Designing Separation Systems in Capillary Electrophoresis Based on the Fundamental Physicochemical Properties of Analytes

by

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B.Sc., University of Toronto, 1994

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY
in
The Faculty of Graduate Studies
Department of Chemistry

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
September 1999
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Date **Sep 30 1994**
Abstract

This thesis strives to develop an integrative approach towards separation design in capillary electrophoresis (CE) based on the fundamental physicochemical properties of analytes. It is divided into four main sections to deal with four important aspects of separation design in CE which include theory, selectivity, assay development and sensitivity.

The use of additives in CE represents one of the most powerful ways to separate analytes in a mixture, since both electric field (mobility) and equilibria (affinity) are used in the separation process. Section A developed a theory to describe the migration behaviour of analytes in CE when using additives in the run buffer. The mobility of an analyte can be predicted at any additive concentration, capillary length, or voltage for rapid and systematic optimization of separation conditions. Section B involved the synthesis, characterization and application of TESMR, a water-soluble aromatic based macrocycle, as a new type of additive in CE. Separation of neutral species is accomplished by imparting an electrophoretic mobility to the analytes via differential complexation with TESMR. The development and validation of two different quantitative assays by CE is presented in Section C. A simple and sensitive method to analyze the \( \gamma \)-carboxyglutamic acid content of protein, urine and plasma is developed using CE with laser-induced fluorescence detection. The second assay involved the development of a robust method for the quantification of epinephrine in fifteen different dental anesthetic solutions. Selective on-line focusing of analytes using a discontinuous electrolyte system is presented in Section D as a facile way to improve concentration sensitivity in CE when using UV detection. Large volumes of a dilute sample can be focused into extremely sharp zones by carefully selecting appropriate sample and background electrolyte conditions. A summary of the results of the thesis and possible future endeavours is also discussed, as well as an integrative framework towards separation design in CE.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>α</td>
<td>-</td>
<td>Selectivity (Separation Factor)</td>
</tr>
<tr>
<td>A</td>
<td>-</td>
<td>Analyte</td>
</tr>
<tr>
<td>AC</td>
<td>-</td>
<td>Analyte-Additive Complex</td>
</tr>
<tr>
<td>ACE</td>
<td>-</td>
<td>Affinity Capillary Electrophoresis</td>
</tr>
<tr>
<td>ACN</td>
<td>-</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Asp</td>
<td>-</td>
<td>Aspartic Acid</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>Complexing Agent (Additive)</td>
</tr>
<tr>
<td>CE</td>
<td>-</td>
<td>Capillary Electrophoresis</td>
</tr>
<tr>
<td>CD</td>
<td>-</td>
<td>Cyclodextrin</td>
</tr>
<tr>
<td>CIEF</td>
<td>-</td>
<td>Capillary Isoelectric Focusing</td>
</tr>
<tr>
<td>CITP</td>
<td>-</td>
<td>Capillary Isoachophoresis</td>
</tr>
<tr>
<td>CZE</td>
<td>-</td>
<td>Capillary Zone Electrophoresis</td>
</tr>
<tr>
<td>δ</td>
<td>-</td>
<td>Relative Dielectric Correction Factor</td>
</tr>
<tr>
<td>dN</td>
<td>-</td>
<td>Deoxyribonucleoside</td>
</tr>
<tr>
<td>dNT</td>
<td>-</td>
<td>Deoxyribonucleotide</td>
</tr>
<tr>
<td>DMF</td>
<td>-</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>-</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>ε_r</td>
<td>-</td>
<td>Relative Permittivity (Dielectric Constant)</td>
</tr>
<tr>
<td>E</td>
<td>-</td>
<td>Electric Field Strength</td>
</tr>
<tr>
<td>EC</td>
<td>-</td>
<td>Electrochemical (detector)</td>
</tr>
<tr>
<td>EOF</td>
<td>-</td>
<td>Electroosmotic Flow</td>
</tr>
<tr>
<td>ESMS</td>
<td>-</td>
<td>Electrospray Mass Spectrometry</td>
</tr>
<tr>
<td>FITC</td>
<td>-</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>FTC-Gla</td>
<td>Fluorescein Thiocarbamyl Derivative of γ-Carboxy-Glutamic Acid</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
<td></td>
</tr>
<tr>
<td>Gla</td>
<td>γ-Carboxyglutamic Acid</td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic Acid</td>
<td></td>
</tr>
<tr>
<td>η</td>
<td>Viscosity</td>
<td></td>
</tr>
<tr>
<td>HP-β-CD</td>
<td>Hydroxypropyl β-Cyclodextrin</td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance (Pressure) Liquid Chromatography</td>
<td></td>
</tr>
<tr>
<td>IEC</td>
<td>Ion Exchange Chromatography</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>Equilibrium Constant</td>
<td></td>
</tr>
<tr>
<td>k'</td>
<td>Capacity Factor</td>
<td></td>
</tr>
<tr>
<td>LIF</td>
<td>Laser Induced-Fluorescence</td>
<td></td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of Detection</td>
<td></td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of Quantitation</td>
<td></td>
</tr>
<tr>
<td>μ_{ep,T}^A</td>
<td>Apparent Electrophoretic Mobility of an Analyte (at Specific Temperature)</td>
<td></td>
</tr>
<tr>
<td>μ_{eo}</td>
<td>Electroosmotic Mobility</td>
<td></td>
</tr>
<tr>
<td>μ_{ep}</td>
<td>Electrophoretic Mobility</td>
<td></td>
</tr>
<tr>
<td>μ_{ep,A}</td>
<td>Ideal State Electrophoretic Mobility of Free Analyte</td>
<td></td>
</tr>
<tr>
<td>μ_{ep,AC}</td>
<td>Ideal State Electrophoretic Mobility of Analyte Additive Complex</td>
<td></td>
</tr>
<tr>
<td>MEKC</td>
<td>Micellar Electrokinetic Chromatography</td>
<td></td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
<td></td>
</tr>
<tr>
<td>m/z</td>
<td>Mass/Charge Ratio (Mass Spectrometry)</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>Number of Observations</td>
<td></td>
</tr>
<tr>
<td>Symbol</td>
<td>Meaning</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>Number of Theoretical Plates</td>
<td></td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
<td></td>
</tr>
<tr>
<td>PDA</td>
<td>Photodiode Array</td>
<td></td>
</tr>
<tr>
<td>$Q_{\text{eff}}$</td>
<td>Effective Charge of an Ion</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>Hydrodynamic Radius of Ion</td>
<td></td>
</tr>
<tr>
<td>Rs</td>
<td>Resolution</td>
<td></td>
</tr>
<tr>
<td>SBE-β-CD</td>
<td>Sulphobutylether-β-Cyclodextrin</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecylsulphate</td>
<td></td>
</tr>
<tr>
<td>TESMR</td>
<td>Tetraethylsulphonate Methylresorcinarene</td>
<td></td>
</tr>
<tr>
<td>$v$</td>
<td>Relative Viscosity Correction Factor</td>
<td></td>
</tr>
<tr>
<td>$\nu_{\mu_{\text{ep},T}}^{A}$</td>
<td>Viscosity Corrected Electrophoretic Mobility of Analyte at Specific Temperature (Ideal State)</td>
<td></td>
</tr>
<tr>
<td>$\nu_{\delta\mu_{\text{ep},T}}^{A}$</td>
<td>Viscosity-Dielectric Corrected Electrophoretic Mobility of Analyte at Specific Temperature (Ideal State)</td>
<td></td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet Absorbance Spectroscopy</td>
<td></td>
</tr>
<tr>
<td>$v$</td>
<td>Velocity of ion</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
<td></td>
</tr>
<tr>
<td>ζ</td>
<td>Zeta Potential</td>
<td></td>
</tr>
</tbody>
</table>
"Only what is separated may be properly joined.

When two things are muddled together,

They need to be separated, distinguished,

And untangled so that they may later,

Be rejoined in a workable synthesis.

Analysis must always serve

Synthesis in order to serve life.

What is taken apart must be

Put back together."

- C. G. Jung quoting a medieval alchemist
People throughout history have strived to understand themselves and the world that surrounds them. From the dawn of early civilizations, philosophers, alchemists and other seekers of truth, have attempted to formulate the essential constituents of matter and apply this knowledge through the transformation of matter to new forms. Two parallel traditions have pervaded the history of western philosophy and the natural sciences as approaches to further understanding: analysis (taking things apart) and synthesis (putting things together). The symbiosis of analysis and synthesis, as formalized through theory and experiment, has remained an essential feature of the continuing development of new fields of knowledge, including modern chemistry. Within the ancient tradition of separation, capillary electrophoresis (CE) is a relatively recent tool used to analyze discrete components of a mixture. Although CE is primarily an analytical technique, the importance of a "synthetic" or integrative perspective is vital for its continued development. This thesis attempts to balance the scales of analysis and synthesis through the modes of theory and experiment. Indeed, experimentation is often nurtured by theory, as is theory developed through careful observation from experiment. Although the work during this thesis often entailed formal theoretical exploration and repetitive experimentation, it became apparent that it necessitated a philosophical approach to derive meaning; that abstract quality which gives vitality and significance to any endeavour. However, the most profound qualities gained from this thesis were in a sense exquisitely personal: a "mixture" of asinine patience, persistent perseverance and the utmost humility (qualities that are best left unseparated). Despite the great
advancement of science and technology, and the abundance of factual knowledge in our society, there still exists (thankfully) phenomena mysterious to even the most brilliant "savants" of our time. Although science has enlightened many riddles that confronted our ancestors, new questions are constantly being formulated. Indeed, the circle is round; it is from this long-standing tradition of inquiry that this thesis begins...
Portions of the following chapters have been previously published elsewhere:


Acknowledgements

"No man is an island": Indeed, I wish to acknowledge the many individuals who have directly or indirectly helped me throughout the undertaking of this thesis. First and foremost, I would like to thank all the present and past members of my research group who have greatly contributed to the fruits of this labour: Xuejun Peng, Michael Bowser, Andrea Kranack, Jamie Morris, Brad Schneider, Judy Wong, Kylie O'Brien, Angela Duso, Xiyun Hou and David McClaren. I would particularly like to thank Xuejun Peng and Michael Bowser for sharing their collective wisdom of capillary electrophoresis during the early part of my research. I would also like to thank all the people in Dr. Sherman's laboratory, especially Naveen Chopra, for allowing me the use of their equipment and for informative discussions regarding resorcinarene chemistry. The help of Alison Paprica (Astra Pharma Canada) is also appreciated for providing samples of epinephrine standards and the dental anesthetic solutions. I would also like to thank Hung Vo for his hard work and diligence throughout our collaborative project involving the Gla assay; hey thanks for the urine!

I would particularly like to acknowledge the wise council of Gwendolyn Bebault who has often enlightened me during my most intense and tumultuous of times; her keen advice, both on personal and academic matters, has been greatly appreciated.

I am indebted to my supervisor, David Chen, for providing me a stimulating environment in which to undertake my doctorate. His passion for scientific understanding, continual encouragement, openness and willingness to explore and share new ideas, has influenced me immensely. He has generously given me the opportunity to be exposed to different milieus, while being involved in interesting projects and collaborations. Under his tutelage, I believe that I have been able to grow and develop both personally and professionally, not only as an analytical chemist, but also as an inquisitive and mature research scientist.

