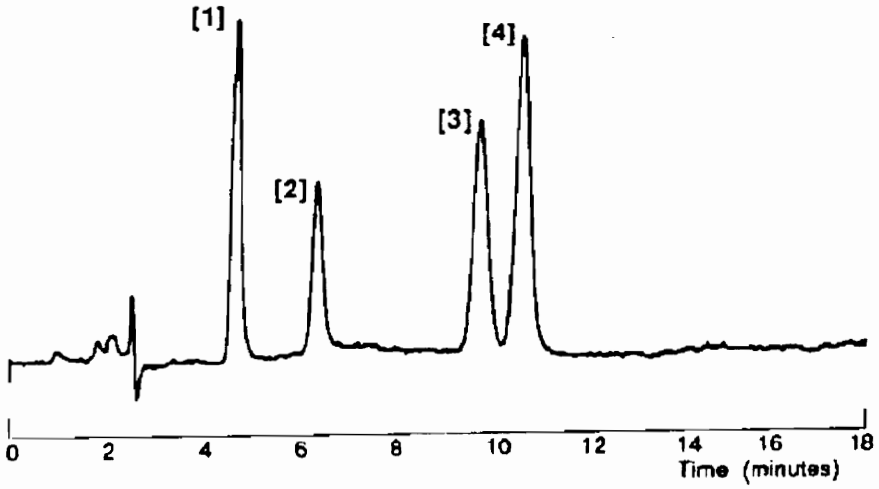
{Each figure represents the average value for 3 chromatographic runs of the mixed standard.}

likely due to a substantial reduction in extracolumn volume. The absence of a suppressor column and the elimination of connective tubing in the packed capillary system provided it with vastly lower extracolumn volume. The use of a suppressor column in these experiments was unnecessary because a UV detector was used in place of the conductivity detector used in the 4mm and 2mm ID ion chromatography experiments. The UV detector did not have the annoying characteristic of being sensitive to the conductive mobile phase medium, and therefore was much simpler to employ than the conductivity detector.

Capacity factors fell within the desired range of 2 to 10, which allowed for the selective retention of the nucleotide analytes without presenting excessively long analysis times. Selectivity between the adjacent CMP and AMP peaks was greater than 1.5 as desired. The selectivity between uridine 5'-monophosphate (UMP) and GMP was less satisfactory, though sufficient for quantitation, as can be seen in figure 32.

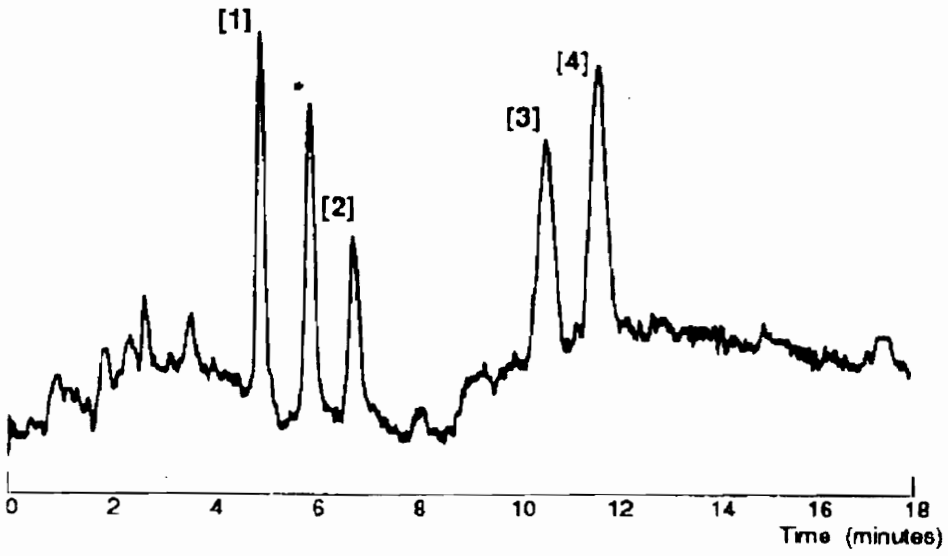
Figure 33 illustrates the utility of the technique in analysis of a mixture of polynucleotides. A rattlesnake venom phosphodiesterase digest of synthetic DNA manufactured using the polymerase chain reaction (PCR), from United States Biochemical Co. (Cleveland, OH, USA), was separated using the method developed for the standard test mixture. The photodiode array detector was used to collect spectra of each nucleotide from the standard test mixture for reference purposes, and spectra of eluting peaks in the nucleic acid digest were collected for the purpose of peak identification. An example of such spectra is shown in figure 34. The presence of an additional peak came as somewhat of a surprise, given that the PCR technique typically yields very

pure short chain nucleic acids. The impurity could not be identified using only the photodiode array detector, but it seems to be in high enough concentration that it could be an additional nucleoside monophosphate. The noisy baseline in figure 33 is evidence that the detector was being used to the fullest extent of its detection capability, as the samples were in low concentration (~50ppb).



(1) CMP; (2) AMP; (3) UMP; (4) GMP

Figure 32. A separation of a nucleoside 5'-monophosphate test mixture by micropacked capillary Ion Chromatography.



(1) CMP; (2) AMP; (3) UMP; (4) GMP

Figure 33. Chromatogram showing a separation of a rattlesnake venom phosphodiesterase digest of synthetic DNA.

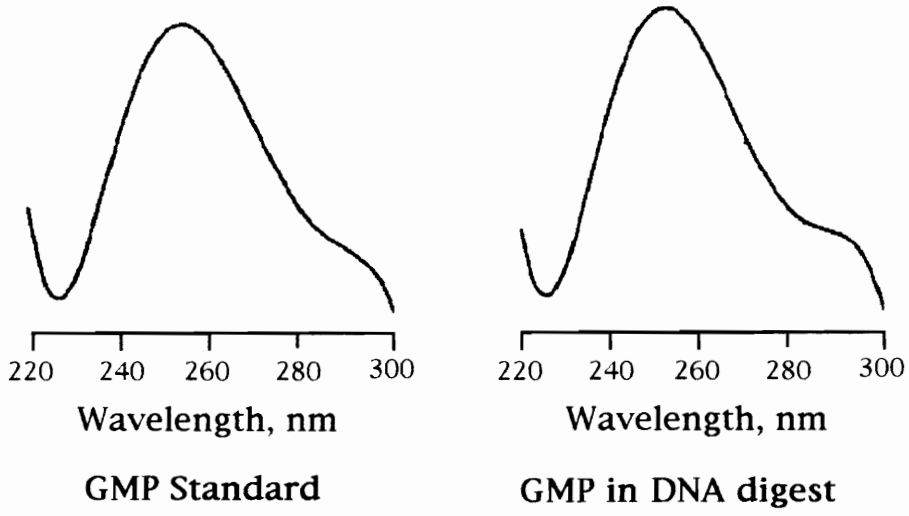


Figure 34. UV spectra of GMP from the mixed standard (A) and a rattlesnake venom phosphodiesterase digest of synthetic DNA (B).

The application of micropacked capillary ion chromatography presented here illustrates the potential which exists for the routine use of microscale HPLC. Without a doubt, more care and attention to detail in system design and operation is necessary when HPLC systems are miniaturized, but successful use of the technique is not an impossibility.

5.3 Pressure-Driven Separations in Packed Capillaries.

Figure 35 is a chromatogram obtained on a 200 micron ID packed fused silica capillary column. This particular column was packed with E.M. Laboratories (Elmsford, NY, USA) 5 μm Lichrosorb RP-18 packing material, and was 208mm long. The optimum linear velocity for this column is given by:

$$u = v\ D_m\ /\ d_p \qquad \{38\}$$

where: **u = the optimum linear velocity;**

D_m = diffusion coefficient of analyte in the mobile phase;

v = the optimum flow, arbitrarily set at 3; and

d_p = the packing material particle diameter.

For a mean diffusion coefficient of 1 x 10^{-9} m^2/sec and 5 μm particles, the optimum linear velocity is 0.6mm/sec. In a 200μm ID column, this corresponds to an approximate volumetric flow rate of 1μl/min. The column was operated at 2μl/min, which roughly corresponds to twice the optimum flow rate. One of

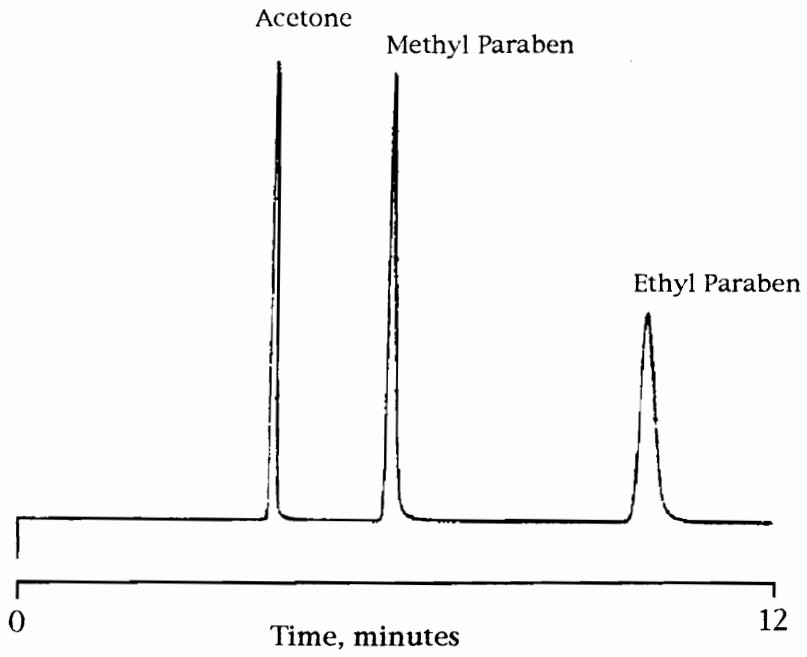


Figure 35. A typical micropacked capillary HPLC separation. The test mixture separated consisted of acetone, methyl paraben, and ethyl paraben.

the most notable features relayed by the chromatogram is the high efficiency which the column yields for each of the analytes, in a relatively short time period and column length. As calculated for the methyl paraben peak, this column generated 18,231 theoretical plates, which corresponds to a plate height of 10.97 microns and a reduced plate height of 2.2. This is extremely close to the theoretical optimum for reduced plate height, which is accepted to be 2 for pressure-driven liquid chromatographic systems[129,130]. The ability to reach these very small reduced plate heights, coupled with the mild operating conditions under which the high efficiency is generated (here, 2050psi), makes packed capillary columns an inviting field of research. In fact, the backpressure which was generated during the operation of this column indicated that it could potentially have been doubled in length without putting excessive demands on the instrumentation. If a similar column of 40 cm length were manufactured, it could potentially have developed nearly 40,000 theoretical plates. Micropacked capillary HPLC seems to offer some important operational benefits. In the ensuing discussion, these benefits are measured using several different parameters as suggested by Knox[131]: efficiency as a function of linear velocity of the mobile phase, resistance to mobile phase flow, and separation impedance. The author prefers to supplement that data with measures of both qualitative and quantitative reproducibility. Additionally, information on peak broadening due to extracolumn effects is presented so that comparison to the extracolumn volume trends in electrochromatography will be possible.

5.3.1 Efficiency of Micropacked Capillary Columns.

In a paper entitled "Standardization of Test Conditions for High Performance Liquid Chromatography Columns," John Knox[132] recommends that reduced parameters be employed in constructing van Deemter curves relating efficiency to mobile phase velocity. According to HPLC theory, smaller particle diameters, greater column lengths, and higher analyte diffusion coefficients in the mobile phase all lend to improvements in efficiency. This means that when two columns which are identical in all respects except for particle diameter are compared, the one with the smaller particle diameter will yield a higher plate count, and therefore will seem to be the better column. In effect, the experimenter becomes biased towards the smaller particles, even though the column packed with the larger particles may in fact yield a better efficiency *relative to its particle size* than the column packed withthe smaller material. By using reduced parameters, these biases are relieved, allowing two columns which are slightly different to be assessed fairly. As stated earlier, the recognized efficiency limit for well packed columns operated in a pressure-driven mode corresponds to a reduced plate height of 2 (h_{min} = 2). This optimum efficiency should theoretically occur at a reduced velocity of about 3 (ν_{opt} = 3)[133]. The van Deemter curves in this section are constructed using the recommendations of Knox, with h being plotted as a function of ν.