Beyond the walls of academia, there have been many individuals who have supported me before and during my thesis whom I would also like to acknowledge. I would like to thank Chris Savva for his help in discussing some philosophical implications of science and education, as well as sharing a number of interesting quotations with me. A formal exhortation of gratitude to my dear friends in Toronto (a.k.a. the Boys), Patrick Klaus, Frank "Tard" Gianonne, Kresimir Milkovic, Walter Wodchis, John DosSantos, Paul Kenning and John "Red Eye" Passalaqua; indeed, their friendship is always close to my heart. The companionship and humour of Mike Roberts and Mike Schmidt are also very much welcomed, especially during my time labouring in the confines of the Organ Grinder: Here's a Red Rocket Dude! The support and guidance of Eva Ray has been an important influence throughout my life; she has been a caring and close confidante, introducing me to many aspects of eastern thought and religion. I also wish to thank my brother, Christian McKibbin, for being my brother; he has matured into a talented and distinguished musician - May the "Pocket Dwellers" dwell in peace! I would like to thank my dad, David McKibbin, for his love and affection, and for showing me the value of good humour.

Last, but certainly not least, I wish to acknowledge the constant love and support of my mother, Rosemarie McKibbin, for instilling in me the values of education and hard-work. She has imparted me with the confidence and determination to realize my dreams. A final thanks to all other people not mentioned (sorry !), who have shaped my life in subtle ways, including the great musicians, poets, philosophers, spiritualists and other bold seekers of truth, or some shade of truth, since time immemorial.
"Of all that is written, I love only what a man has written with his blood. Write with blood, and you will experience that blood is spirit...

Whoever writes in blood and aphorisms does not want to be read, but to be learnt by heart. In the mountains, the shortest way is from peak to peak: but for that one must have long legs."

- F. Nietzsche, "Thus spoke Zarathustra"
Chapter I:

Introduction to Separation and Capillary Electrophoresis

"The impulse to separate:
From copper purification to nucleic acid analysis.
The art and science of separation."
I: Introduction to Separation and Capillary Electrophoresis

1.1 A Brief History of Separation Science: Why Separation?

"If we have our own why of life, we shall get along with almost any how."
- F. Nietzsche, "Twilight of the Gods"

The natural world is an enormously complex, interconnected and diverse environment in which most material objects consist of heterogeneous mixtures. Indeed, only a handful of elements (copper, gold, silver, mercury, and the noble gases) can be found in their pure state in nature. Minerals, plants, animals, and man himself, are composed of a multitude of combinations of elements, molecules and macromolecules. Amidst this thoroughly mixed environment, humankind, armed with the double-sword of intellect and self-awareness (albeit to varying degrees), has strived to devise ways to create, sustain, destroy and master his environment. Separation is currently one of the most important modes of analysis used by science to simplify the various facets of nature in order to promote knowledge and understanding. An important consequence of scientific knowledge (not necessarily understanding) is the direct manipulation and control of natural processes for the benefit and to the chagrin of humankind.

Separation can be generally described as a process to isolate discrete components from a mixture. The origins of the impulse to separate can be traced far back into history. Since the early days of metallurgy, textiles and cosmetics (the predecessors of modern chemistry), civilizations have realized the enormous benefits and potential dangers, derived from separating and purifying raw materials found in nature. During the Copper Age (circa 4000 BC), in ancient Mesopotamia, people learned ways to separate molten copper from copper ore (malachite). The separative processes behind metallurgy were of such great importance to early civilizations, that modern scholars have classified ages in history by the predominant
metal: Copper, Bronze and Iron Ages. The fabrication of iron weapons of war, grand bronze artforms, royal purple dyed clothing, and rose-scented perfumes, greatly depended upon early separation techniques to provide pure components and their recombination to form new materials.

"When the Ten Thousand Things are viewed in their oneness, we return to the Origin and remain where we have always been."

- Sen Ts'en, from Aldous Huxley's "The Perennial Philosophy"

Beyond these pragmatic creations, there also has existed a more sublime inspiration behind the impulse to separate. Humanity has always paid reverence to an ultimate, pure state of being. Humankind's innate compulsion to seek unity, oneness and purity, while living in a seemingly disordered and chaotic world, is in effect, the same aim as separation. This motif is common to many peoples throughout history and it is the prime motivation central to many of humanity's greatest endeavours. Both the theoretical physicist attempting to formulate a grand unified theory of the universe and the spiritual practitioner aspiring to experience a state of "awakening" use different means to accomplish the same goal: A unified perspective of oneself and the world around us. Indeed, although the nature of the arrow varies, the bow still aims.

Analysis is the dominant mode of thought central to separation. Moreover, analytical thought (scientific, psychological, political, legal, etc.) is the dominant paradigm of our current technological society for being the primary, if not only, means of progress (i.e., positivism). Analysis seeks to obtain information of the most essential structural and functional aspects of an object. Its goal is to formulate an objective sense of truth and a clearer picture of reality. The raison-d'être is the belief that analysis can impart knowledge of the object of interest and therefore understanding. Indeed, it is this ancient belief that stimulates the efforts of mankind (past and present) towards probing the ever-receding
microscopic and sub-atomic world of particles and energy, as exemplified by Democritus' theory of the atom and the void (circa 500 B.C.). Many other disciplines, cloaked in the veneer of different languages, share a similar bond. The humanities, philosophy and religion also attempt to derive understanding of the phenomenal world through a technique of "separation", shared by even scientists of the most materialistic persuasion. An example of this common approach is the mystical tradition of alchemy, the predecessor of modern chemistry. Early practitioners of this discipline, which combined Greek philosophy and Egyptian technology, made use of various filtration and distillation devices for their quest of the enigmatic philosopher's stone, "magnum opus". Many of these early separation techniques are still in use today. The transmutation of base metals to gold was not only a material goal, but also a figurative transformation of the psyche, through a process of rigorous purification of the base levels of ignorance towards rarer forms of wisdom. In fact, the process of writing this thesis was a similar process of continuing refinement (although some may disagree whether it is veritable gold) from the raw initial draft.

With the advent of modern science and the rise of reductionism, rationalism and industrialization in Europe, the early separative techniques were further refined. These separation technologies included the methods of precipitation, filtration, distillation and extraction. During the Enlightenment, alchemy and other pre-scientific traditions were gradually dismissed as nonsense or "primitive magic", while being assimilated into the modern mechanistic rationale of scientism. Indeed, like so many previous revolutions of mankind, the new dominant mode of thought (paradigm) is often intimately influenced by its discarded vestiges of the past. The impulse to separate and analyze remained.

During the last century, several new separation techniques were developed in order to handle the complexity of mixtures that required analysis: some of these include chromatography, centrifugation, and electrophoresis. The growing prominence of
microbiological, pharmaceutical, clinical, environmental and forensic sciences in today’s society, have placed increasingly greater demands on separation and analysis.

Three important trends are evident in the field of separation science today. First, the demand for increasing selectivity for separation of complex matrices. Secondly, the separation of enormous numbers of different samples rapidly and economically. Lastly, the ability to perform separations in a micro-scale format to reduce sample sizes and probe microscopic environments. Three prime examples of these trends may be highlighted: the bold attempt to sequence the entire human genome (Human Genome Project), the almost certain identification of suspects involved in nefarious crimes through DNA fingerprinting (thus separating possible suspects from actual perpetrators of a crime), and the never-ending search for new drugs to improve humanity’s (and the company’s) lot, led by numerous pharmaceutical multinational corporations and their subsidiaries. These scientific fields are rapidly becoming popular images in the media and the general public because of their potential to influence humanity and the world in ways never seen before. Their common bond is their dependence on efficient separation technologies, such as capillary electrophoresis.

Capillary electrophoresis (CE) has evolved and continues to evolve in this current cultural environment, with the hope that this new form of technology may help solve today’s greatest separative challenges. It is interesting to note that despite the tremendous technological development in the field of separation science during the last century, the same universal maxim applies to both modern scientists and their predecessors: To separate the individual components that comprise an object of interest, so that one may understand the object as a whole.
1.2 Separation Philosophy: How to Separate?

Separation, by definition, requires difference, uniqueness, variety and diversity of traits among the individual components (elements, molecules, macromolecules, cells, macroparticles, etc.) that make a mixture. It is precisely these differences which permit their possible separation. The *how* of separation essentially relates to specific means used to induce separation against the tendency of *mixing*, as dictated by the natural laws of energy and entropy.

"Separation is the art and science of maximizing separative relative to dispersive transport"

As the quote by Calvin Giddings eloquently describes, two fundamental transport processes are operative with any separation system: separative and dispersive transport. These two opposing forces, the "ying and yang" of separation, must be properly considered when designing or improving separations. Separative transport is the result of the differential interaction of analytes with their surrounding environment. Separative mechanisms are based upon differences in energetic and/or kinetic processes due to differences in specific physicochemical properties of solutes. Differences in the melting point, particle size, boiling point, solubility, density, equilibrium (affinity), molecular size and charge of solutes may be used to achieve separation. For example, chromatographic separations are generally based upon differences in equilibria (free energy), whereas electrophoresis and centrifugation achieve separation from differences in solute mobility or sedimentation rates (kinetic processes) in their environment. In either case, each solute must possess unique transport properties in order for separation to occur.

In direct opposition to separation is dispersive transport or band broadening. Diffusion, convection and resistance to mass transfer are three processes that contribute to dispersive transport. Hence, the principle idea in separation science is to maximize factors enhancing
separative transport (maximize separation selectivity) and to minimize sources of band 
broadening (maximize separation efficiency). The specific concepts of separation selectivity 
and efficiency, as related to CE, are discussed later in the introduction.

Superimposed on the two mechanistic transport processes are two functional or 
pragmatic aspirations of separation: resolution and analysis time. Ideally, one strives to 
obtain high resolution (high selectivity and efficiency) in the separation in a minimum 
amount of time. Generally, there is a trade-off between the amount of resolution desired and 
the length of time required to achieve it. Thus, one optimizes the separation conditions in 
order to obtain a rapid and sufficiently resolved separation of components in a mixture. 
Figure 1.1 depicts the separation philosophy in terms of dominant processes, mechanisms 
and goals, including the roles of various separation techniques.

CE is currently one of the most powerful separation technologies used to optimize both 
transport mechanisms to produce highly efficient separations. The flat flow profile of 
electroosmosis (minimize axial diffusion), the efficient heat dissipation of narrow bore 
capillaries (minimize convection), and the lack of a stationary phase (eliminate resistance to 
mass transfer), result in extremely efficient separations for CE, in comparison to established 
chromatographic techniques. Moreover, CE has the unique advantage among separation 
techniques, to utilize both equilibria (free energy) and mobility (kinetics) to enhance 
separative transport, with the use of additives (complexation agents) in the run buffer during 
electromigration. Thus, two entirely different mechanisms may be used simultaneously to 
produce highly selective separations. The general principles and specific factors important 
for separation in CE is introduced throughout this portion of the thesis.
Figure 1.1 Schematic representation of the separation philosophy illustrating the two complementary forces of separative and dispersive transport, and the separation optimization principles of resolution and analysis time.
1.3 Capillary Electrophoresis: A Historical Perspective

Electrophoresis is a separative process based upon differential movement, "phoresis" of charged analytes under an applied electric field, "electro". Although Kohlrausch formally described the principle of electrophoresis and ionic migration in an electrolyte solution as early as 1897, it was Arne Tiselius, in his doctoral thesis, who first demonstrated the technique experimentally in 1930. Tiselius discovered that by placing a protein mixture in a tube filled with buffer solution under an applied voltage, sample components migrated in a direction and velocity determined by their effective charge and size. Despite the promising achievement of this technique, a number of deficiencies arose from conducting electrophoresis in free solutions. Dispersive processes due to convection and thermal diffusion contributed to significant band broadening, which deteriorated the separation.