Column inside diameter is not a <u>direct</u> component of the rate equation in any of the forms presented in this document. Column diameter <u>can</u> have an influence on efficiency, though, and this relationship has been explored

previously in large diameter (530μm) fused silica capillaries[134]. Examination of the effect of column inside diameter on efficiency in very small capillaries (20 to 50μm ID) has also been conducted[135]. Interestingly, the range of column sizes which was overlooked in these studies is the most popular range of sizes used today in both packed capillary HPLC and in Capillary Electrophoresis. Fused silica capillaries in the region of 50 to 320 microns in inside diameter represent a good compromise between sample capacity, detection simplicity, and ease of handling.

Packed capillary HPLC in columns of <50μm ID is complicated by the difficulty of packing such columns as well as the special requirements these systems pose in terms of detection and mobile phase pumping. The 20 to 50μm ID capillary work alluded to above, performed by Robert Kennedy and James Jorgenson at the University of North Carolina, required the use of a specially-built micro amperometric detector and a home-built micro pumping system. Additionally, columns of very small inside diameter such as these have extremely small sample capacities, making injection and detection both even more problematic.

Packed capillary HPLC in very large ID fused silica columns is made difficult by the fact that these columns are fairly brittle and inflexible. Some work performed by the author in 530μm ID fused silica capillaries also pointed out the difficulty of manufacturing a stable, low volume column termination in capillaries of this large dimension. Finally, the use of very large inside diameter capillaries in an electrochromatographic mode is not recommended, as heat dissipation from these large volume, low surface area tubes is not as facile as in the smaller capillaries. Although the electrochromatography is

not a direct concern in this section of this dissertation, it will become important in comparing the pressure-driven packed capillary HPLC discussed here to the electroosmotically driven work described later.

Figure 36 illustrates the relationship between column inside diameter and reduced plate height as a function of reduced linear velocity. All of the columns used in the construction of this plot were packed with a 10μm irregular, RP-18 derivatized packing material. The data indicates that 50μm and 100μm ID capillary columns offer improved efficiency over the larger 250 and 320μm ID columns. The h_{min} values for the 320 and 250 micron ID columns are essentially the same, being 2.6 and 2.5, respectively, while h_{min} for the 100μm ID column is 2.1. The highest efficiency per unit column length is indicated in the 50μm ID column, which yielded an h_{min} of 2.0 (actually 1.9_5). This is at the theoretical optimum for reduced plate height. Additionally, the minima in the h vs. v curves appear to shift toward higher velocities as column ID is decreased. For the 50 micron column, the optimum reduced velocity is about 4.1, indicating that it can be operated at a high mobile phase velocity relative to the larger columns without sacrificing large amounts of efficiency. Put simply, the smaller columns can yield more theoretical plates in a shorter analysis time than their larger counterparts. Figure 37 illustrates that the trend is carried also with analytes having higher capacity factors than the unretained acetone test probe.

In addition to collecting data on different column inside diameters, data was generated for several different particle sizes in 220μm ID capillary columns. Figure 38 shows the well established relationship between particle

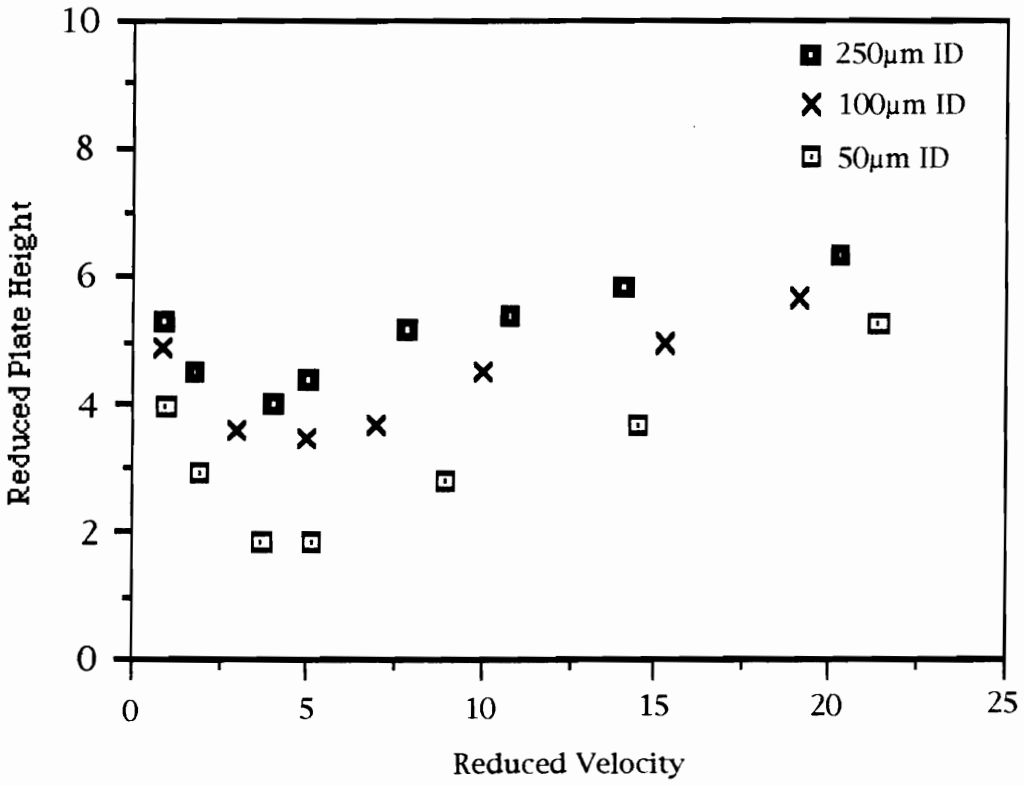


Figure 36. Enhancement of efficiency through reduction of the column inside diameter in micropacked capillary HPLC. h is measured using an unretained test probe (acetone).

diameter and efficiency. Here, the h vs. v plot shows that as particle diameter is decreased, reduced plate height is likewise decreased.

5.3.2 Resistance to Mobile Phase Flow.

Knox[136] has defined a flow resistance parameter for liquid chromatography as:

$$\phi = \Delta P \; d_p^2 \; / \; \mu \; L \; \eta \qquad \{39\}$$

where: ϕ = **a dimensionless flow resistance parameter;**

ΔP = **the backpressure generated by the column;**

d_p = **the particle diameter of paking material;**

μ = **the velocity of the mobile phase;**

L = the column length; and

η = **the viscosity of the mobile phase.**

The flow resistance parameter is useful in that it can convey information about how long a column can be made before pressure drop across the column becomes so great as to preclude its' operation. Longer columns generate a larger number of theoretical plates, so it is desirable to have columns which have low ϕ values. In section 1.2 of this dissertation, it was presented that smaller column inside diameters lead to lower ϕ values at constant particle diameter. In effect, packed capillary columns, which give rise to lower

resistance to flow than larger columns, can be made very efficient by simply increasing their length. Table 9 lists the flow resistance parameters calculated for the columns used in constructing the van Deemter plot in figure 36. Clearly, as column diameter decreases while holding particle diameter constant, resistance to flow decreases. Not only does this mean that longer columns could be manufactured for higher efficiency, but it also indicates that a difference exists in the manner in which the particles within the column are packed. A less obstructed flow path is available for the mobile phase. This in turn means that the differences in flow path geometry and therefore flow velocity across the inside diameter of the column are smaller than in a larger column where the packing structure is tighter and less homogeneous.

5.3.3 Separation Impedance.

In the same article in which he suggested the use of the dimensionless flow resistance parameter in the evaluation of columns for HPLC, Knox[137] presented the possibility of evaluating the column as a whole in a single mathematical relationship. This parameter is referred to as the separation impedance, and therefore decreases as column performance improves. The separation impedance is measured as:

$$E = [t_R \ \Delta P] \ / \ [N^2 \ \eta \ (1+k')] = h^2 \ \phi \qquad \{40\}$$

Table IX. Flow resistance parameters (ϕ) calculated for columns of several different inside diameters, all packed with 10 micron irregular RP-18 derivatized packing material.*

Column ID (μm)	Column Number	ϕ (Dimensionless)
320	1	854
320	2	831
320	3	845
250	1	842
250	2	826
200	1	832
200	2	848
100	1	773
100	2	791
100	3	767
50	1	648

* Each ϕ is the average of at least two analyses.

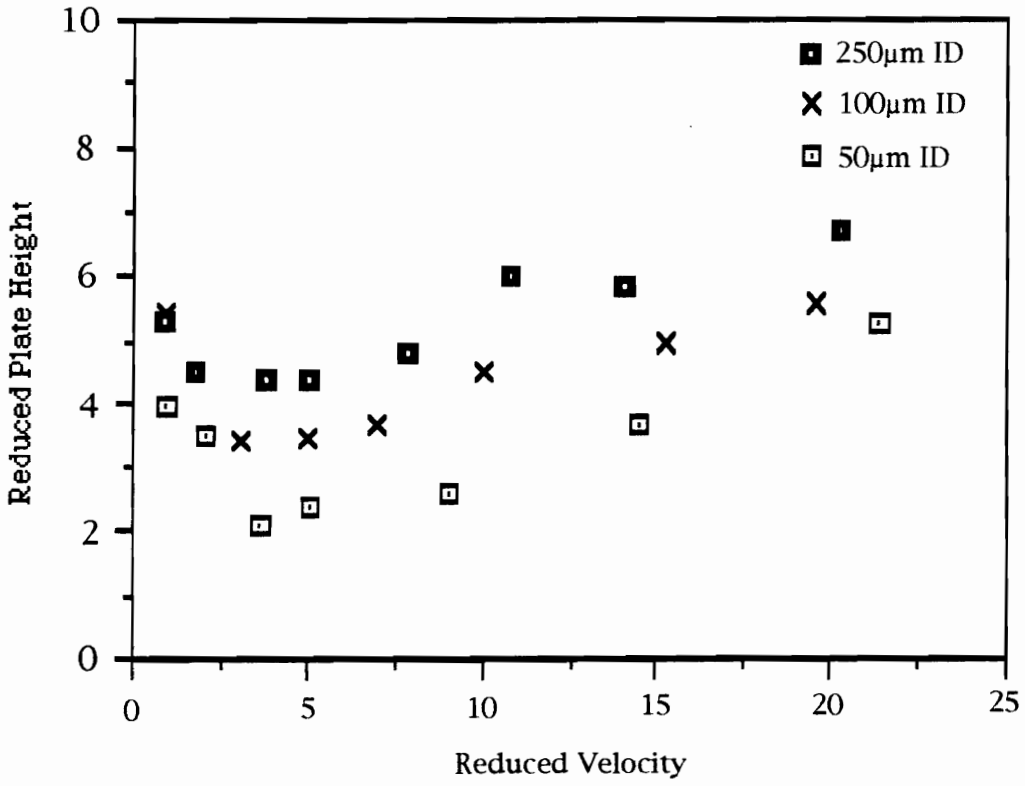


Figure 37. Plate height as a function of linear velocity for several micropacked capillary HPLC columns. h is measured for methyl paraben.