During the forties and fifties, anti-convective support media, such as paper or gel, were introduced to electrophoresis to suppress convection due to Joule heating. Paper electrophoresis was applied to the separation of small charged molecules, whereas gels made from polyacrylamide and agarose, acted as unique sieving matrices to influence the separation of large charged macromolecules. Gel electrophoresis gradually developed into the central separation technique of biochemistry for the separation of complex matrices derived from proteins and nucleic acids. Despite the potential separation power demonstrated by the technique, time-consuming and labour intensive processes involved with gel preparation, sample application, staining and quantification of zones, limited gel electrophoresis, and electrophoresis in general, to the recluse domain of biochemists.

During this same period, the extensive use of chromatographic separations by researchers and the development of fully automated and commercial instrumentation by industry, such as HPLC, GC and IEC, postponed the need for new separation modes based on
electrophoresis. On the other hand, the new chromatographic technology also enabled the development of CE, with the introduction of narrow capillaries in GC and on-line detectors in HPLC. As the complexity of sample matrices increased, along with the reduction in the amount of sample available (sub-microgram levels) with the advent of biotechnology, the requirement for rapid and robust micro-separation systems complementary to established chromatographic separations, provided favorable conditions for CE development. CE is a classic example of a method ahead of its time, requiring the right timing and technology necessary for its proper implementation and maturation.

Despite the success of gel electrophoresis, many attempts were made to perform electrophoresis in free solution without any stabilizing media to overcome convection. In 1967, Hjertén, a student of Tisilius, performed electrophoresis in free solution in tubes of quartz glass with inner diameters of 1-3 mm. The glass tubes were coated with methylcellulose to prevent electroosmosis and were rotated along the longitudinal axis during separation in order to minimize thermal convection. Despite the complexity of the apparatus, Hjertén's work provided inspiration to perform electrophoresis in smaller tubular formats, later adapted by Mikkers and co-workers in narrow Teflon tubes. It was not until Jorgenson and Lukacs in 1981, who performed electrophoresis in narrow glass capillaries (20-100 μm inner diameter), that CE truly started to blossom. Figure 1.2 depicts a typical narrow bore, fused-silica capillary used in CE. The polyimide coating is used to provide increased flexibility and durability for the normally fragile glass capillary.

Many benefits arise from the introduction of narrow-diameter glass capillaries. It allows efficient heat dissipation caused by Joule heating due to the large surface-to-volume ratio and ease of heat transport. Better heat dissipation through capillaries permits use of higher
Figure 1.2 Cross-sectional view of a fused-silica capillary.

electric fields, resulting in more rapid and efficient separations. In addition, the small capillary dimensions require the injection of only nanoliter amounts of sample, making CE an exquisite micro-separation technique for the analysis of trace amounts of material. Online detection is possible since a small detection window can be made on one end of the capillary by burning off the protective polyamide coating. Despite excellent mass sensitivity due to small sample volumes injected, concentration sensitivity is limited because of the extremely small optical pathlength across the capillary. Soon after the initial report by Jorgenson and Lukacs, the development and application of CE increased dramatically throughout the eighties. The first fully automated CE instruments were manufactured in 1989 and continued to be refined throughout the nineties. Presently, after almost twenty
years of rapid development, the use of CE is still generally limited to academic research facilities. This is primarily due to the wide acceptance and long-standing practice of conventional chromatography in most industrial laboratories. As separation performance, robustness and reliability continues to improve, along with increased understanding of the fundamental principles governing CE, it is only a matter of time until industry adopts it as a viable and useful complement to chromatography.

1.4 Capillary Electrophoresis: Instrumental Set-up

CE is an efficient micro-separation technique composed of relatively simple and inexpensive components. The system consists of a high voltage power supply, buffer reservoirs, electrodes, a separation capillary, an on-line detector and a microprocessor control. Two ends of a narrow bore fused-silica capillary are immersed in buffer reservoirs (inlet and outlet). The buffer is vital to electrophoretic separations to conduct electricity and to provide buffering capacity. The electrodes are placed in the buffer reservoirs to make electrical contact between voltage supply (0 - 30 kV) and capillary. Sample is loaded into the capillary by replacing the inlet buffer with a sample reservoir and applying either an electric field (electrokinetic injection) or an external pressure (hydrodynamic injection) for a fixed time period. Sample injection volumes are typically nanolitres (e.g., 10 nL). After replacing the sample reservoir with the run buffer, the voltage is applied and separation is performed. Detection is performed at the opposite end of the capillary through the capillary wall, (UV, PDA and LIF), or by coupling the capillary with other detectors (MS and EC), through an interface. Figure 1.3 depicts the instrumental set-up of a typical capillary electrophoresis system. The arrival of commercial instruments provide additional features such as capillary temperature control, multiple detector formats, autosamplers, fraction collectors and data
Figure 1.3 General instrumental set-up of a capillary electrophoresis system.

processing software. These features improve the ease of use, reliability and performance of CE.

1.5 Electrophoretic Mobility

Electrophoretic separations are based on differential movement of charged particles under an electric field.\(^7\)\(^8\) The velocity of an ion, \(v\) is dependent upon the magnitude of the applied electric field, \(E\) and its electrophoretic mobility, \(\mu_{ep}\) which is an intrinsic property of the ion in a particular environment:

\[
v = \mu_{ep} \cdot E \quad (1.1)
\]
Ideally, two opposing forces act upon a charged particle as it migrates through a fluid under a potential difference. In a homogeneous electric field, the charged particle is accelerated by an electric force $F_e$:

$$ F_e = Q_{eff} \cdot E \quad (1.2) $$

where $Q_{eff}$ is the effective charge of the analyte in a specific medium. The effective charge of an ion is nearly always lower than its nominal charge because of the formation of a counter-ion double layer around the particle in an electrolyte solution. Opposing the motion of the ion is the viscous drag force, $F_d$ which acts on the moving particle as it migrates through a viscous medium:

$$ F_d = 6\pi \cdot \eta \cdot R \cdot v \quad (1.3) $$

where $\eta$ is the viscosity of the solution, $R$ is the hydrodynamic radius of the particle (Stoke's radius) and $v$ is the migration velocity of the ion. At equilibrium, both forces acting on the charged ion are balanced and the particle travels at a constant electrophoretic mobility given by:

$$ \mu_{ep} = \frac{v}{E} = \frac{Q_{eff}}{6\pi \cdot \eta \cdot R} \quad (1.4) $$

The units used for mobility are typically cm$^2$ V$^{-1}$s$^{-1}$. The electrophoretic mobility represents the average velocity of a charged ion per unit of electric field strength. It is a characteristic property of the ion in a given medium and is dependent primarily on its effective charge to hydrodynamic radius. A number of assumptions are made with the derivation of this equation. Namely, the ion is assumed to be a rigid spherical particle surrounded by a relatively large Gouy-Chapman electric double layer. Also, the effects of electrophoretic relaxation and retardation due to the surrounding double layer, both of which tend to slow the motion of the ion, are neglected. In addition, the electric field is homogeneous and the viscosity is assumed to be independent of electric field strength. Hence, separations in
electrophoresis can be ideally attributed to differences in the effective charge and/or size of the ion.

1.6 Electroosmotic Mobility

The electroosmotic mobility, also known as the electroosmotic flow (EOF), is a phenomenon central to CE. It can be generally described as the relative motion of a liquid to a fixed charged surface caused by an electric field. The magnitude and direction of the EOF depends on the composition of the capillary and the nature of the solution. Fused-silica is the most common material used for the production of capillaries in CE. When silica is in contact with an aqueous solution, its surface hydrolyzes to form silanol surface groups. The groups are generally either neutral SiOH or negatively charged SiO\(^{-}\) depending upon the pH of the surrounding electrolyte. Since the silanol groups are acidic (pK\(_a\) = 2.5), the surface of the capillary wall is negatively charged above pH 3. The anionic charge on the capillary surface results in the formation of an electric double layer with the ions in the solution. Counter-ions (cations) tend to absorb onto the silica wall by electrostatic attractions to balance the surface charge.

According to Stern's model, a rigid double layer of absorbed ions (Stern layer) is adjacent to a diffuse layer (Gouy-Chapman layer) that permits diffusion of ions by thermal motion. The excess negative charges at the capillary wall induces an electric potential at the interface between the silica surface and the electrolyte solution, which decreases linearly within the Stern layer and then drops exponentially throughout the diffuse layer. The potential of the diffuse layer is referred to as the zeta potential, \(\zeta\).
**Figure 1.4** Stern model of the electric double layer occurring at the interface between an electrolyte solution and the silica capillary wall.
The pH and the ionic strength of the electrolyte are two factors which greatly influence the zeta potential of the fused-silica surface. The pH of the buffer affects the overall charge on the capillary surface that leads to greater zeta potential with increasing pH. After the titration of the silanols is completed at pH > 8, the zeta potential no longer changes significantly with higher pH. The ionic strength influences the zeta potential by modifying the negative surface charge through electrostatic attraction of cations. The charge of the capillary surface is balanced with increasing numbers of cations at higher ionic strengths, thereby reducing the zeta potential. Consequently, the zeta potential is the greatest at high pH and/or low ionic strength buffer conditions. The thickness of the Stern layer lies in the molecular range, whereas the thickness of the diffuse layer (3 to 300 nm) varies substantially with ionic strength and pH of the electrolyte, with potentials typically ranging from 10 to 100 mV. Figure 1.4 depicts the idealized Stern model of the electric double layer formed on the capillary surface and the resultant zeta potential.7,8

When a voltage is applied across the capillary, the mobile cations in the diffuse layer migrate in the direction of the cathode. Since the ions are solvated by water, the bulk fluid within the capillary is dragged along with the migrating ions, thereby generating an EOF. The net direction of the EOF is towards the cathode because of the excess number of cations in the diffuse layer required to charge balance the negatively charged capillary wall. Although the diffuse layer is relatively small in comparison to the diameter of the capillary, the EOF is transmitted throughout the capillary by hydrogen-bonding interactions. One of the inherent advantages of the EOF is its flat plug profile, in contrast to the laminar flow of pressure driven processes in HPLC. The liquid flow of the EOF shows a flat profile since the driving force is uniformly distributed across the capillary. Hence, band dispersion due to longitudinal diffusion is minimized in CE due to the nature of the EOF. Figure 1.5 depicts
Figure 1.5 Flow profiles of pressure-driven systems (A) and electrically-driven systems (B). Arrows indicate flow velocity vectors.

The two different flow profiles generated by an electric field (EOF in CE) and an external pressure (HPLC).

The velocity of the EOF is proportional to the magnitude of the electric field and the electroosmotic mobility, \( \mu_{eo} \), which is given by:

\[
\nu_{eo} = \mu_{eo} \cdot E
\]

(1.5)

The electroosmotic mobility is dependent on properties of the capillary and electrolyte solution such as the zeta potential \( \zeta \), the dielectric constant (relative permittivity) \( \varepsilon_r \) and the viscosity \( \eta \) of the medium:

\[
\mu_{eo} = \frac{\varepsilon_r \cdot \zeta}{4\pi \cdot \eta}
\]

(1.6)

The assumptions made with eq. 1.6 include a homogeneous distribution of charge on the capillary wall and the independence of both viscosity and dielectric of the solution with electric field strength. The units used for the electroosmotic mobility are the same as those used to describe electrophoretic mobility, typically \( \text{cm}^2\text{V}^{-1}\text{s}^{-1} \).
1.7 Apparent Mobility

Normally, two distinct mobilities are operative in a CE separation. The apparent mobility of an analyte, $\mu^A$ is a result of the superimposition of both its electrophoretic mobility and the electroosmotic mobility of the bulk solution:

$$\mu^A = \mu_{ep} + \mu_{eo}$$ (1.7)

The electrophoretic mobility of an ion can be calculated experimentally by measuring the apparent migration time of the analyte and the migration time of a neutral analyte (as a measure for $\mu_{eo}$), referred to as the EOF marker, as follows:

$$\mu_{ep} = \frac{L_c \cdot L_d}{V} \cdot \left( \frac{1}{t_A} - \frac{1}{t_{eo}} \right)$$ (1.8)

where, $L_c$ is the capillary length, $L_d$ is the length to the detector, $V$ is the applied voltage, and $t_A$ and $t_{eo}$ are the apparent migration times of the analyte and the EOF, respectively. The electrophoretic mobility is a characteristic property of the analyte, which is dependent on specific conditions of the medium such as buffer type, pH, ionic strength, dielectric constant, viscosity, and temperature.

1.8 Capillary Zone Electrophoresis

Traditionally, CE can be performed under three different formats or modes of operation: capillary zone electrophoresis (CZE), capillary isotachophoresis (CITP), and capillary isoelectric focusing (CIEF). These CE modes are determined primarily on the nature and homogeneity of the run buffer in the capillary. CITP and CIEF are band focusing techniques which utilize discontinuous buffer systems, whereas CZE uses a continuous, homogeneous buffer during the separation where normal band broadening operates. CZE is by far the most common mode of operation because of its wide applicability, versatility and ease of use. The
Separations in CZE typically involve three steps: 1) pre-rinse of the capillary with a homogeneous background electrolyte (run buffer), 2) hydrodynamic or electrokinetic injection of sample, and 3) application of the voltage and separation of sample components. Initially, the sample is injected as a sharp zone at the capillary inlet. Upon application of the voltage, the ionic components of both the run buffer and sample migrate towards the corresponding electrode: cations to the cathode and anions to the anode. Sample components migrate independently from the background electrolyte with specific electrophoretic mobilities determined by their effective charge to size ratio. The apparent mobility of each species is determined by the superimposition of the analytes electrophoretic mobility and the EOF. After some time, analytes with different mobilities separate into distinct zones. The relative positions and band shapes of the analytes change with time. The monitoring of the detector signal at the other end of the capillary as a function of time generates an electropherogram, which is similar in appearance to a chromatogram. If the EOF is larger in magnitude than the individual electrophoretic mobilities of the negatively charged analytes, then all sample components are swept towards the cathode (outlet) across the detector window. The order of migration (increasing migration time) in an electropherogram is typically: small cations < large cations < neutral analytes < large anions < small anions. Normally, neutral analytes cannot be separated in CZE and thus co-migrate with the EOF. Figure 1.6 depicts the separation process in CZE and the resultant electropherogram that is generated. Since CZE is the most widely used mode in CE, the terms CE and CZE are often used interchangeably.
Figure 1.6  The separation process of capillary zone electrophoresis, depicting (A) the initial sample injection of a complex mixture, (B) the separation of components into distinct zones upon application of the voltage, according to their effective charge:size ratio and (C) the resultant electropherogram generated as the components migrate past the detector window.
1.9 Performance Criteria in CE

1.9.1 Efficiency

The efficiency of the separation is one of the most frequently used criterion used to emphasize the tremendous separation potential of CE. The efficiency, or the number of theoretical plates (N), is a quantitative measure to describe the tendency of a particular column to produce band broadening, originally developed for chromatography. General dispersive transport mechanisms responsible for band broadening in separations were discussed in chapter 1.2. In CE, band broadening or peak variance, \( \sigma^2 \) is the result of a number of effects which contribute additively to the total spreading of the peak, \( \sigma_T^2 \):

\[
\sigma_T^2 = \sigma_D^2 + \sigma_I^2 + \sigma_J^2 + \sigma_E^2 + \sigma_A^2 + \sigma_w^2 + \sigma_o^2
\]

(1.9)

where terms on the right side of eq. 1.9 represent variances caused by diffusion (\( \sigma_D^2 \)), injection length (\( \sigma_I^2 \)), Joule heating (\( \sigma_J^2 \)), electrophoretic dispersion (\( \sigma_E^2 \)), adsorption (\( \sigma_A^2 \)), width of detection zone (\( \sigma_w^2 \)) and other effects (\( \sigma_o^2 \)), respectively. The various band broadening processes may not be totally eliminated in CE, but their effects can be significantly minimized through proper design and consideration of the separation conditions.

Under optimum conditions, the major contribution to peak variance in CE is longitudinal diffusion which can be described by the Stokes-Einstein equation:

\[
\sigma^2 = 2D\ t
\]

(1.10)

where \( \sigma^2 \) is the peak variance, \( D \) is the diffusion coefficient for the species and \( t \) is its migration time. Generally, small molecules (large \( D \)) that reside in the capillary for an extended time show greater peak variance than larger molecules that migrate rapidly through the system. In the rate theory of band broadening in chromatography, \( N \) can be defined as:

\[
N = \frac{L\sigma^2}{\sigma^2}
\]

(1.11)
where $L_d$ is the capillary length to the detector. Assuming that longitudinal diffusion is the major source of band broadening in the separation, efficiency in CE can be approximated by:

$$N = \frac{L_d^2}{2Dt} = \frac{L_d^2}{2D[\frac{L_d^2}{(\mu_{e\rho} + \mu_{e\sigma})V}]} = \frac{(\mu_{e\rho} + \mu_{e\sigma})V}{2D}$$

(1.12)

Eq. 1.12 demonstrates that the efficiency of a separation in CE is enhanced by large electrophoretic and electroosmotic mobilities, high voltages and low sample diffusion coefficients. These factors contribute to faster separations and reduced band broadening due to longitudinal diffusion. For a given analyte, the most facile way to improve the efficiency theoretically is by utilizing high voltages. However, there is a reasonable limit in which voltages can be increased, since higher voltages generate higher ion conductances and increased Joule heating, leading to increased peak dispersion. In practice, an Ohm plot of the measured current generated with the specific buffer at increasing voltages is used to determine the optimum voltage for separation. The maximum voltage within the linear portion of the Ohm plot is selected as the optimum voltage.

Experimentally, the efficiency can be calculated from an electropherogram as follows:

$$N = 5.54 \cdot \left(\frac{t}{W_{1/2}}\right)^2$$

(1.13)

where $W_{1/2}$ is the peak width at half maximum. It is important to note eq. 1.13 and other equations derived from the rate theory of band broadening assume a Gaussian peak shape. Efficiencies in CE often range from $10^5$ to $10^6$ theoretical plates under optimum conditions.

1.9.2 Selectivity

The selectivity of a separation is another essential measure of the performance of a separation. Separation selectivity is related to the specific sources of differential separative
transport mechanisms as was discussed in chapter 1.2. Differences in the mobilities and/or equilibria towards additives in the run buffer are responsible for selectivity in CE. Separation selectivity, $\alpha$ can be described generally as the ratio of the migration rates (velocities) of the two migrating components, A and B (where, $v_A > v_B$) by the following equation:

$$\alpha = \frac{v_A}{v_B}$$ (1.14)

In CE without complexation additives, the ratio of migration rates can be described by the ratio of the mobilities of the analytes since the electric field is the same for all analytes. Thus, the migration rates can simply be replaced by the net mobilities of the analytes, and the separation factor is defined as:

$$\alpha = \frac{\mu_A}{\mu_B} = \frac{\mu_{ep, A} + \mu_{eo}}{\mu_{ep, B} + \mu_{eo}}$$ (1.15)

Separation optimization in terms of selectivity is limited to modification of the electrolyte system in CE. The selectivity can be tuned through variation of the buffer type, pH, and ionic strength of the run buffer. These buffer properties can effect the separation through changes in the effective charge to size ratio of analytes, thereby modifying their electrophoretic mobility. Also, organic modifiers (water immiscible organic solvents such as methanol, acetonitrile and dimethylsulfoxide) can be added to the run buffer to improve sample solubility and modify analyte mobility. One of the most versatile ways to tune the selectivity in CE separations is through the use of complexation "additives" in the run buffer. Specific examples and applications of additives in CE is discussed in the next section of the introduction.
1.9.3 Resolution

The resolution between two adjacent migrating peaks is also another performance criterion used to assess a CE separation. Resolution takes into account the effects of both band broadening and band separation in a separation system as was introduced in chapter 1.2. The resolution, $R_s$, of two peaks is defined as the quotient of the distance between two peak centers, $\Delta x$, and the mean of the two standard deviations of the peaks:

$$R_s = \frac{\Delta x}{4\sigma}$$  \hspace{1cm} (1.16)

Eq. 1.15 can be rewritten in terms of mobilities and efficiency for $\Delta x$ and $\sigma$ respectively:

$$R_s = \frac{\sqrt{N}}{4} \cdot (\gamma - 1)$$  \hspace{1cm} (1.17)

The resolution equation given by eq. 1.17 shows that the resolution is determined by the column efficiency and the selectivity for a separation. Hence, resolution is a measure of the additive effects of band broadening and separation selectivity. Experimentally the resolution can be calculated by measuring the relative difference in migration times ($t$) and the summation of the base peak widths ($w$) of species 1 and 2:

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2}$$  \hspace{1cm} (1.18)

The minimum resolution required for baseline separation of two adjacent peaks is $R = 1.5$. Besides resolution, the other constraint considered important for separation optimization is analysis time. Generally, one aspires sufficient resolution of components in a mixture under a minimum amount of time as is depicted in the philosophical approach of separations in Figure 1.1. Thus, resolution much greater than 1.5 is deemed unnecessary since it generally requires longer analysis times.
1.10 Additives in CE

Buffer additives can be used to modify the EOF, solubilize solutes, or reduce solute-capillary wall interaction. However, their major application to date is to modify the mobility of analytes via differential analyte-additive interaction. The use of additives in CE represents one of the most elegant ways to tune the selectivity of a separation. One disadvantage of electrophoresis is its lack of selectivity for neutral and enantiomeric compounds. However, since Terabe et al.\textsuperscript{10} first added micelles in the run buffer to separate a mixture of neutral components, the use of additives in CE has greatly expanded.