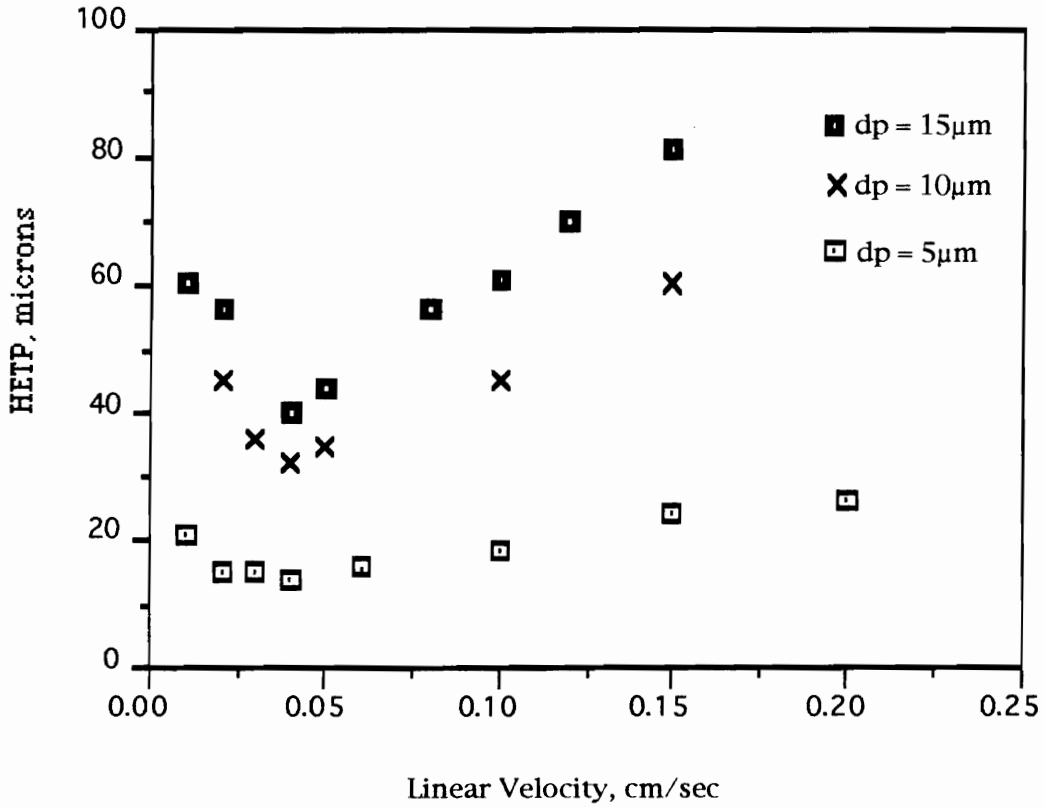


Figure 38. H versus μ plot for several different particle sizes in 220 micron ID fused silica capillaries. h calculated using the unretained acetone test probe.

According to Knox, "Optimisation of the kinetic performance of a chromatographic system involves minimizing the separation impedance, and optimum performance represents the best combination of plate height and permeability to flow." These two parameters, plate height and permeability, have been shown in this study to improve with decreasing column inside diameter when 10μm particles are used in small ID (50 to 200μm ID) fused silica capillary columns. Figure 39 shows the separation impedance for the series of columns packed with 10μm particles. The separation impedance value is smallest when the Knox-Parcher ratio (ρ) is at lower values. The Knox-Parcher ratio is therefore not only a measure of packing geometry, but also relates important information on flow stream homogeneity, which is dependent on packing geometry in pressure-driven microscale HPLC.

5.3.4 Qualitative & Quantitative Reproducibility.

No system is of any utility unless it is capable of demonstrating a high degree of both quantitative and qualitative reproducibility. Quantitative reproducibility is fairly easy to achieve in HPLC through the use of rotary sample injection valves. In the experimentation conducted here, a Rheodyne (Cotati, CA, USA) model 7520 internal loop micro injection valve with a 200nl sample loop was employed in sample measurement and delivery. This volume, as small as it is, was excessive in the case of the 50, 100, and even the 200μm ID packed capillary columns. In order to offset the derogatory effects of the large extracolumn volume introduced by this sample size, the samples were

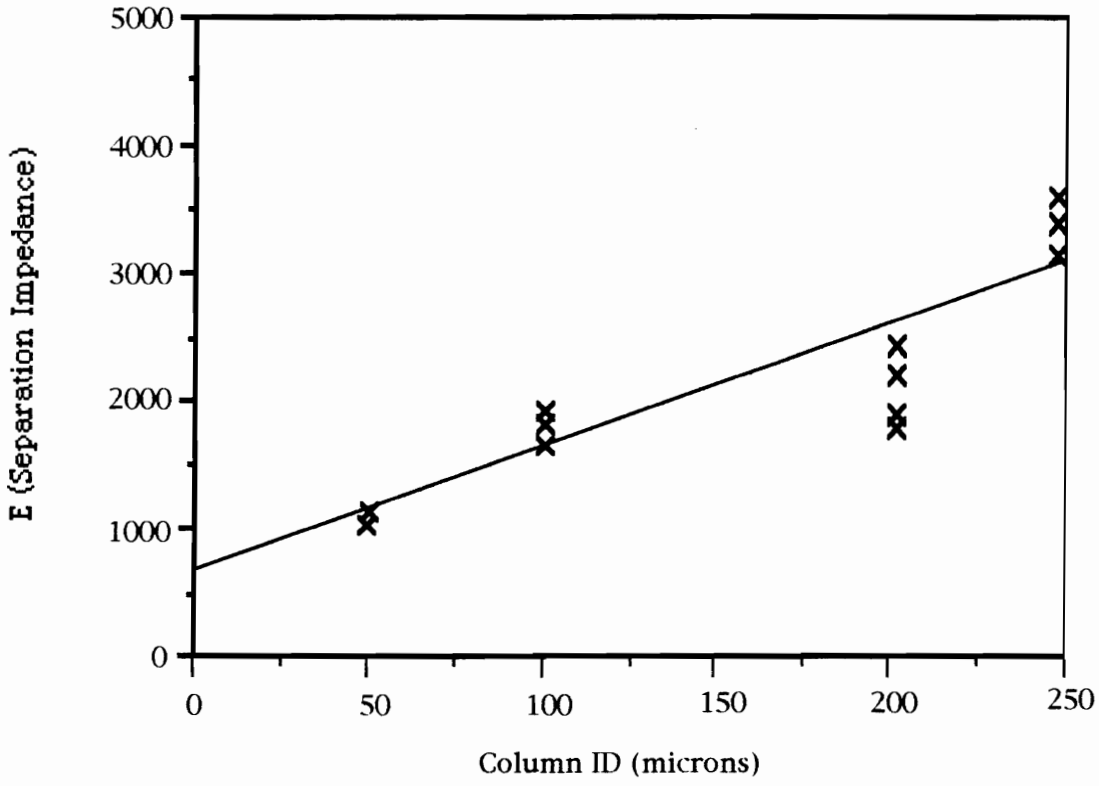


Figure 39. Separation impedance as a function of column inside diameter for columns packed with 10 micron irregular RP-18 derivatized packing material. E is calculated for acetone.

prepared in a 25% acetonitrile/75% water solution, which was a weaker elution solvent than the chromatographic mobile phase. This resulted in a focussing of the analyte bands at the head of the column. Using this methodology, the band dispersion due to the large injection volume was minimal. Use of the sample valve resulted in excellent quantitative reproducibility, as evidenced by figure 40. A relative standard deviation in peak area for 23 analyses of 1.85% was measured.

Qualitative reproducibility is in the largest part dependent on flow velocity reproducibility. Using an acetone t_o marker, a relative standard deviation of 1.6% over 23 measurements in a 100mm ID packed fused silica capillary was calculated. Figure 41 graphically represents the flow reproducibility, and shows and interesting oscillation pattern in flow velocity. The difficulty of reproducing very low volumetric flow rates has been widely recognized, and the achievment of a very low RSD of flow velocity in these experiments was encouraging.

5.3.5 Extracolumn Volume Effects on Bandwidth.

Extracolumn volume was addressed in detail in the Theory section of this dissertation. In brief, that section indicated that system volume outside the column had the effect of increasing band width through several processes. Diffusion in the mobile phase was one contributor to band spreading, but the radial flow inhomogeneities inherent in pressure-driven liquid flow were seen to be an extremely large contributor to the loss of efficiency.

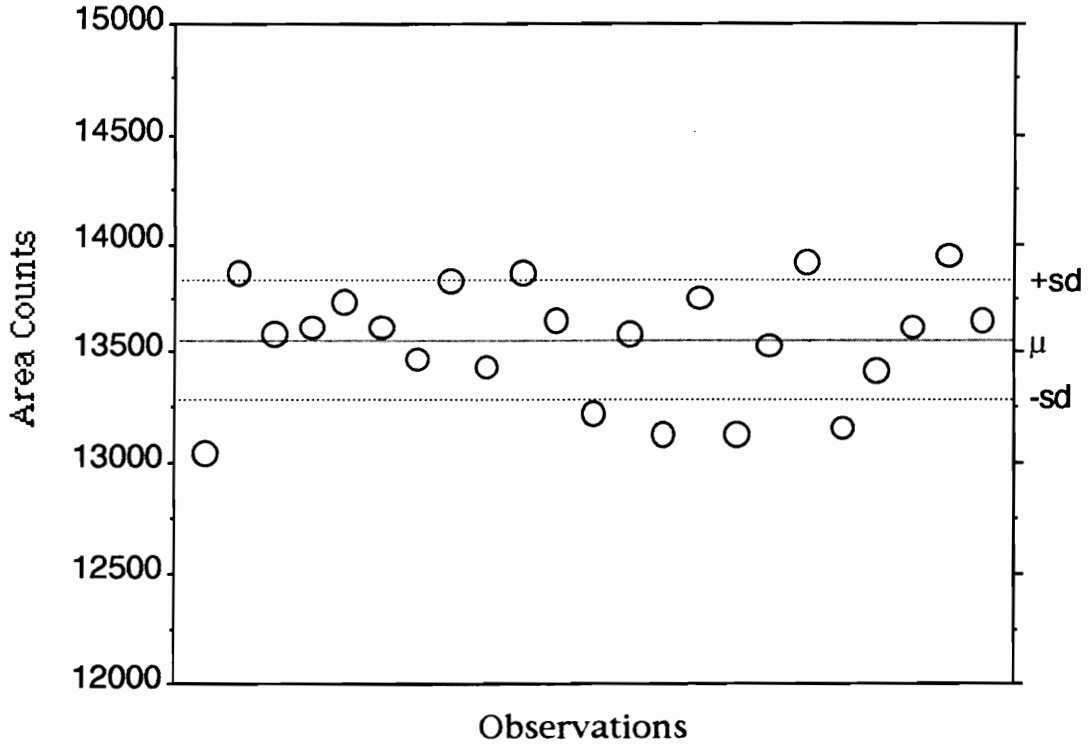


Figure 40. A scattergram showing the quantitative reprodicibility achieved in pressure-driven micropacked capillary HPLC.

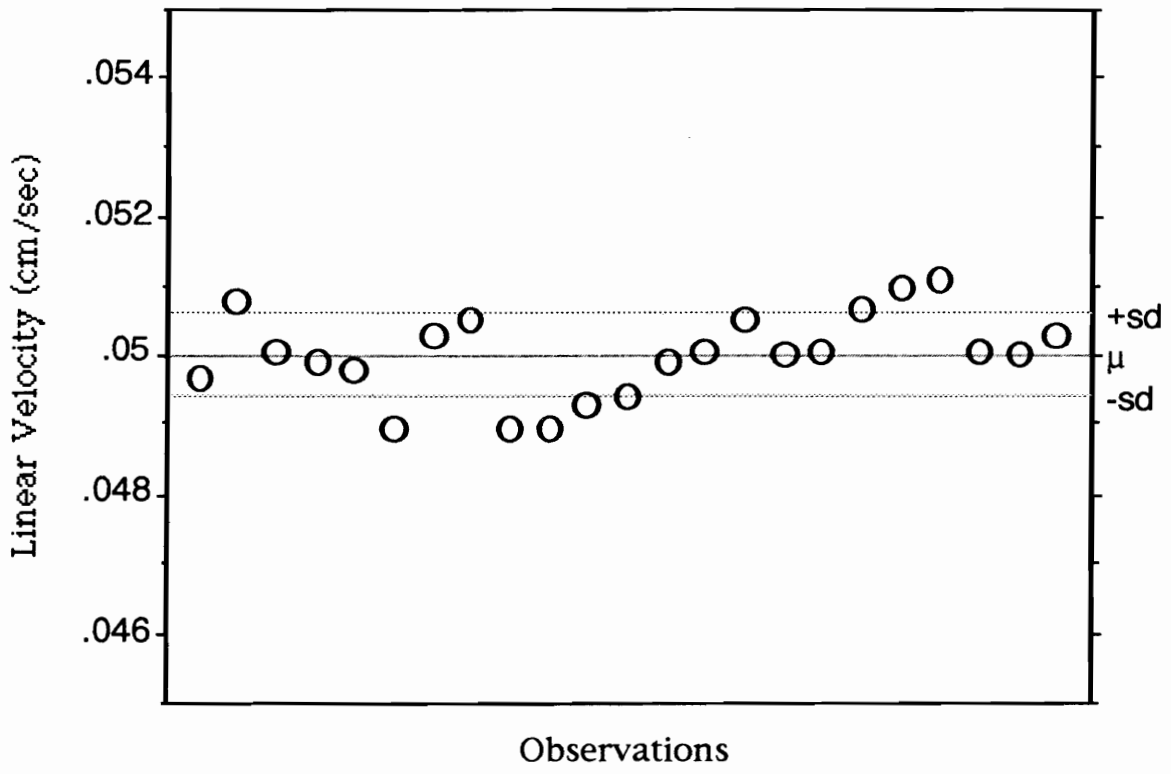


Figure 41. A scattergram representing the flow velocity reproducibility achieved in pressure-driven micropacked capillary HPLC.