Neutral analytes are separated in CE through differential interaction (equilibria) with charged additives in the run buffer, such as SDS micelles. Association of the neutral analyte with a charged additive results in the induction of a charged state on the analyte-additive complex, which permits its separation by differential electromigration in the capillary. Similarly, the differential interaction of enantiomeric analytes with chiral additives, such as cyclodextrins, may result in the formation of diasteromeric analyte-additive complexes (different equilibria), allowing their separation. Additives may also be used to further improve the separation of charged, achiral analytes as well. Thus, CE with additives represents a flexible and versatile approach to enhance the selectivity, permitting the separation of diverse types of species, including charged, neutral, and enantiomeric molecules.

Additives share a similar function to the different types of stationary phases in chromatography used to modify the selectivity of the separation. However, CE with additives offers much greater flexibility than stationary phases, since one or more additives can be easily added to the run buffer. In addition, the small volume requirements of CE results in lower consumption of additive or run buffer reagents, which offers economic and
Figure 1.7 Schematic of some of the diverse types of additives used to improve the separation selectivity in CE.
environmental advantages in comparison to HPLC methods.

The types and numbers of additives that may be used to modify separation selectivity in CE is enormous. Additives ranging in size and complexity from metals to molecules to macromolecules may be selected when appropriate. Separations may be based on differential metal complexation, molecular ion pairing, inclusion complexation with molecular hosts, partitioning within apolar micelles, or binding to biospecific macromolecules. Indeed, hydronium or hydroxide ions (buffer pH) can also be considered one of the most widely used types of additives in CE, that selectively modify the mobility of weakly acidic or basic molecules. Figure 1.7 depicts common types of additives that have been used in CE to date. One may consider this list as an "additive arsenal" at the disposal to the discerning analytical chemist. Indeed, the "armed" analyst, with judicious and wise selection, may greatly enhance separation selectivity to its theoretical maximum, through the use of multiple, complementary additive systems.

The application of additives in CE has been so great during the past decade that entirely new modes of CE have been designated according to the type of additive used. For example, micellar electrokinetic chromatography (MEKC) and capillary affinity electrophoresis (CAE), are widely applied modes of CE using micelles and biomolecules, respectively. Rational design of separation systems in CE through proper choice and application of additives is a vital theme throughout much of this thesis. Chapters II & III of this thesis (Section A) attempts to unify the various modes of CE, irrespective of the type of additive, through a comprehensive dynamic complexation theory. Chapter IV (Section B) of the thesis explores the use of a novel type of charged aromatic macrocycle (TESMR), as an additive in CE. The development of a quantitative assay for epinephrine from dental anesthetic solutions (Chapter VI - Section C) depended on additive (borate) complexation for separation. In addition, improvements in CE sensitivity based on selective focusing of
catecholamines (Chapter VII - Section D) also relied on borate complexation. Separation design based on proper selection and application of buffer conditions (including additives) in CE is the overall goal of this thesis. Consequently, CE with additives is a potent micro-separation technique that can separate a wide variety of samples with high selectivity and efficiency, as well as permitting improvements in concentration sensitivity.

1.11 Research Objectives

"Good scientists, study the most important problems they think they can solve. It is, after all, their professional business to solve problems, Not merely to grapple with them."  

- Peter B. Medawar, from E. F. Schumacher's "A Guide for the Perplexed"

Traditionally, there has existed a fundamental dichotomy in science: that of natural scientific research and understanding, often expressed as theory and philosophy, and that of applied science and engineering, most commonly manifested as "useful" creations, devices and technology. Ideally, it is believed that a mutual balance of these approaches is most beneficial for scientific progress and the eventual beneficiaries of this progress, humankind. However, there is a growing emphasis within the scientific disciplines today for rapid, pragmatic research, that may be patented as intellectual property or applied to the economic demands of industry. Although the reasons behind this trend are complex, the decreasing support from individuals and government of the more abstract fields of natural sciences is one apparent cause. There is an increasing demand and belief in technological solutions to answer both mankind's sublime pleasures and painful dilemmas. Ironically, there now seems to be more problems to be solved than ever before, despite the fact that many scientists are becoming expert problem solvers. Clearly defined objectives and predetermined outcomes
are gradually becoming the norm. The prolonged effects of this cultural emphasis on the commercialization of scientific research is still too early to ascertain. The lack of formal ethical and philosophical training of scientists in many universities (despite the connotation of Ph.D.) certainly does not promote a balanced personal development of the individual to meet these challenges. A recent case involving a University of Toronto researcher, Dr. Nancy Olivieri, who was funded by a local pharmaceutical company, Apotex Inc., may highlight the increasing pressure experienced by scientists who strive not to compromise their scientific objectivity in order to please clients or disrupt potential markets (*The Vancouver Sun*, Friday, January 8, 1999). Although, it is not the purpose of this thesis to study the issue of scientific objectivity and the independence of academic research, it can be said that my experiences throughout the thesis presented many challenges to my preconceived objectives and the *ideal* of objectivity.

During much of the work in this thesis, it was realized that the *reality* of novel research rarely obeys the *hypothesis* or linear framework one may impose. An ability to continuously verify assumptions experimentally, and maintain complete objectivity (the scientific *maxim*), was (and still is) tremendously challenging. The creation and refinement of objectives and theories often underwent a non-linear time schedule. Many new questions were posed after noticing "unusual" phenomena and not prior to the observations. In fact, much of the work during the thesis required good *fortune* and right *timing*; two unpredictable elements common to many of life's endeavours.

Although CE has enjoyed great success in the field of separation science over the past decade, there still remain vital issues that postpone its acceptance by the well-established legion of chromatographers in research and industry. Beyond the "hype" sometimes preached by its most loyal proponents, a number of deficiencies still exist with CE. The work in this thesis attempts to encompass four main areas in CE that require substantial
refinement: A) theoretical understanding of fundamental parameters involving separation, B)
enhancement of separation selectivity, C) greater reliability and performance with assays
required by academia and industry, and D) improved concentration sensitivity; which may be
referred to as the "Achilles' heel" of CE. Hence, the stated objectives of this research span
several different aspects of capillary electrophoresis, in an attempt to remedy these problems.
The objectives are ordered sequentially as chapters in this thesis, and divided into four main
sections, which may be paraphrased as:

Section A: The study, development and application of a comprehensive theory to describe
the fundamental properties operative in CE separations; 1) Prediction of the migration
behaviour of analytes in CE based on three fundamental parameters, Chapter II and 2)
Accurately describing weak analyte-additive interactions in CE, Chapter III.

Section B: The synthesis and application of a charged macrocycle, based on
tetraethylsulfonated resorcinarene (TESMR), as a novel additive to improve separation
selectivity in CE, Chapter IV.

Section C: The development of reliable and quantitative assays for academia and industry;
1) The analysis of gamma-carboxyglutamic acid by CE-LIF in protein, urine and plasma,
Chapter V, and 2) Quantitative assay for epinephrine from dental anesthetic solutions by CE,
Chapter VI.

Section D: The development and application of on-line focusing techniques to improve
concentration sensitivity in CE; 1) Selective focusing of catecholamines and weakly acidic
analytes in CE via a dynamic pH junction, Chapter VII and 2) On-line focusing of nucleic
acid monomers in CE, Chapter VIII.
Each objective is often interconnected and related to the other. Together, these objectives strive to evolve a general separation philosophy that permit the rational design of separation systems in CE (chapter IX of this thesis). Figure 1.8 gives an overview of the organization and direction of the research in this thesis, in terms of its main sections and their corresponding chapters.
1.12 References


(2) Tiselius, A. Almquist and Wiksell, Uppsala, 1930.


Section A:

Understanding of the Fundamental Parameters Governing Separation in CE.
Chapter II:

Prediction of the Migration Behaviour of Analytes in CE Based on Three Fundamental Parameters.

"CE: From an empirical art to a rational separation science."
II: Prediction of the Migration Behavior of Analytes in Capillary Electrophoresis Based on Three Fundamental Parameters.

2.1 Introduction

One of the inherent advantages of CE is the ease with which the composition of the background electrolyte can be adjusted in order to modify the analyte mobility. The use of additives in CE has led to dramatic increases in separation power. Micelles, complexing agents, affinity ligands, and ion pairing agents are various types of additives that have been used to control the selectivity of the separation. Systematic optimization using one or more additives, based on the fundamental understanding of the determinants of migration behavior, would represent a significant advancement in CE. Indeed, a deeper theoretical understanding of the parameters effecting the mobility of an analyte in CE, would help guide researchers towards rapid optimization of separation conditions and the evolution of rational design strategies, based on the appropriate selection of buffer additive(s).

In chromatography, the migration behavior of an analyte is primarily determined by the velocity of the mobile phase and the fraction of analyte in the mobile phase, because the velocity of the stationary phase is zero. However, in CE separations that utilize additives in the background electrolyte, the analyte mobility is determined by three parameters: the electrophoretic mobilities of the free ($\mu_{ep,A}$) and complexed analyte ($\mu_{ep,AC}$), and the equilibrium constant ($K$), or the capacity factor ($k'$), of the analyte-additive interaction. It is often sufficient in chromatography to concentrate on improving the resolution of the most difficult pair of analytes for the optimization of separation conditions. Whereas in CE, because the mobility of an analyte is controlled by the three parameters, the optimum conditions depend not only on specific pairs of analytes, but also on the migration behavior
of other analytes. For a complicated mixture, it is essential to model the migration behaviour of all analytes before optimum conditions can be predicted.

Since the first description of CE with additives by Terabe and co-workers, there have been several theoretical models developed for specific additive types. Models to describe chiral separations, affinity CE, complexation CE, and micellar CE have been described. Our group has amalgamated and extended the existing theories by proposing to use the theory based on dynamic complexation between analytes and additives to describe the migration behavior of all types of open tubular CE. The primary goals of the unified dynamic complexation model in CE is: 1. to better understand the three fundamental physicochemical parameters, \( \mu_{ep}, A, \mu_{ep, AC} \) and \( K \), influencing a separation, 2. to apply the model for the systematic separation optimization of mixtures and the prediction of analyte behaviour, and 3. to permit the rational design of effective separation strategies for specific sample matrices based on appropriate selection of additive(s).

There have only been a few reports devoted to the prediction of analyte migration behaviour in CE. Khaledi and co-workers utilized an empirical/semi-empirical model to predict mobilities and migration times. This type of approach can be used for rapid separation optimization based on a limited set of experimental conditions. Wren et al. developed a theoretical model based on physicochemical parameters to describe and optimize chiral separations. The model was supported by experimental results and provided a systematic way to optimize additive concentration to achieve maximum resolution between enantiomers. Similar models for chiral separations have been expanded by Goodall et al. and Sepaniak et al. to optimize chiral separations for enantiomeric drugs and amino acids, respectively. The dynamic complexation model can be applied to the separations of all types...
of analytes (charged or neutral, chiral separations, etc.), in all types of background electrolytes (aqueous or nonaqueous), with any number of additives. The aim of this work is to examine the generality of this theoretical model by describing the migration behavior of 12 deoxyribonucleotides using β-CD as the additive. An extensive study of the predictive capabilities of the model under a variety of constraints is investigated in order to assess its strengths and limitations. In addition, molecular modeling of analyte-additive interactions confirmed the trends observed in the equilibrium constants obtained experimentally.

2.2 Theory

When an additive is used in the buffer of a CE system, the equilibrium in a homogeneous system, assuming a 1:1 molecular interaction between the analyte and additive can be described by:

\[ A + C \rightleftharpoons AC \]  \hspace{1cm} (2.1)

where A is the analyte and C is the additive or complexing agent (such as a surfactant, host molecule or biomolecule, etc.), and AC is the analyte-additive complex. The equilibrium constant for eq. 2.1 is:

\[ K = \frac{[AC]}{[A][C]} \]  \hspace{1cm} (2.2)

where [AC] is the analyte-additive concentration, [A] is the analyte concentration, and [C] is the concentration of the additive.

The capacity factor, \( k' \), also called the mass distribution coefficient, is defined in chromatography as the ratio of the analyte in the stationary phase compared to the amount of analyte in the mobile phase. A similar concept can be used in CE, where one defines the
capacity factor as the ratio of the amount of analyte-additive complex to the amount of free analyte. Since the additive and the analyte are in the same phase, the capacity factor is:

\[ k' = \frac{AC}{C} = \frac{[AC]}{[A]} = K[C] \]  

(2.3)

In CE, the capacity factor is proportional to the additive concentration. The fraction of the free analyte, \( f \), is defined as \([A]/[A]_0\) (\([A]_0\) is the original analyte concentration). When \([C]_0\), the initial additive concentration, is much greater than \([A]_0\), \([C]\) is equal to \([C]_0\). Therefore, substituting \([A]_0\) by \([A] + [AC]\) gives:

\[ f = \frac{1}{1 + k'} \]  

(2.4)

For a 1:1 analyte-additive interaction, the electrophoretic mobility of an analyte is described by:

\[ v\mu_{ep,T}^A = f\mu_{ep,A} + (1 - f)\mu_{ep,AC} = \frac{1}{1 + k'}\mu_{ep,A} + \frac{k'}{1 + k'}\mu_{ep,AC} \]  

(2.5)

where \( \mu_{ep,T}^A \) is the apparent electrophoretic mobility of the analyte at a specific temperature, and \( v\mu_{ep,T}^A, \mu_{ep,A}, \) and \( \mu_{ep,AC} \) are the ideal state electrophoretic mobilities of the analyte, free analyte, and the analyte-additive complex, respectively. Hence, the apparent mobility of an analyte is determined by the relative fractions of the mobilities of the free analyte and analyte-additive complex. Since the viscosity of the solution is often changed with additive concentration, a viscosity correction factor, \( v (\nu = \eta/\eta^0, \) the viscosity of buffer with the additive compared with the viscosity without the additive), is therefore required to convert measured apparent mobilities to the ideal state where the additive concentration is approaching zero. This ensures that any changes to the observed mobilities of the analyte is due only from specific molecular interaction with the additive and not to changes in the bulk properties of the medium.

Because the three parameters are intrinsic properties of the analyte-additive pair in a
defined system, the electrophoretic mobility of an analyte is a function of additive concentration only. Once the values of these three parameters are known and the viscosity effect is taken into account, the migration behavior of the analyte can be predicted for all additive concentrations. However, it is important to realize that these parameters can be affected by temperature as well. Goodall et al. reported that the neglect of viscosity and temperature corrections combine to underestimate K significantly. The use of a thermostatted CE instrument, that is operated at a low enough voltage to minimize Joule heating, will increase the accuracy and the precision of the parameters.

The mobility of the free analyte, $\mu_{\text{ep},A}$ can be determined directly by measuring the mobility without any additive present. However, the values of K and $\mu_{\text{ep},AC}$ must be obtained from the regression analysis using the measurements of the apparent mobility of the analyte at various concentrations of additive. Eq. 2.5 can be transformed into various linear equations for the calculation of K and $\mu_{\text{ep},AC}$. Recently, various plotting methods used to determine binding constants, which included nonlinear curve fitting and three linear plotting methods (double reciprocal, y-reciprocal, and x-reciprocal plots) have been discussed for both aqueous and nonaqueous CE. Theoretically, the various plotting methods should give identical results. In practice, the results may vary because of the way that errors associated with the variables are transformed. Although the equilibrium constant can be calculated using nonlinear regression, the linear presentation of data can be useful for detecting trends and deviations from the presumed stoichiometry of the interactions. Bowser et al. compared these plotting methods in a nonaqueous buffer system. Eq. 1 can be rearranged to:

$$\frac{[C]}{(V\mu_{\text{ep},T} - \mu_{\text{ep},A})} = \frac{[C]}{(\mu_{\text{ep},AC} - \mu_{\text{ep},A})} + \frac{1}{(\mu_{\text{ep},AC} - \mu_{\text{ep},A})K}$$

(2.6)
Figure 2.1 Structures of the deoxyribonucleotides used as analytes (A) and a schematic of their 1:1 dynamic complexation with β-CD (C), showing the three parameters that influence the analyte's mobility. In addition, the electroosmotic mobility and the viscosity correction factor are required to predict the migration time of analytes in CE.
A plot of \( \frac{[C]}{(v\mu_{ep,T} - \mu_{ep,A})} \) versus [C] should give a straight line, therefore the values of \( \mu_{ep,AC} \) and K can be obtained from the slope and the intercept, respectively. Once these values are determined, the ideal state mobility of an analyte can be calculated by substituting the values into eq 2.5. However, in order to compare these theoretical values with the measured values, the viscosity correction factor has to be incorporated.\(^4\)\(^5\) The migration times of the analytes can then be calculated once the electroosmotic mobility has been measured. The optimum additive concentration(s) occur where there is the least overlap in the analyte mobility curves.

Figure 2.1 depicts a schematic of the 1:1 dynamic complexation of the deoxyribonucleotides with \( \beta \)-CD, highlighting the fundamental parameters effecting the analyte's mobility.

### 2.3 Experimental

#### 2.3.1 Apparatus

All experiments were performed on a Beckman P/ACE 5500 automated CE system using System Gold software (Beckman) with a 386 PC computer. Fused silica capillaries (Polymicro Technologies, Phoenix, AZ) with inner diameters of 50 \( \mu \)m, outer diameters of 375 \( \mu \)m, and lengths of 27 cm, 37 cm, and 47 cm were used. New capillaries were first rinsed with 0.1 M NaOH for 3 minutes, then rinsed with deionized water for 5 minutes and finally washed with a 160 mM borate buffer, pH 9.0, for 10 minutes and equilibrated overnight before being used. The sample was introduced using a pressure injection for 3 seconds. All separations were carried out at 20 °C, and the UV absorption was monitored at 254 nm. Voltages of 5 kV, 6.85 kV and 8.7 kV were used with the 27 cm, 37 cm, and 47 cm capillaries respectively, maintaining a relative electric field of 185 V/cm. Relative viscosity measurements were performed by a 2 second injection of a 1\% benzene plug onto the
capillary inlet, UV detection at 214 nm using a 57 cm capillary, and measuring the time needed to push the plug to the detector under a constant pressure of 20 psi. Molecular modeling was performed on CS Chem 3D version 3.2 (CambridgeSoft Corporation, Cambridge, USA). Molecular structures were originally drawn on CS ChemDraw version 3.5 and their structures minimized on CS Chem 3D. Qualitative complexation studies of the deoxyribonucleotides with β-CD were performed by successively minimizing the energy of the complex, then running a molecular dynamics (a form of temperature gradient) and repeating this procedure until an energy minimum was observed. Energy minima are determined by both steric and noncovalent intermolecular forces of dipole/dipole, charge/dipole, and van der Waal forces. Solvation effects and charge (electrostatic) interactions are neglected in the modeling.

2.3.2 Chemicals and Procedures

Borax (Na₂B₄O₇·10H₂O), β-CD (cycloheptaamylose) and the 2'-deoxyribonucleotides, (dNTs): deoxyadenosine mono-, di-, and triphosphate (dAMP, dADP, and dATP), deoxyguanosine mono-, di-, and triphosphate (dGMP, dGDP, and dGTP), deoxycytosine mono-, di-, and triphosphate (dCMP, dCDP, and dCTP) and deoxyuridine mono-, di-, and triphosphate (dUMP, dUDP, and dUTP), were all purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol was purchased from Fisher Scientific (Nepean, Ont., Canada). Stock solutions of 160 mM borate were prepared in deionized water. A stock solution of 20 mM β-CD was prepared by dissolving the appropriate amount of β-CD in borate buffer. The appropriate volumes of stock solutions were mixed with buffer to make the 160 mM borate, pH 9.0 with concentrations of β-CD ranging from 5 to 20 mM. A stock solution of the analytes was prepared by dissolving approximately equal concentrations (1 x 10⁻³ M) of
each deoxyribonucleotide in deionized water. This stock solution was further diluted to a concentration of approximately $5 \times 10^{-5}$ M before injection onto the capillary. Methanol (1.7% v/v) was used as the EOF marker in the sample. Peaks were identified by spiking the sample solution with standard solutions of each deoxyribonucleotide. Before experiments were started, an Ohm plot was performed with the 27 cm capillary and the run buffer in order to select a voltage at which Joule heating was minimal. This was necessary to ensure the heat generated during the separation was efficiently dissipated. A voltage of 5 kV was used and this voltage is well within the linear region of the Ohm plot.

2.4 Results and Discussion

2.4.1 Determination of the Three Parameters and Their Influence on the Separation

Figure 2.2 shows the effect of changes in the β-CD concentration on the migration behavior of the analytes. When no additive is used, only ten resolved peaks can be observed for the twelve dNTs. The negative electrophoretic mobility of the nucleotides generally increases from mono- to di- to triphosphate. However, for a given degree of phosphorylation, the dNTs of guanine and uridine show larger negative electrophoretic mobilities when compared to the similarly sized dNTs of adenine and cytosine, respectively. Both guanine and uridine have an acidic hydrogen ($pK_a \approx 9.5$) that is partially ionized in the borate buffer (pH 9.0). Thus, the absolute value of $\mu_{ep,L}$ generally increases with increasing phosphorylation and increases in the order $U > C > G > A$. When 5 mM β-CD is used in the buffer, significant changes in the migration order of the dNTs is observed. By analyzing the changes in the migration times of the analytes with changes in cyclodextrin concentration, it can be seen that the dNTs of adenine exhibit the most dramatic decreases in the migration time, compared to slow decreases in migration time for the dNTs of guanine, uridine, or
cytosine. These trends can be explained by eq. 2.5 using the $\mu_{ep,A}$, $\mu_{ep,AC}$, and K values.

It was observed that the data points were increasingly nonlinear, using eq 2.6, for weakly binding analytes (K < 10). The nonlinearity is a result of the limited range of the fraction of analyte in the complex form. Ideally, the fraction of analyte complexed with an additive ($f_{AC}$) should range from 0.2 to 0.8 in order to minimize the error during the calculation process. To meet this requirement, the concentration of $\beta$-CD should change from 20 to 80 mM, when the K value is $10 \text{M}^{-1}$. Unfortunately, the limited solubility of $\beta$-CD in aqueous
solution restricts the upper limit of the concentration to 20 mM, so a concentration range varying from 7.5 mM to 20 mM β-CD was used for the calculation. In cases where it is impossible to obtain the parameters directly from a linear regression, reasonable estimates for K and $\mu_{ep,AC}$ were used. Comparison of the values of K and $\mu_{ep,AC}$ obtained without viscosity correction to the measured mobilities shows not only a significant underestimate of the K values (varying up to 60% for weakly binding analytes), but also some unrealistic values for the complex mobilities (which are either seriously underestimated, or result in positive mobilities for weakly binding analytes, see Table 2.1).