One of the major driving forces for research into microscale liquid chromatography is interest in interfacing the technique to information rich detection devices such as mass spectrometers. This requires the use of a length of transfer line which serves to connect the column outlet to the mass spectrometer: a transfer line which contributes to extracolumn band broadening. It is important to describe how much transfer line can be used in interfacing micro-LC to an exotic detector before excessive bandspreading ruins the separation. Equation {26} conveyed the contribution of connective tubing to bandspreading as:

$$\sigma^2_t = 2000\ d_t^4\ L_t\ F \qquad \{41\}$$

in terms of the connective tubing inside diameter and length, and the flow rate of the mobile phase. In figure 42, the theoretical prediction of band spreading due to connective tubing as a function of tubing length is plotted along with an experimental determination. Here, theory and practice agree well, as witnessed by two nearly overlapping lines. This should not come as a surprise, but in the upcoming discussion of Capillary Electrokinetic Chromatography it will be an important feature to note.

5.4 Electroosmotically-Driven Separations in Packed Capillaries.

The performance enhancement which was achieved in the pressure-driven packed capillary HPLC experiments just described was attributed to a

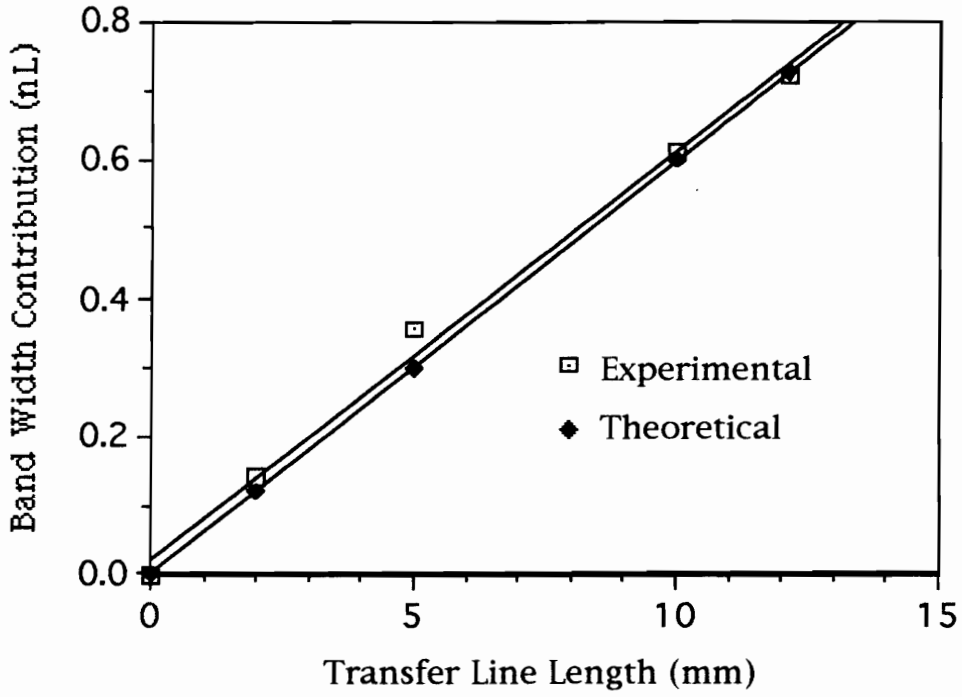


Figure 42. Experimental and theoretical values representing band broadening as a function of the amount of connective tubing used in a pressure-driven packed capillary HPLC system.

decrease in *transcolumn* flow velocity inhomogeneity. Another factor which lends to band dispersion in *transchannel* flow velocity inhomogeneity. A method which could reduce both transcolumn and transchannel velocity inhomogeneities would forseeably yield higher efficiencies than any previous effort in Liquid Chromatography. In an effort to produce a system with a negligible amount of transchannel induced band spreading, the pump which provided the motive force for the mobile phase in section 5.3 was replaced by a high voltage power supply, which was used to generate electroosmotic flow in a packed capillary HPLC column. Electroosmotic flow has a plug flow profile, rather than a laminar one (as discussed earlier) and therefore does not produce the transchannel flow velocity inhomogeneity which is typical of a pressure-driven, laminar flow system. Using packed capillary columns of the same basic dimensions, constructed using the same methodologies, the electrochromatographic system was evaluated. The criteria selected for describing system performance were identical to those in the previous pressure-driven micro-LC study, so that conclusions could be drawn on the potential benefits of using electroosmosis as the motive force for HPLC separations.

5.4.1 Efficiency of Micropacked Capillary Columns in CEC.

In interpreting these results, reduced parameters are again used such that all bias towards columns packed with smaller diameter particles is alleviated. Control over the mobile phase flow velocity was exerted by

changing the applied potential delivered axially to the micropacked capillary column. The effect of potential on linear velocity is shown in figure 43 for several particle sizes. The columns used in gathering this data were 100μm in inside diameter, and were operated using the conditions described in the Experimental section of this dissertation. The experimental data agree well with Pretorius' equation relating electroosmotic flow velocity to applied potential as:

$$v_{eo} = [\varepsilon \zeta / 4 \pi \eta] E \qquad \{42\}$$

described in detail earlier in the Theory section. In the limit that resistive heating is eliminated so that solution viscosity remains constant, E and v_{eo} *should* be related in a linear fashion.

Figure 44 illustrates the relationship between column inside diameter and reduced plate height as a function of reduced linear velocity. All of the columns used in the construction of this plot were packed with a 10μm irregular, RP-18 derivatized packing material, as they were in the pressure-driven micro-LC studies previously described. The data indicates that 50μm and 100μm ID capillary columns do *not* offer any improvement in h_{min} over the larger 150 μm ID columns, whereas in the pressure-driven micro-LC experiments the smaller ID columns presented noticable improvements in efficiency. Remember that in CEC, transcolumn effects are eliminated in all capillary ID dimensions and particle diameter dimensions because the bulk flow profile exists throughout the cross section of the column, so long as no viscosity changes due to resistive heating are encountered. The 320μm ID

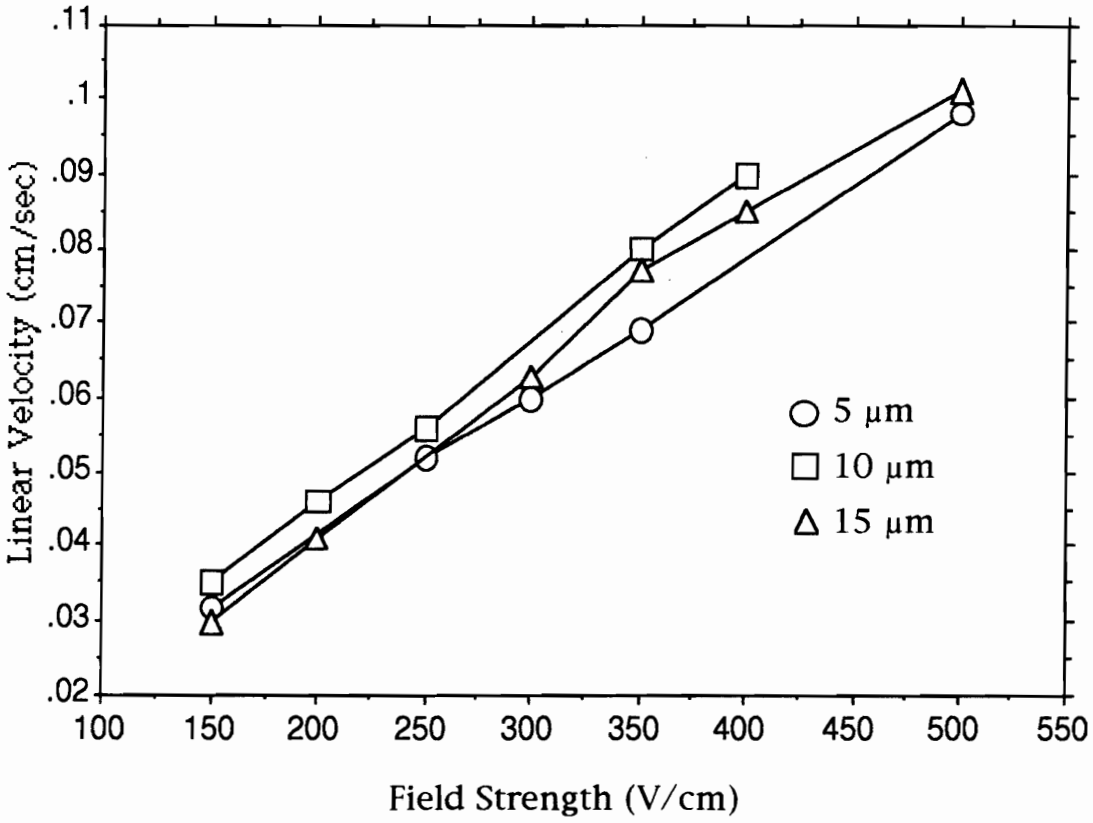


Figure 43. The effect of applied potential on electroosmotic flow velocity in Capillary Electrokinetic Chromatography.

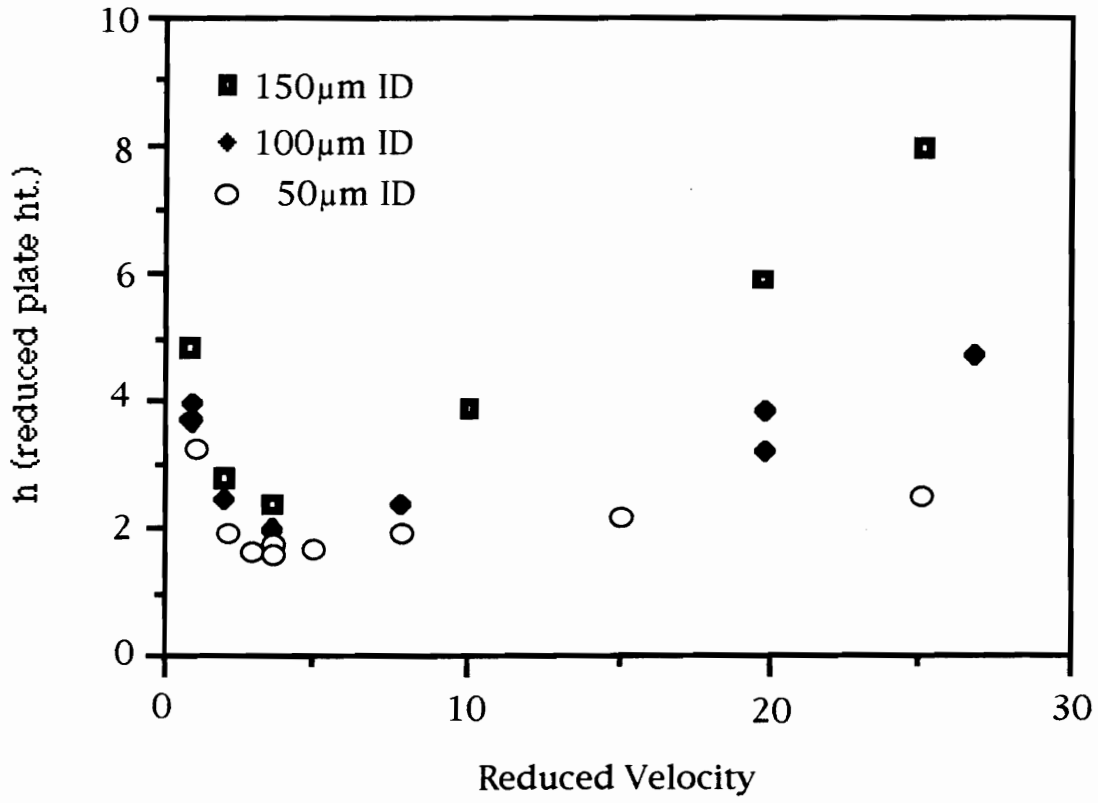


Figure 44. A plot of h versus ν for several different column inside diameters in CEC.

columns used in the pressure-driven experiments could not be used in the electrochromatography experiments because of the excessive Joule heating which degraded the separation quality significantly.