Table 2.1 shows the values of $\mu_{ep,A}$, $\mu_{ep,AC}$ and K for each analyte. Certain trends can be seen in the magnitudes of the binding constants and complex mobilities which can help explain the observed migration behavior of the analytes in the electropherograms of Figure 2.2. To compare the effect of complexation on analytes with different values of $\mu_{ep,A}$, the difference in mobility ($\mu_{ep,AC} - $ $\mu_{ep,A}$) or the relative change in mobility ($\mu_{ep,AC} - $ $\mu_{ep,A}$)/$\mu_{ep,A}$ can be used. The analytes differ in the structure of the base and the degree of phosphorylation. The base on the nucleotide significantly influences the magnitude of the binding constant. Nucleotides with adenine bases show the largest affinity for β-CD (dAMP has the largest K, 63.8 M$^{-1}$). The dNTs of guanine and uridine have similar, yet lower affinities towards β-CD, while the dNTs of cytosine have extremely weak interactions. These calculated K values are consistent with the measurements of the binding constants of ribonucleotides with β-CD made by Formoso using circular dichroism and, Hoffman and Bock using UV absorption spectroscopy. Steric effects and the functional groups on the bases can be used to rationalize the observed trend. Molecular models show that the pyrimidine bases of U and C are too small for significant interactions to occur within the
### Table 2.1

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**Notes:**
- \( V_{4 \text{arb}}^{V_{4 \text{arb}}} \) is the fundamental parameter influencing the moduli of an admixture in CE.
apolar cyclodextrin cavity. Adenine was found to have a greater affinity towards β-CD than the larger guanine because insertion of guanine leads to distortion of the cyclodextrin cavity. The acidity of the guanine base can also explain the decreased binding affinity towards cyclodextrin compared to the neutral adenine base. However, it is not clear why U has a greater affinity than C.

The magnitude of the interaction between the dNTs and β-CD is also dependent on the degree of phosphorylation, with monophosphorylated dNTs exhibiting the largest affinity. The association constant is observed to be drastically reduced going from mono- to diphosphates (up to a 50% reduction in K values), while the difference between di- to triphosphates is less substantial. Increasing the phosphorylation of each nucleotide increases the charge and the solvation sphere, resulting in a weaker interaction with the apolar cavity of β-CD. Consequently, the magnitude of K is observed to decrease dramatically from mono- to diphosphates. The much less significant decrease in K between the di- and triphosphates can be explained by comparing the relative changes in the free mobilities. Despite an overall increase in the charge of the analyte from di- to triphosphates, the relative increase in mobility is significantly lower than from mono- to diphosphates. For example, for the nucleotide series dGMP, dGDP and dGTP, the μ_{ep,A} are: -0.2359, -0.2748, and -0.2793 × 10^{-3} cm²V⁻¹s⁻¹, while the K values are 18.2, 11.9, and 10.1, respectively. This suggests that the di- and triphosphates have comparable charge to size ratios. This trend is supported by work done by Uhrová et al. which demonstrated that the ionization of nucleoside triphosphates is incomplete due to the weaker acidity of the terminal OH groups of polyphosphates.

The net mobility of an analyte is also dependent on the magnitude of μ_{ep,AC}. Although the differences in the K values have generally been considered as the most dominant factor in separations, a comparison of the K values for dGMP and dUMP demonstrate that even when
the K values are similar, separation can still be achieved if the magnitudes of the complex mobilities ($\mu_{\text{ep,AC}}$) or the relative changes in mobilities ($\mu_{\text{ep,AC}} - \mu_{\text{ep,A}}$) are different. This property demonstrates the fundamental difference between CE and chromatography. However, it is important to note that there is no direct relationship between the K values and the complex mobilities. The affinity of the analyte for this additive is determined by both steric factors and the strength of the hydrophobic interactions. The value of $\mu_{\text{ep,AC}}$ is determined by the overall shape of the complex, assuming negligible changes in the charge of the analyte upon complexation. For instance, although dAMP and dGMP have drastically different values for K, their complex mobilities are quite similar. In contrast, dGMP and dUMP, which have similar binding affinities to $\beta$-CD, possess significantly different values for $\mu_{\text{ep,AC}}$. Another way to investigate the nature of the interaction between an analyte and an additive, as suggested by Goodall and co-workers,\textsuperscript{22} is to use the ratio of $\mu_{\text{ep,AC}} / \mu_{\text{ep,A}}$ as a measure of the relative change in hydrodynamic radius (Stoke's radius). Again, a similar trend is observed with respect to the type of nucleotide base and the degree of phosphorylation.

Table 2.1 also shows the values of the complex mobility estimated by:

$$\mu_{\text{ep,AC}} \approx \left( \frac{M_A}{M_{AC}} \right)^{2/3} \mu_{\text{ep,A}}$$

(2.7)

where $M_A$ and $M_{AC}$ is the molecular weight of the analyte and additive respectively, and $M^{2/3}$ is a measure of the surface area of the molecule.\textsuperscript{29,30} This type of estimation of the complex mobility neglects the important effects of solvation in aqueous solutions. A comparison of the $\mu_{\text{ep,AC}}$ values calculated by eq. 2.6 with the experimentally determined values of $\mu_{\text{ep,AC}}$ clearly demonstrates that by neglecting solvation effects on the overall size of the analyte-additive complex, the complex mobility is significantly underestimated, resulting in an overestimated $\mu_{\text{ep,A}} / \mu_{\text{ep,AC}}$. Moreover, estimation of complex mobilities by molecular
Figure 2.3 3-D computer molecular models of the lowest energy configurations of the inclusion complexes: dAMP:β-CD and dCMP:β-CD

weights cannot take specific steric effects into account. Therefore, the complex mobilities must be determined experimentally until a more rigorous theory is available.

2.4.2 Computer Modeling of the Analyte-Additive Complexes

Computer modeling of inclusion complexes may indicate the most probable binding orientation upon complexation, and can be used to estimate the change in free energy. The complex formation of dAMP and dCMP with β-CD were modeled based on noncovalent
interactions while neglecting the effects of charge interactions and solvation. dAMP and dCMP were best suited for modeling because their bases are electrically neutral and were observed to exhibit widely different affinities. A comparison of the complexation of dAMP and dCMP with β-CD revealed that the most stable configuration for dAMP is when the adenine base is located within the apolar cavity of β-CD, while the cytosine base of dCMP is most stable when located outside the cavity, along the rim of the cyclodextrin (see Figure 2.3). This observation supports previous arguments regarding ribonucleotide affinities for cyclodextrin given by Hoffman and Bock. Since the relative size and hydrophobicity of the base are the most important factors determining the strength of binding, clearly dAMP demonstrates greater affinity for β-CD than dCMP, which is also confirmed experimentally.

2.4.3 Prediction of Analyte Migration Behaviour

Incorporation of the three fundamental parameters into eq. 2.5 should be able to describe the nonlinear change of analyte mobility at various additive concentrations. Figure 2.4 depicts the predicted ideal state electrophoretic mobilities of the 12 dNTs (solid lines) as a function of β-CD concentration, by using the values of $\mu_{ep,A}$, $\mu_{ep,AC}$, and K from Table 2.1. There is an excellent correlation between the calculated values and the experimentally measured mobilities with a relative error of less than 1%. The mobility of dAMP (K = 63.8, $\mu_{ep,AC} = -0.1227 \times 10^{-3}$ cm²/Vs) drastically changes with cyclodextrin concentration. In contrast, the mobilities of weakly interacting analytes, such as dCMP, dCDP, and dCTP, change minimally with increasing concentration of additive. This type of plot gives an overview of all possible mobilities for each analyte, showing the additive concentration(s) where the overlap of the analyte mobilities is the least. For example, dGMP-dCMP and
Figure 2.4 Simulated ideal electrophoretic mobilities (solid lines) of 12 dNTs as a function of [β-CD] based on the three fundamental parameters using eq. 2.5. The markers represent the average measured mobilities at 5, 7.5, 10, 12.5, 15 and 20 mM β-CD. The nucleotides are numbered the same as in Fig. 2.1.

dATP-dGTP are overlapping peaks without additive, but they are separated at higher concentrations of β-CD due to the differential interaction with the neutral additive.

Moreover, the actual order of migration may change substantially, with peaks shifting at different rates and eventually overlapping and passing analytes that were originally eluted earlier. It is important to simultaneously model the migration behavior of all the analytes to choose the optimum separation conditions.

In order to translate the ideal state mobilities into migration times, the relative viscosity of the buffer solution and its electroosmotic mobility have to be considered. With the mobility values shown in Figure 2.4, the viscosity correction, and the measured EOF, the
Figure 2.5 Simulated apparent migration times (solid lines) of 12 dNTs as a function of [β-CD] based on the three fundamental parameters, the viscosity correction factor and the electroosmotic mobility. The markers represent the average migration times at 5, 7.5, 10, 12.5, 15 and 20 mM β-CD. The nucleotides are numbered the same as in Fig. 2.1.

Migration times of all 12 dNTs can be plotted as a function of the β-CD concentration, as shown in Figure 2.5. Again, there is excellent correlation between simulated migration times (solid curves) and measured average migration times, with a relative error of less than 1.5%. The plots of the simulated migration time can be used to predict the concentration of β-CD (14 mM in this case) required to achieve optimum separation.

The goal of this work was to verify that the plots in Figures 2.4 and 2.5 can be used to accurately predict the analyte migration behavior at any usable concentration of β-CD under a variety of conditions. The calculated and experimental mobilities and migration times of all 12 dNTs were compared at β-CD concentrations of 2.5 mM, 6 mM, 14 mM (separation
optimization), and 17.5 mM. These concentrations were chosen since they were not used originally in the model and encompass a wide range of β-CD concentrations. Excellent agreement between predicted and observed mobilities and migration times is shown (Table 2.2) by the linearity of the correlation plots and the low relative error. The migration time predictions have a slightly larger error than the mobility predictions. This is reasonable because the EOF often varies from run to run. Figure 2.6(A) shows the separation optimization of all 12 dNTs at the predicted optimum condition (14 mM β-CD). The predicted migration times (marked by × in Figure 2.6) of all 12 dNTs agree well with the obtained electropherogram.

2.4.4 Predictions Using a Different Capillary and a Different Batch of Buffer

Because the values of $\mu_{ep,A}$, $\mu_{ep,AC}$, and K are intrinsic properties of the analyte molecules and their environment, they should remain constant in a different capillary or in a new batch of buffer. A new batch of borate buffer with 6 mM and 14 mM β-CD were used to verify the calculated parameters. It was observed that the electroosmotic mobilities were 1 to 2% lower, and the currents were 0.8 to 1.0 µA lower than in the original buffer and capillary system. Despite these differences, the overall appearances of the electropherograms were similar. There is excellent correlation between the theoretical and the observed mobilities (Table 2.2). The relative error between the measured and the predicted mobilities is under 1%. In order to predict the migration times of the 12 dNTs, the average measured electroosmotic mobilities of the new solutions were used. Again, excellent correlation was observed between predicted and measured migration times, with a relative error ranging from 0.2 to 2.0%. Therefore, this dynamic complexation model is able to accurately predict the mobilities and migration times of analytes, even when the buffer and capillary are changed.
2.4.5 Predictions Using Different Capillary Lengths

Capillary lengths of 47 cm and 37 cm were used with the new buffer solutions to determine whether the model can still accurately describe the migration behaviour of analytes. Voltages of 6.85 kV and 8.70 kV were applied to 37 cm and 47 cm long capillaries so that the electric field is the same as in the other experiments. An advantage of using longer capillary lengths is that analytes spend a longer time in the electric field, resulting in an increased difference in mobilities, hence greater resolution. Correlation plots of electrophoretic mobilities and migration times for 6 mM and 14 mM β-CD solutions for both the 37 cm and 47 cm capillaries revealed a high degree of correlation between observed and predicted values (see Table 2.2). Figures 2.6B and 2.6C show the electropherograms of the 12 dNTs at 14 mM β-CD using the 37 cm and the 47 cm capillaries. The predicted migration times of the dNTs (×) agree well with those measured from the electropherograms (a relative error of less than 1.5%). Consequently, the dynamic complexation theory can be used to simulate optimal separation conditions at any concentration of additive and capillary length.