The Joule heating problem manifested itself in two ways. First, the radial temperature profile, which is typified by a high core temperature and an increasingly cooler temperature as the wall is approached, resulted in band spreading due to the imposition of a laminar flow profile. This occurs because of the more facile flow of the warmer, less-viscous mobile phase at the center of the column. Additionally, the diffusion coefficients of analytes in this less viscous region are increased, leading to an increase in band spreading due to diffusive phenomena. The second manifestation of the Joule heating problem came in the form of vapor bubble formation. The elution of these bubbles caused a number of spikes to be recorded on the chromatogram, and rendered the data both ugly and useless. Resistive heating raised problems in the 200 and 150μm ID columns as well, although a few experiments were painstakingly conducted so that the effect of column inside diameter on efficiency in CEC could be studied. The author does <u>not</u> recommend the use of capillaries larger than 100μm ID in an electrochromatographic mode.

The h_{min} values for the 200 and 150 micron ID columns are coincident, being 1.9, while h_{min} for the 100μm and 50μm ID columns is 1.7. It should be noted that these numbers are not statistically different, which is not entirely unexpected. When a packed capillary is operated electrokinetically the flow profile will be bulk in nature, regardless of position in the capillary both with respect to the column cross section (transcolumn) and with regard to position within a channel in between particles of packing material (transchannel). As

stated in the Theory section, this arises because of the lack of a geometric constraint in the formulae describing the formation of an electrochemical double layer and electroosmosis.

The most notable feature of the analyses conducted in the study of the effect of column ID on efficiency in CEC is that all of the h_{min} values tabulated were below the theoretical optimum for reduced plate height. Whereas the minima in the h vs. v curves appeared to shift toward higher velocities as column ID was decreased in the pressure-driven experiments, in the electroosmotically driven work the minima remained near a reduced velocity of 3, the theoretical optimum. As in pressure-driven micro-LC smaller packed capillary columns tend to yield better experimental results in CEC, but for different reasons. In pressure-driven micro-LC the improvements delivered through the use of 50 and 100μm ID columns were an increase in efficiency through improvements in flow homogeneity and a potential decrease in separation time through the use of faster mobile phase flow velocities. In CEC the benefit of working with smaller columns comes in the form of reduced resistive heating (actually, more facile cooling) which aids in maintaining a bulk flow profile, and improvements in system reliability.

In addition to collecting data on different column inside diameters, data was generated for several different particle sizes in 100μm ID capillary columns. Figure 45 shows the relationship between particle diameter and efficiency in CEC. As in all other chromatographic techniques, a decrease in particle diameter results in efficiency enhancement. The most interesting feature of the van Deemter plots generated for the CEC columns is the decreasing slope of the far right (high velocity) portion of the curve as

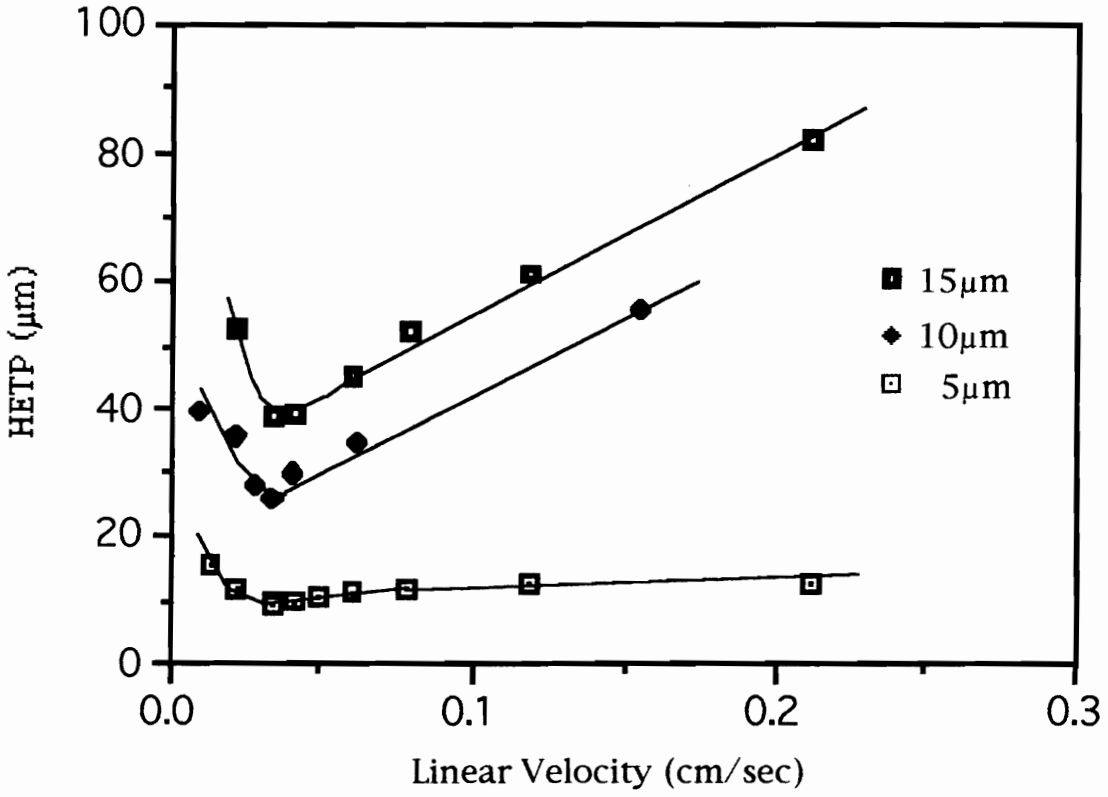
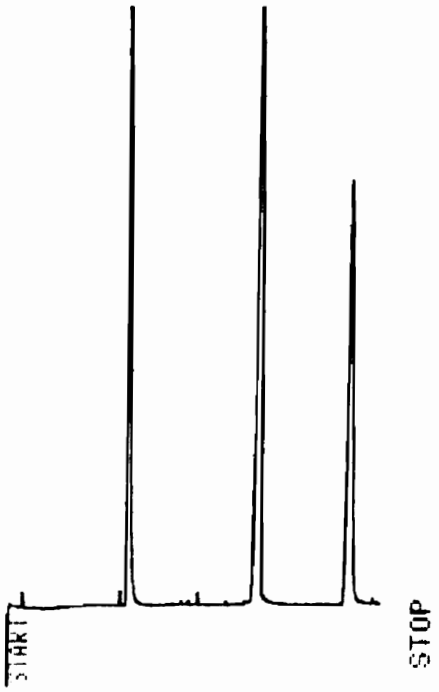


Figure 45. van Deemter plots illustrating the effect of decreasing particle diameter on plate height in CEC. As in all chromatographic methods, smaller particles result in lower plate heights.

particle size is decreased. This portion of the van Deemter curve is traditionally dominated by resistance to mass transfer effects ("C" term effects). The relief of transchannel inhomogeneity in CEC seems to result in a marked decrease in this contribution to plate height, meaning that ultra-fast liquid chromatographic separations with high efficiencies may be a possibility through CEC. Figure 46 is a typical CEC chromatogram.

5.4.2 Resistance to Mobile Phase Flow in CEC.

The flow resistance parameter is useful in pressure-driven systems in that it can be used to determine how long a column can be before the backpressure it generates is too high for the column to be used. In CEC, no pressure is generated along the length of the column. This means that the flow resistance parameter is not applicable to CEC: columns can be made as long as the experimenter desires. The only limits on column length are: analysis time, which increases with column length; power supply capacity; and resistive heating. As column length is increased, higher potentials are required to generate the same field strength per unit length of column. Most power supplies currently in use for capillary electrophoresis are limited to ca. 30kV. For a typical CEC field strength of 400V/cm, this limits the total column length to 75cm. Resistive heating is the largest limiting factor in column length selection: longer columns require higher potentials and therefore are more prone to resistive heating by Ohms' Law:



1. Acetone
2. Methyl Paraben
3. Ethyl Paraben

Figure 46. A typical Capillary Electrokinetic Chromatography chromatogram.

$$E = iR \qquad \{42\}$$

where: **E = applied potential;**

i = current; and

R = resistance.

External cooling of the packed capillary column in CEC by forced air convection or by cooling with a water jacket could extend the range of column lengths which would be practical in CEC. It is doubtful, though, that capillaries much longer than one metre will be necessary. The use of submicron particles in a one metre column could theoretically produce one million theoretical plates[138], which along with the selectivity of CEC should be sufficient to solve most liquid-phase separation problems.

5.4.3 Separation Impedance.

The separation impedance parameter is directly related to the pressure drop along the column, and therefore to the flow resistance parameter, ϕ. Given that essentially no measureable pressure drop exists along a CEC column in use, the ΔP term of the impedance equation must be dropped. In truth, more radical modifications to the equation will be required in the future, but the elimination of the ΔP term will suffice in this application. A separation "impedance factor" in CEC can be *estimated* as:

$$E = [t_R] \; / \; [N^2 \; \eta \; (1+k')] \qquad \{43\}$$

Figure 47 shows a separation "impedance factor" for the series of columns packed with 10μm particles.

5.4.4 Qualitative & Quantitative Reproducibility in CEC.

Quantitative reproducibility was fairly easy to achieve in HPLC through the use of rotary sample injection valves. In the experimentation conducted in CEC, valve injections were not possible. Instead, electrokinetic injections were made just as they are employed in capillary electrophoresis.

Electrokinetic injections are made by replacing the appropriate mobile phase reservoir with a vial containing the sample, applying a low voltage for a specific time period, and replacing the mobile phase reservoir to start the analysis. The volume of sample introduced is given by:

$$S = (\mu_{el} + \mu_{eo}) \; E \; t_S \; \pi \; r^2 \qquad \{44\}$$

where: μ_{el} = **the electrophoretic velocity of a given analyte;**

μ_{eo} = **the electroosmotic flow velocity;**

E = the field strength per unit length of column;

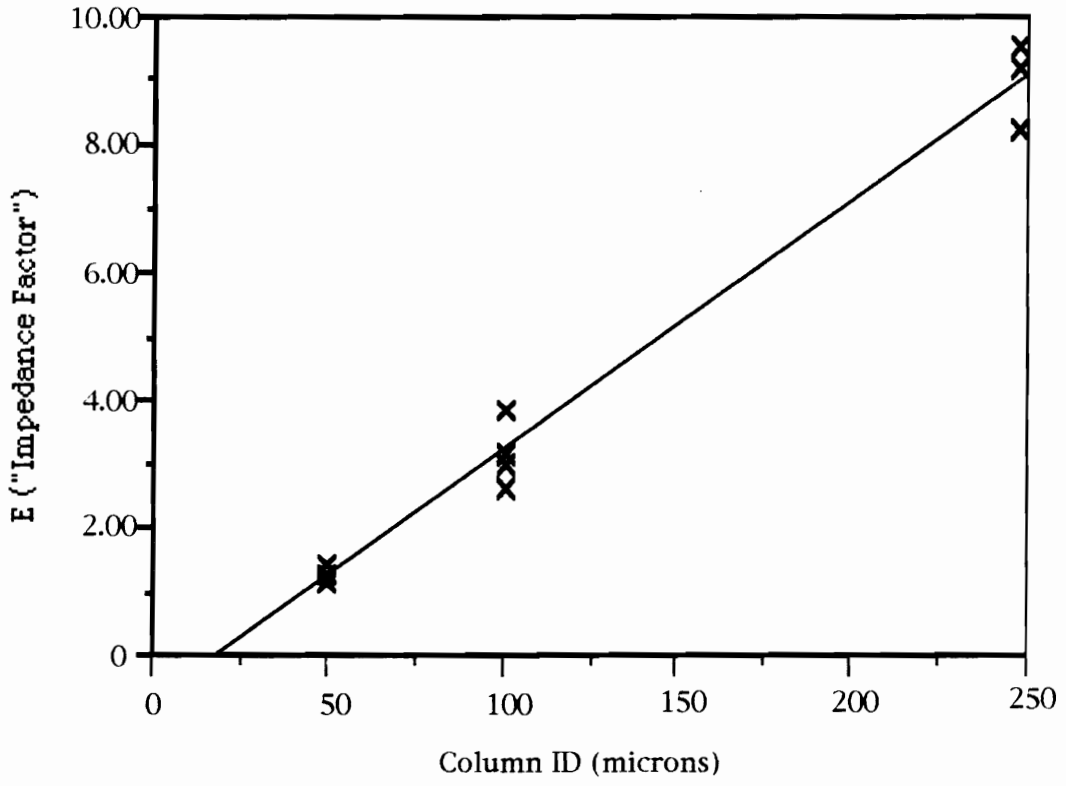


Figure 47. Separation "impedance factor" as a function of packed capillary column inside diameter in CEC.

t_S = the sampling time interval; and

r = the column radius.

Clearly, careful control over the applied potential and the time duration over which it is applied are required if quantitative reproducibility is to be good. In the limit that CEC is applied to unionized species, sample will always be introduced at the anodic end of a fused silica capillary column packed with silica particles and discrimination will not occur. When ionized species are introduced, respect must be paid to their electrophoretic mobilities such that they are not excluded from introduction to the point of being undetectable.

The instrumentation used in conducting these experiments was manually controlled, such that a 10-turn potentiometer had to be used to adjust the output of the power supply. Returning this potentiometer to the *exact* same position every time an injection was made was a physical impossibility, and is one factor responsible for the poor quantitative reproducibility of the technique. A second limiting factor was timing of the injection duration. Manual switching of the on/off toggle on the power supply was also a contributor to irreproducibility. Computer control over applied potential and switch actuation would have improved the quantitative reproducibility of the technique. Finally, any substantial change in the surface of the packing material or the capillary itself may change the electroosmotic flow velocity and therefore affect injection volume. A scatter plot in figure 48 illustrates the relative standard deviation in peak area for an unretained test probe (acetone) over 21 measurements as 12.8%.

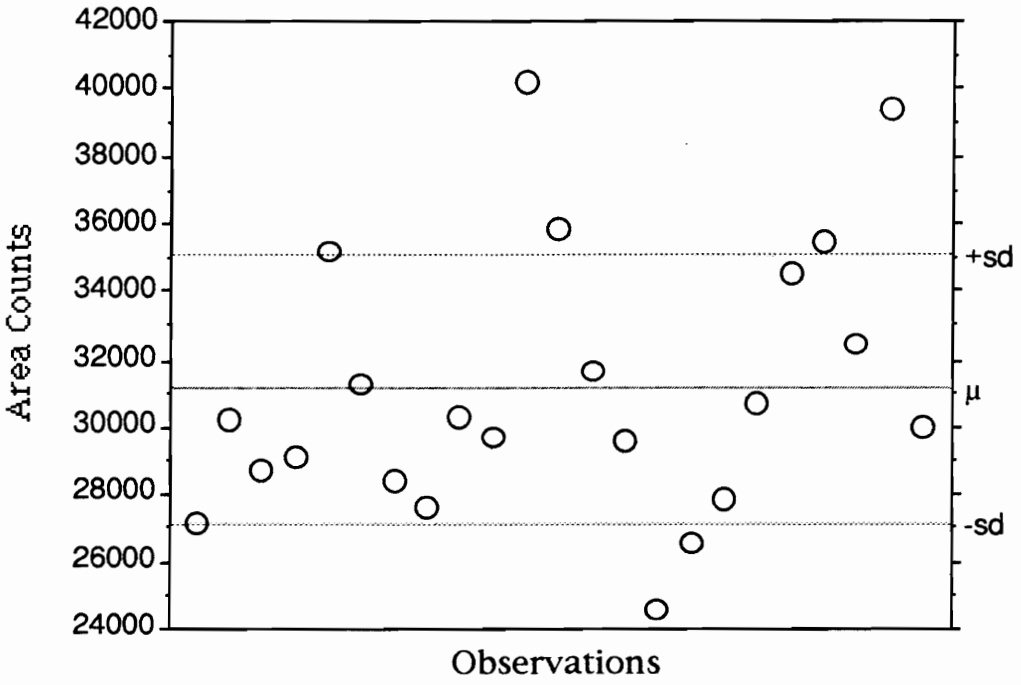


Figure 48. A scattergram relating the quantitative reproducibility of CEC.

Qualitative reproducibility is in the largest part dependent on flow velocity reproducibility, as was the case in pressure-driven microseparations. Using an acetone t_o marker, a relative standard deviation of 4.6% over 21 measurements in a 100μm ID packed fused silica capillary was calculated. Figure 49 graphically represents the flow reproducibility. The achievement of flow velocity reproducibility in CEC is dependent on returning the potentiometer dial to the exact same setting for each run, which again is not a likely event. Additionally, since it is the surfaces of the capillary and the packing material which give rise to electroosmosis, these surfaces must be nearly identical from run to run. This also is unlikely. The measured flow reproducibility of 4.6% RSD is therefore quite acceptable given that the instrument is manually controlled.

5.4.5 Extracolumn Volume Effects on Bandwidth in CEC.

System volume outside the column has the effect of increasing band width through several processes in pressure-driven micro-LC. Diffusion in the mobile phase is one contributor to band spreading, but the radial flow inhomogeneities inherent in pressure-driven liquid flow are an extremely large contributor to the loss of efficiency. Radial flow velocity inhomogeneity is not a problem in electrokinetic methods.

Equation {26} conveyed the contribution of connective tubing to bandspreading as:

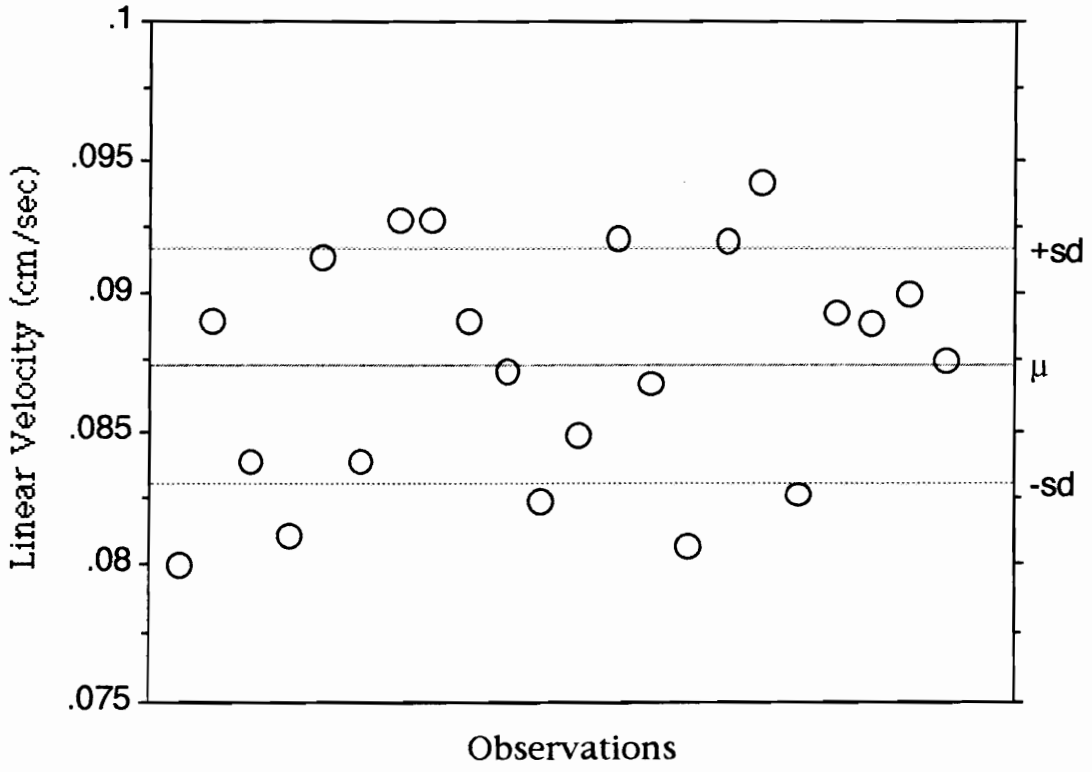


Figure 49. A scattergram illustrating the flow velocity reproducibility in CEC.

$$\sigma^2_t = 2000\ d_t^4\ L_t\ F \qquad \{45\}$$

in terms of the connective tubing inside diameter and length, and the flow rate of the mobile phase. In figure 50, the theoretical prediction of band spreading due to connective tubing as a function of tubing length is plotted along with an experimental determination for Capillary Electrokinetic Chromatography. Whereas theory and practice agreed well in the pressure-driven micro-LC experiments on extracolumn band spreading, CEC results indicate that the use of long transfer lines will not as appreciably decrease efficiency. This should not come as a surprise, since the bulk flow profile which occurs within the packed bed of the CEC system is also characteristic of the open tubular transfer line. This makes diffusion in the mobile phase the sole contributor to band dispersion in CEC transfer lines. The slow diffusion of analytes in the liquid phase supports the results on extracolumn effects presented herein. The use of long transfer lines in interfacing CEC to MS and other information rich detection devices seems more efficient than for pressure-driven systems.

5.4.6 Practical Considerations in CEC.

At this point it should be mentioned that the problem of bubble formation was both persistent and irritating, and occurred regardless of what conditions the system was operated under. The bubbles seemed to form at the interface of the packed bed and the open tubular portion of the separation

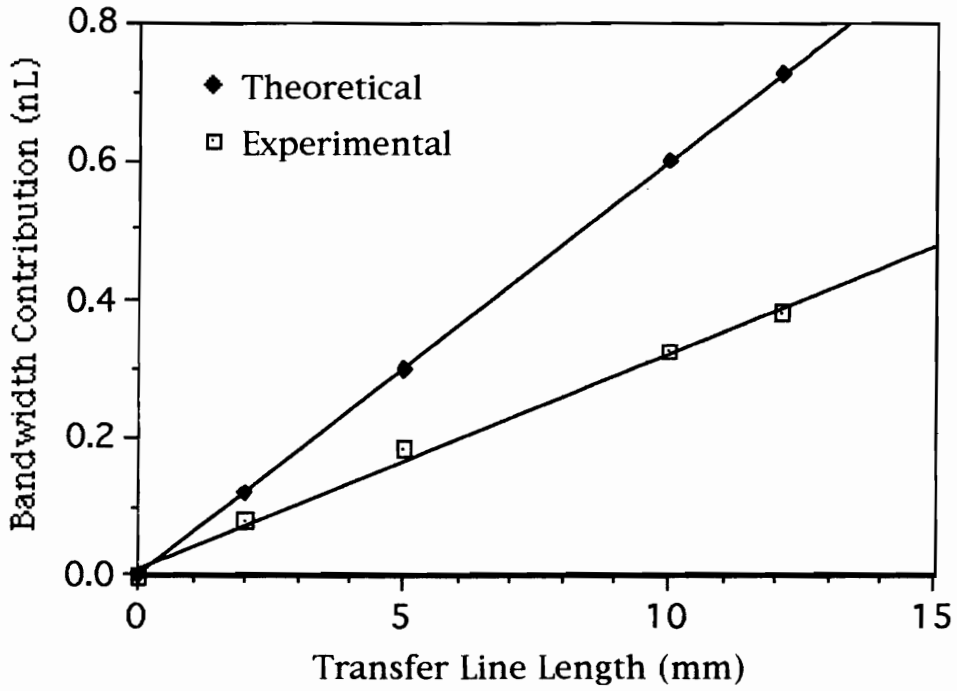


Figure 50. Experimental and theoretical values relating band broadening to the amount of connective tubing added in a CEC system.

capillary. Given that each of these regions has a vastly different internal volume, but the same electroosmotic flow velocity, it seems likely that the bubbles form because of the inability of the packed portion of the capillary to "keep up" with the volumetric demand for mobile phase placed on it by the open tubular portion of the column. Perhaps a slight vacuum is formed at the interfacial region which allows for any dissolved gases in the mobile phase to collect here and later be carried as a distinct bubble through the capillary and past the detector. After one to three "runs," the bubble formation problem became so substantial that the column outlet had to be connected to a syringe pump, and mobile phase forced through the column under pressure in order to re-wet the entire column. In-packing detection by laser-induced fluorescence would eliminate the need for an open-tubular detection region after the packed bed, and could therefore eliminate the bubble formation problem. Alternatively, using the column under slightly elevated pressure could improve the operating character of the system. In any event, the bubble problem was not at all trivial, and was crippling to the process of data collection.

CHAPTER VI
CONCLUSIONS

As stated at the beginning of this dissertation, the ultimate goal of most separations is the achievement of high resolving power in a minimum of analysis time. In order to recognize this goal, the theory of High Performance Liquid Chromatography was consulted to find those parameters in system design which could be adjusted to provide the optimum resolving power achievable in an LC separation. Optimization of flow dynamics seemed the most logical route to improved efficiency, and therefore this was the focus of the majority of the work performed.

Research objectives were to convey the potential benefits of: 1) downsizing Liquid Chromatographic instrumentation and; 2) applying an alternative method of moving mobile phase through the column such mobile phase flow dynamics were optimized. Experimental results were presented which suggested that, when carefully and properly applied, micro-LC was capable of delivering important benefits over conventional analytical-scale HPLC systems. The performance enhancement which was achieved in packed capillary columns for LC operated in an electrochromatographic mode indicated that Capillary Electrokinetic Chromatography can be to liquid phase separations what capillary GC is to gas phase separations: an efficient, rapid, flexible, and practical separation technique.

The semi-micro IC studies served as an introduction to small scale liquid phase separations, and illustrated several important points regarding the benefits of micro-LC. First, it was demonstrated that an apparent improvement

in sensitivity results when a 2mm ID column is used in place of a 4mm ID column. This phenomenon was only observed when injection volume and detector cell volume are unchanged from those in conventional IC. Numerically, the sensitivity improvement was reflected in a 2:1 peak height increase and a 1.6:1 peak area increase. The increase in peak height and area was easily explained. In both analyses the injection volume was unchanged. The use of a lower flow rate in the semi-micro separation indicated that the volume of sample injected would be diluted into a smaller volume of mobile phase in the same time of analysis as for the conventional system. An equal mass of analyte was eluted in one fourth of the volume of mobile phase. Since the conductivity detector used was a concentration dependent detector, the resulting signal was larger for the microseparation than for the conventional one. This phenomenon would not have been observed if the injection volume had been scaled down along with column ID, fairly comparing the two different dimensions.

Decreases in the physical dimensions of HPLC columns must be accompanied by decreases in the volume of all system components if high efficiency is to be maintained. Keeping these facts in mind, experimentation was conducted which indicated that semi-micro IC on conventional IC systems was a practical means of increasing sensitivity and lowering detection limits. In order to verify this, a microconductivity cell was constructed and used in place of the conventional detector cell. The sensitivity enhancement observed after making this modification was nearly identical to that measured using the conventional cell. The much smaller volume of the microcell was shown to

improve system efficiency, though, and indicated the importance of regulating extracolumn volume in microseparations.

Finally, it was demonstrated that semi-micro IC columns could be successfully manufactured in a reproducible manner. A statistical comparison of the 2mm ID columns in terms of their efficiency indicated that they were not significantly different from one another at the 95% confidence interval. A practical application of micropacked capillary IC in 530 micron ID columns was also demonstrated.

The pressure-driven micro-LC experiments developed the relationship between column inside diameter and reduced plate height as a function of reduced linear velocity. The data indicated that 50μm and 100μm ID capillary columns can offer improved efficiency over the larger 250 and 320μm ID columns. The h_{min} values for the 320 and 250 micron ID columns were essentially the same, being 2.6 and 2.5, respectively, while h_{min} for the 100μm ID column was 2.1. The highest efficiency per unit column length was indicated for a 50μm ID column, which yielded an h_{min} of 2.0 (actually 1.9_5). This was at the theoretical optimum for reduced plate height.

Minima in the h vs. v curves appeared to shift toward higher velocities as column ID decreased. For the 50 micron column, the optimum reduced velocity was about 4.1, indicating that it could be operated at a high mobile phase velocity relative to the larger columns without sacrificing large amounts of efficiency. In addition to this, it was shown that column permeability for these very small ID columns is higher than for their larger analytical-scale counterparts. Lower pressure drops can translate into further efficiency enhancement through the use of longer columns.

The flow dynamics of an HPLC column were shown to play a major role in the determination of total system efficiency. As a more uniform column cross section was approached, plate heights decreased. This effect was caused by a decrease in *transcolumn* flow velocity inhomogeneity, one of the five major flow contributors to band spreading as described by Giddings[20].

The performance enhancement which was achieved in the pressure-driven packed capillary HPLC experiments just described was attributed to a decrease in transcolumn flow velocity inhomogeneity. Another factor which lends to band dispersion in *transchannel* flow velocity inhomo-geneity. A method which could reduce both transcolumn and transchannel velocity inhomogeneities was demonstrated by removing the pump which provided the motive force for the mobile phase in pressure-driven micro-LC and replacing it with a high voltage power supply. Application of a high potential to a fluid-filled packed capillary HPLC column resulted in electroosmosis. The electroosmotic flow profile, being flat faced rather than laminar, did not produce the transchannel flow velocity inhomogeneity which is typical of a pressure-driven system. Using packed capillary columns of the same basic dimensions, constructed using the same methodologies, the electrochromatographic system was evaluated.

Data indicated that 50μm and 100μm ID packed fused silica capillary columns do *not* offer any improvement in efficiency over the larger 150μm ID columns in CEC except in cases where resistive heating of the larger capillaries is excessive, whereas in the pressure-driven micro-LC experiments the smaller ID columns presented noticable improvements in efficiency due to improvements in flow dynamics. The h_{min} values for the 150 micron ID CEC

columns were about 1.9, while h_{min} for the 100μm and 50μm ID columns was 1.7. The most notable feature of the analyses conducted in the study of the effect of column ID on efficiency in CEC was that all of the h_{min} values tabulated were below the theoretical optimum for reduced plate height.

As in pressure-driven micro-LC smaller packed capillary columns tend to yield better experimental results in CEC, but for different reasons. In pressure-driven micro-LC the improvements delivered through the use of 50 and 100mm ID columns were an increase in efficiency due to a decrease in flow velocity inhomogeneity, and a potential decrease in separation time through the use of faster mobile phase flow velocities. In CEC the benefit of working with smaller columns comes in the form of reduced resistive heating (actually, more facile cooling) which aids in maintaining a flat flow profile, and associated improvements in system reliability.

In addition to collecting data on different column inside diameters, data was generated for several different particle sizes in 100μm ID capillary columns. A decrease in particle diameter resulted in efficiency enhancement. The most interesting feature of the van Deemter plots generated for the CEC columns was the decreasing slope of the far right (high velocity) portion of the curve as particle size is decreased. This portion of the van Deemter curve is traditionally dominated by resistance to mass transfer effects ("C" term effects). The relief of transchannel inhomogeneity in CEC seemed to result in a marked decrease in this contribution to plate height, meaning that ultra-fast liquid chromatographic separations with high efficiencies may be a possibility through CEC.

The chief goal of the research enumerated in this text has been to provide a separation technique which yields high resolving power in the liquid phase. A significant portion of this research was therefore dedicated to generating improved efficiency in LC systems by decreasing analyte band broadening, through: 1) changing the column geometry, or; 2) providing a different means of analyte transport such that the rate of band broadening is decreased.

LITERATURE CITATIONS

1 Ishii, D. (ed.), Introduction to Microscale Liquid Chromatography, VCH Publishers, New York, 1988.

2 Jinno, K., and Fujimoto, C., *LC-GC*, **7** (1989) 328.

3 Ishii, D., Tsuda, T., and Takeuchi, T., *J. Chrom.*, **185** (1979) 73.

4 Novotny, M., and Tsuda, T., *Analytical Chemistry*, **50** (1978) 271.

5 Hirata, Y., Novotny, M., Tsuda, T., and Ishii, D., *Analytical Chemistry*, **51** (1979) 1807.

6 Ishii, D., Hibi, K., Asai, K., and Jonokuchi, T., *J. Chrom.*, **151** (1978) 147.

7 Wilson, W., McNair, H., Maa, Y., and Hyver, K., *J. High Res. Chrom.*, **13** (1990) 18.

8 Bowermaster, J., Ph.D. Dissertation, VPI & SU.

9 Jinno, K., and Fujimoto, C., *LC-GC*, **7** (1989) 328.

10 Ishii, D., Ed., in Introduction to Microscale High Performance Liquid Chromatography, VCH Publishers, New York (1988).

11 Knox, J., and Parcher, J. F., *Analytical Chemistry*, **41** (1969) 1599.

12 Wilson, W. H., Ph.D. Dissertation, VPI & SU.

13 Jorgenson, J. W., and Lukacs, K. D., *Analytical Chemistry*, **53** (1981) 1298.

14 Pretorius, V., and Bertsch, W., *J. High Res. Chrom.*, **6** (1983) 64.

15 Hjerten, S., and Zhu, M.-D., *J. Chrom.* ,**327** (1985) 157.

16 Verheggen, Th. P. E. M., van Ballegooijen, E. C., Massen, C. H., and Everaerts, F. M., *J. Chrom.*, **64** (1972) 185.

17 Terabe, S., Otsuka, K., and Ando, T., *Analytical Chemistry*, **56** (1984) 111.

18 Knox, J. H., *Chromatographia*, **26** (1988) 329.

19 Braithwaite, A., and Smith, F., Chromatographic Methods, Chapman & Hall, London (1985).

20 Giddings, J. C., Dynamics of Chromatography, Marcel Dekker, New York (1965).

21 Translation to English by: Strain, H. , and Sherma, J., *J. Chemical Education*, **44** (1967) 238.

22 Kuhn, R., Winterstein, A., and Lederer, *Hoppe-Seyler's Z. Physiol. Chem.*, **197** (1931) 141.

23 Martin, A. J. P., and Synge, R. L. M., *Biochem J.*, **35** (1941) 1358.

24 Craig, L. C., *Analytical Chemistry*, **22** (1950) 1346.

25 Van Deemter, J. J., and Zuiderweg, F. J., and Klinkenberg, A., *Chem. Eng. Sci.*, **5** (1956) 271.

26 Golay, M.J.E., in Gas Chromatography 1958, D.H. Desty (ed.), Butterworths, London (1958) 36.

27 Golay, M.J.E., in Gas Chromatography, V.J. Coates, H.J. Noebels, and I.S. Fagerson (eds.), Academic Press, New York (1958) 1-13.

28 Spackman, D. H., Stein, W. H., and Moore, S., *Analytical Chemistry*, **30** (1950) 1190.

29 Giddings, J. C., *J. Chem. Phys.*, **31** (1959) 1462.

30 Giddings, J. C., Dynamics of Chromatography, Marcel Dekker, New York (1965).

31 Knox, J., *Anal. Chem.*, **38** (1966) 253.

32 Horvath, C., Preis, B., and Lipsky, S., *Anal. Chem.*, **39** (1967) 1422.

33 Snyder, L. R., and Kirkland, J. J., Introduction to Modern Liquid Chromatography, 2nd Ed., Wiley-Interscience, New York (1979).

34 Kirkland, J., *J. Chrom. Sci.*, **7** (1969) 7.

35 Knox, J., and Parcher, J. F., *Analytical Chemistry*, **41** (1969) 1599.

36 Ishii,D., *Jasco Report*, **11** (1974) no.6.

37 Scott, R. P. W., and Kucera, P., *J. Chrom.*, **125** (1976) 251.

38 Ishii, D., Asai, K., Hibi, K., and Jonokuchi, T., *J. Chrom.*, **144** (1977) 156.

39 Ishii, D., Asai, K., Hibi, K., and Jonokuchi, T., *J. Chrom.*, **151** (1978) 147.

40 Ishii, D., Asai, K., Hibi, K., and Nagaya, M., *J. Chrom.*, **152** (1978) 341.

41 Webber, T., and McKerrell, E., *J. Chrom.*, **125** (1976) 251.

42 Dandeneau, R. D., and Zerenner, E., *J. High Res. Chrom. & Chrom. Commun.*, **2** (1979) 351.

43 Yang, F., *J. High Res. Chrom.*, **3** (1980) 589.

44 Yang, F., *J. High Res. Chrom.*, **4** (1981) 84.

45 Ishii, D., and Takeuchi, T., *J. Chrom.*, **213** (1981) 25.

46 Gluckman, J., Hirose, A., McGuffin, V., and Novotny, M., *Chromatographia*, **17** (1983) 303.

47 Wiedemann, G., *Pogg. Ann.*, **104** (1858) 166.

48 Helmholtz, H. V., *Wiedemanns Ann. (Ann. Phys. Leipzig)*, **7** (1877) 337.

49 Hittorf, J. W., *Pogg. Ann.*, **89** (1853) 177.

50 Nernst, W., *Zeitschrift Electrochem.*, **3** (1897) 308.

51 Kohlrausch, F., *Wiedemanns Ann., (Ann. Phys. Leipzig)*, **62** (1897) 209.

52 ibid.

53 Tiselius, A., *Trans. Faraday Society*, **33** (1937) 524.

54 Svedberg, T., and Rinde, H., *J. Am. Chem. Soc.*, **46** (1924) 2677.

55 Tiselius, A., *Trans. Faraday Society*, **33** (1937) 524.

56 Hardy, W. B., *J. Physiol. (London)*, **33** (1905) 251.

57 Michaelis, L., *Biochem. Zeitschrift*, **16** (1909) 81.

58 Kendall, J. and Crittenden, E. D., *Proc. Nat. Acad. Sci. USA*, **9** (1923) 75.

59 Kendall, J., *Science*, **67** (1928) 163.

60 Everaerts, F., Beckers, J., and Verheggen, P., Isotachophoresis-Theory, Instrumentation, and Applications, Elsevier, Amsterdam, (1976).

61 Hjerten, S., *Chromatogr. Reviews*, **9** (1967) 122.

62 Arlinger, L., and Routs, R. J., *Sci. Tools*, **17** (1970) 21.

63 Verheggen, Th. P. E. M., van Ballegooijen, E. C., Massen, C. H., and E veraerts, F. M., *J. Chrom.*, **64** (1972) 185.

64 Terabe, S., Otsuka, K., and Ando, T., *Analytical Chemistry*, **56** (1984) 111.

65 Jorgenson, J. W., *Analytical Chemistry*, **58** (1986) 743A.

66 Mikkers, F. E. P., Everaerts, F. M., and Verheggen, P. E. M., *J. Chrom.*, **169** (1979) 11.

67 Jorgenson, J. W., and Lukacs, K. D., *Analytical Chemistry*, **53** (1981) 1298.

68 Terabe, S., Otsuka, K., and Ando, T., *Analytical Chemistry*, **56** (1984) 111.

69 Wallingford, R., and Ewing, A., *Anal. Chem.*, **59** (1987) 1792.

70 Green, J., and Jorgenson, J., *J. Chrom.*, **352** (1986) 337.

71 Pretorius, V., Hopkins, B., and Schieke, J., *J. Chrom.*, **99** (1974) 23.

72 Jorgenson, J., and Lukacs, K., *J. Chrom.*, **218** (1981) 209.

73 Knox, J., and Grant, I., *Chromatographia*, **32** (1991) 317.

74 Knox, J., *Chromatographia*, **26** (1988) 329.

75 Fallon, A., Booth, R., and Bell, L., Applications of HPLC in Biochemistry, Elsevier, New York (1987).

76 Giddings, J. C., Dynamics of Chromatography, Marcel Dekker, New York (1965).

77 Eisenberg, D., and Crothers, D., Physical Chemistry, Benjamin/Cummings, California (1979).

78 ibid.

79 ibid.

80 Giddings, J. C., Dynamics of Chromatography, Marcel Dekker, New York (1965).

81 Knox, J., *Analytical Chemistry*, **38** (1966) 253.

82 Giddings, J. C., Dynamics of Chromatography, Marcel Dekker, New York (1965).

83 Bard, A., and Faulkner, L., Electrochemical Methods, Wiley & Sons, New York (1980).

84 Bolt, G., *J. Phys. Chem.*, **61** (1957) 1166.

85 Rice, C., and Whitehead, R., *J. Phys. Chem.*, **69** (1965) 4017.

86 ibid.

87 Knox, J., and Grant, I., *Chromatographia*, **32** (1991) 317.

88 Knox, J., and Scott, H., *J. Chrom.*, **316** (1984) 311.

89 Knox, J., and Grant, I., *Chromatographia*, **32** (1991) 317.

90 ibid.

91 Knox, J., *Chromatographia*, **26** (1988) 329.

92 Terabe, S., Otsuka, K., Ichikawa, A., & Ando, T., *Anal. Chem.*, **56** (1984) 111.

93 Terabe, S., Otsuka, K., and Ando, T., *Anal. Chem.*, **57** (1985) 834.

94 Rasmussen, H. T., Ph.D. Dissertation, VPI & SU.

95 Bakalyar, S., Olsen, K., Spruce, B., and Bragg, B., *Technical Notes 9*, Rheodyne, Cotati, California, 1988).

96 Reese, C., and Scott, R., *J. Chrom. Sci.*, **18** (1980) 479.

97 Lochmuller, C. H., and Summer, M., *J. Chrom. Sci.*, **18** (1980) 159.

98 Bakalyar, S., Olsen, K., Spruce, B., and Bragg, B., *Technical Notes 9*, (Rheodyne, Cotati, California, 1988).

99 Knox, J., and Parcher, J. F., *Analytical Chemistry*, **41** (1969) 1599.

100 Dolan, J., *LC-GC Magazine*, **10** (1992) 20.

101 ibid.

102 Sternberg, J., *Adv. Chrom.*, **2** (1971) 206.

103 Coq, B., Cretier, G., Rocca, J., and Porthault, M., *J. Chrom. Sci.*, **19** (1981) 1.

104 Bowermaster, J., Ph.D. Dissertation, VPI & SU.

105 Slais, K., Kourilova, D., and Krejci, M., *J. Chrom.*, **282** (1983) 363.

106 Small, H., Ion Chromatography, Plenum Press, New York (1989).

107 ibid.

108 Small, H., Stevens, T., and Bauman, W., *Anal. Chem.*, **47** (1975) 1801.

109 Oi, D., Okada, T., and Dasgupta, P., *Anal. Chem.*, **61** (1989) 1383.

110 Hoffman, S., and Blomberg, L., *Chromatographia*, **24** (1987) 416.

111 DeWeerdt, M., Dewaele, C., Verzele, M., *J. High Res. Chrom.*, **10** (1987) 553.

112 Verzele, M., et al, *J. High Res. Chrom.*, **11** (1988) 317.

113 Jinno, K., *Chromatographia*, **25** (1988) 1004.

114 Cortes, H., Pfeiffer, C., Richter, B., and Stevens, T., *J. High Res. Chrom.*, **10** (1987) 446.

115 Specifications Sheet, Linear Instruments Corp, Reno, NV.

116 Lehninger, A., Biochemistry, 2nd Ed., New York, Worth Publishers (1981)

117 Cortes, H., Pfeiffer, C., Richter, B., and Stevens, T., *J. High Res. Chrom.*, **10** (1987) 446.

118 Borra, C., Soon, M., Novotny, M., *J. Chrom.*, **385** (1987) 75.

119 Knox, J., and Grant, I., *Chromatographia*, **32** (1991) 317.

120 Lux, J., Hausig, U., Schomburg, G., *J. High Res. Chrom.*, **13** (1990) 373.

121 McNair, H. M., "ACS Basic HPLC Slide Notebook," VPI & SU Press, Blacksburg, VA, USA (1990).

122 Meyer, V., *J. Chrom.*, **334** (1985) 197.

123 Small, H., Ion Chromatography, Plenum Press, New York (1989).

124 Snyder, L. R., and Kirkland, J. J., Introduction to Modern Liquid Chromatography, 2nd Ed., Wiley-Interscience, New York (1979).

125 Knox, J., *J. Chrom. Sci.*, **15** (1977) 352.

126 Kennedy, R., and Jorgenson, J., *Anal. Chem.*, **61** (1989) 1128.

127 Belenkii, B., et al, *HRC & CC*, **13** (1990) 185.

128 Biddlingmeyer, B., and Warren, F., *Anal. Chem.*, **56** (1984) 1583A.

129 Snyder, L. R., and Kirkland, J. J., Introduction to Modern Liquid Chromatography, 2nd Ed., Wiley-Interscience, New York (1979).

130 Knox, J., *J. Chrom. Sci.*, **15** (1977) 352.

131 Knox, J., and Bristow, P., *Chromatographia*, **10** (1977) 279.

132 ibid.

133 Meyer, V., *J. Chrom.*, **334** (1985) 197.

134 Wilson, W., and McNair, H., *Proceedings of the 10th International Symposium on Capillary Chromatography*, Riva del Garde, Italy (1989).

135 Kennedy, R., and Jorgenson, J., *Anal. Chem.*, **61** (1989) 1128.

136 Knox, J., and Bristow, P., *Chromatographia*, **10** (1977) 279.

137 ibid.

138 Knox, J., *LC-GC Magazine*, **10** (1992) 12.

APPENDIX

LIST OF SYMBOLS

D_m = diffusion coefficient of solute in the mobile phase

D_{sz} = diffusion coefficient of solute within the particles

d_p = particle diameter

E = applied potential

e = solvent dielectric constant

$H_{mtm} = \{\omega\ d_p^2\ \mu\ /\ D_m\}$

K = thermodynamic partition coefficient

k' = capacity factor

n = solvent viscosity

R_R = retention ratio (= t_m/t_r)

t_r = retention time of an eluting analyte

z = zeta potential

a = distance from the center of the capillary

r = the radius of the capillary

I_o = a zero order Bessel function of the first kind

e = solvent dielectric constant

Δt = time

q = configuration factor

d_f^2 = film thickness

D_s = diffusion coefficient

L_t = the tubing length in mm

F = the flow rate of the mobile phase in ml/minute

q = configuration factor

t_m = dead time

β = phase ratio

σ = standard deviation of the analyte distribution

λ = packing factor

γ = obstruction factor

μ = average linear velocity of the mobile phase

ω = packing factor

x^2_{avg} = mean square distance

ζ= the zeta potential

η= solution viscosity

ε_o= permittivity of a vacuum

ε_r= dielectric constant of the separation medium

ε = the dielectric constant of the mobile phase

ζ = the potential across the solution/surface interface

κ_d = the reciprocal of the thickness of the double layer

z = zeta potential

σ^2_{tot} = the total measured peak variance

σ^2_{inj} = the variance caused by the injector

σ^2_{tub} = the variance contribution of connective tubing

σ^2_{col} = the columns' contribution to variance

σ^2_{det} = the variance due to the detector cell

d_c^2 = square of the column diameter

ε_{tot} = total porosity of the packing material

r_c^2 = square of the column radius

t_r = the retention time of an analyte

σ^2 = the variance of the peak representing that analyte

H_{opt} = best (lowest) achievable plate height

h_{opt} = the optimum reduced plate height, accepted[124,125] to be 2

u = the optimum linear velocity

v = the optimum flow, arbitrarily set at 3

ϕ = a dimensionless flow resistance parameter

ΔP = the backpressure generated by the column

L = the column length

η = the viscosity of the mobile phase

i = current

R = resistance

μ_{el} = the electrophoretic velocity of a given analyte

μ_{eo} = the electroosmotic flow velocity

VITA

Vincent Thomas Remcho III was born on July 23, 1967 in Tampa, Florida. He graduated from Summerville High School in Summerville, South Carolina in 1985. He began his studies toward a Bachelor of Science degree in 1985 at the Virginia Polytechnic Institute and State University (VPI & SU). In December of 1989 he received early admission to the Graduate School at VPI & SU and began his studies in Chemistry. In May of 1989 he was awarded a B. S. in Biochemistry and Nutrition with a minor in Chemistry. He completed the requirements for the Doctor of Philosophy degree in Chemistry in April of 1992. During his tenure at VPI & SU he participated in the instruction of many American Chemical Society sponsored short courses in both Gas and Liquid Chromatography, and with Dr. Harold McNair taught a number of separations short courses at various industrial sites. He spent two summers as a research technician with Westvaco while in college, and two summers with the Tennessee Eastman Company in Kingsport, Tennessee while in graduate school. As a graduate student, he received scholarships to present his research at conferences in Monterey, California and Riva del Garde, Italy. In 1991 he received a Teaching Excellence award as an instructor in instrumental analysis. After completing his graduate studies, Vincent Thomas Remcho III accepted employment as a postdoctoral fellow with Professor J. Calvin Giddings at the University of Utah in Salt Lake City.

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