2.5 Conclusion

Analyte migration behaviour can be quantitatively described using the dynamic complexation model. The model was tested using different batches of buffer, alternate capillaries, and various capillary lengths. It was concluded that this model can predict the electrophoretic mobility and migration time of an analyte when the temperature and pH are well controlled. The three parameters: $\mu_{ep, A}$, $\mu_{ep, AC}$, and $K$, in conjunction with viscosity correction and $\mu_{eo}$, can be used to systematically design a separation. Optimum separation conditions can be determined by simulating the migration time (or electrophoretic mobility)
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<th>Constraint</th>
<th>Correlation Plots - Predicted Vs. Observed</th>
<th>N</th>
<th>Rel. Error</th>
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<tr>
<td></td>
<td>A. Mobility (cm²V⁻¹s⁻¹)</td>
<td>B. Migration Time (min)</td>
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<tr>
<td>1. Same Capillary (27cm) / Same Buffer</td>
<td>$y = 0.9968x + 0.0002$</td>
<td>$y = 0.9854x + 0.1411$</td>
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<td>$R^2 = 0.9995$</td>
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<td>$y = 0.9979x - 0.0001$</td>
<td>$y = 0.9895x + 0.074$</td>
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<td>$R^2 = 0.9987$</td>
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<td>2. New Capillary (27cm) / New Buffer</td>
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<td>$y = 1.0017x - 0.0252$</td>
<td>72</td>
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<td>3. Capillary Length 37cm</td>
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<td>$y = 1.0089x - 0.1586$</td>
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<td>$R^2 = 0.9992$</td>
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Figure 2.6 Comparison between the observed migration times in the electropherograms and the predicted migration times (x), showing separation optimization of 12 dNTs using 14 mM β-CD using a (A) 27 cm, (B) 37 cm and (C) 47 cm capillary.
as a function of additive concentration, and selecting a concentration that produces the least overlap of migration time or mobility values. This understanding leads to more efficient method development through the selection of additives based on their affinity with the analytes, the mobility of the complexes, and the optimum concentration(s) of the additive. Electrophoretic parameters and equilibrium constants, combined with molecular modeling, can assist in the design of CE separation systems.

2.6 References

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Chapter III:

Accurately Describing Weak Analyte-Additive Interactions in Capillary Electrophoresis.

"Refinement of theoretical model to reflect experimental observations."
III: Accurately Describing Weak Analyte-Additive Interactions in Capillary Electrophoresis

3.1 Introduction

Cyclodextrins (CDs) and their derivatives are one of the most commonly used additives for chiral separations in CE. CDs are toroidally-shaped oligosaccharides that possess a relatively apolar hydrophobic interior cavity and a hydrophilic exterior. One of the inherent advantages of CDs is their ability to form stable inclusion complexes with a wide variety of small molecules. In addition, their solubility and inclusion properties can be easily modified through derivatization with a wide variety of substituents, ranging from neutral to ionic functionalities. Since Fanali employed CDs as chiral selectors in free-solution CE in 1989, there have been numerous applications of enantiomeric separations in CE utilizing both native and derivatized CDs. However, much less attention has been directed towards applying CDs as an additive to induce the separation of an achiral mixture. The separation of geometric isomers of substituted aromatics, nucleotide diastereomers and structural isomers of pharmaceutical drugs and their impurities demonstrate that CDs can also be used most effectively as a general approach towards achiral separations.

Several research groups have developed theoretical models for specific additive types in CE to describe chiral, immuno-affinity, ligand-affinity, and micellar based separations. Our group has amalgamated the existing theories and proposed to use a general theory based on dynamic complexation between analytes and additives to describe the migration behavior of all types of open tubular CE, as is described in chapter II of this thesis. In CE separations that utilize additives in the background electrolyte, the mobility of an analyte is determined by three parameters: the electrophoretic mobilities of the free and complexed analyte and the equilibrium constant of the analyte-additive interaction. The
predictive capabilities of the dynamic complexation model (chapter II of this thesis) has been tested to verify the accuracy of the theory to describe the migration behaviour of analytes which interact with an additive.\textsuperscript{21} To date, the majority of theories have modeled analytes which exhibit very strong to intermediate interactions with a specific additive(s) of interest. However, the limits to which the theory can quantitatively model extremely weak analyte-additive interactions in CE has not been fully examined. Moreover, current theoretical models in CE assume that the charge to size ratio of the free analyte remains unchanged with additive addition. This assumption may not hold when working with charged (micelles, CDs) or high concentrations of neutral additives which alter the electric environment of the the analyte. In order to derive accurate values for binding constants and mobilities, it is necessary to correct for all changes in solute mobility that are not due to specific binding.

The focus of this paper is three-fold: 1) to assess the ability of the dynamic complexation model to quantitatively describe weak analyte-additive interactions in CE, 2) to estimate and correct for changes in solute mobility not due to direct analyte-additive binding, and 3) to examine the usefulness of exploiting differential weak interactions of an achiral mixture of deoxyribonucleotides using hydroxypropyl \(\beta\)-CD (HP-\(\beta\)-CD) as an additive for separation optimization. HP-\(\beta\)-CD is used as an additive in this study because of its greater aqueous solubility in comparison to the native \(\beta\)-CD, which is used in chapter II of the thesis. This permits the study of analyte-additive interactions throughout a much larger additive concentration range required for accurate measurement of the binding isotherm.
3.2 Theory

3.2.1 The Study of Analyte-Additive Binding Interactions

The electrophoretic mobility of an analyte exhibiting a 1:1 interaction with an additive can be described by the following equation, which is developed in chapter II of the thesis:

\[
\mu_{ep,T} = \frac{1}{1 + K[C]} \mu_{ep,A} + \frac{K[C]}{1 + K[C]} \mu_{ep,AC}
\]  

(3.1)

where \( K \) is the equilibrium constant of the analyte-additive interaction, \([C]\) is the additive concentration, \( \mu_{ep,T} \) is the apparent electrophoretic mobility of the analyte, \( \mu_{ep,A} \), and \( \mu_{ep,AC} \) are the ideal state electrophoretic mobilities of the free analyte, and the analyte-additive complex, respectively. The ideal state refers to situations where varying additive concentration does not affect the physicochemical properties of the background electrolyte, which is normally referenced to zero additive concentration. Before this equation can be used to extrapolate accurate values for binding constants and mobilities, several conditions have to be satisfied. Two general factors required to model molecular interactions, regardless of the specific instrumental technique (NMR, UV-vis spectroscopy, etc.), are: 1) an appropriate concentration range of additive is selected (where, \([C] >> [A]\)) to obtain significant proportions of both free and complexed analyte so that an accurate representation of the binding isotherm is examined, and 2) the stoichiometry of the analyte-additive interaction is verified, especially when using new analyte-additive combinations or high concentrations of additive. In addition, there are three factors specific to CE (variables that influence analyte mobility other than selective molecular interaction) that require attention when modeling analyte-additive interactions, 3) the influence of viscosity on the apparent mobility of an analyte due to addition of a viscous additive; the need for a relative viscosity correction factor, 6,13 4) the effect of temperature on measured mobilities; the use of thermostated instruments that are operated under low voltages to minimize Joule heating and
to ensure that the capillary is equivalent to the coolant temperature, and 5) the assessment of possible changes in the dielectric properties of the buffer medium with additive addition that may influence analyte mobility. Thus, the apparent mobility of an analyte is a function of temperature, viscosity and dielectric properties of the background electrolyte when using additives in CE. These factors need to be taken into account when modeling analyte-additive interactions by CE.

3.2.2 Electrophoretic Mobility and the Electric Double Layer: The Effect of Solution Composition

Currently, theoretical models have corrected for changes in bulk viscosity while operating at constant temperatures, while assuming negligible changes in the dielectric properties of the buffer and its influence on the mobility of an analyte, when using increasing amounts of additive. The most simple theoretical approach to describe the electrophoretic mobility of a spherically charged particle as derived in chapter I of the thesis:

$$\mu_{\text{ep, A}} = \frac{Q_{\text{eff}}}{6\pi \eta R}$$  \hspace{1cm} (3.2)

where, $Q_{\text{eff}}$ is the effective charge of the molecule, $\eta$ is the bulk viscosity of the medium and $R$ is the effective hydrodynamic radius of the molecule. Eq. 3.2 neglects the influence of both electrophoretic retardation and relaxation contributed by the counter-ions that form the electric double layer around the charged particle. In the limit where ionic atmospheric effects are negligible (for small molecules), eq. 3.2 may be used to describe electrophoresis. A more accurate description of the electrophoretic mobility of a spherically charged analyte, which includes the influence of the double layer, can be described by the Hückel-Onsager equation:
\[ \mu_{\text{ep.A}} = \frac{\varepsilon_r \cdot \zeta}{6\pi\eta} \]  

(3.3)

where, \( \varepsilon_r \) is the relative permittivity of the solution, \( \zeta \) is the zeta potential (electrokinetic potential at the slipping plane of the double layer) and \( \eta \) is the viscosity of the solution. Eq. 3.3 is based on the Stern-Gouy-Chapman model of the undistorted electrical double layer surrounding a spherically charged particle in which the net mobility is determined by both the electrophoretic mobility and retardation (electrophoretic relaxation is not taken into account). In fact, eq. 3.3 is very similar to that derived by von Smoluchowski to describe the electroosmotic mobility (see eq. 1.6). It is interesting to note that in eq. 3.3, neither the size nor the charge of the particle is shown explicitly if the mobility is expressed as a function of the zeta potential.

The electrokinetic charge of a spherical particle, \( Q \) is related to its surface potential, \( \psi_0 \) by the following:

\[ Q = 4\pi\varepsilon_0\varepsilon a(1 + \kappa a)\psi_0 \]  

(3.4)

where,

\[ \kappa = (\frac{2e^2}{\varepsilon_0\varepsilon kT})^{1/2} \]  

(3.5)

where \( Q \) is the electrokinetic charge, \( \varepsilon_0 \) and \( \varepsilon_r \) are the permittivity of the vacuum and the relative permittivity of the solution respectively, \( a \) is the radius of the spherical particle, \( 1/\kappa \) is the Debye length (thickness of the double layer), \( e \) is the electric charge, \( k \) is the Boltzmann constant and \( I \) is the ionic strength. Eq. 3.4 is only valid for relatively low surface potentials. Under these constraints, the magnitude of the charge of a particle is directly related to its surface potential and the composition of the solution (reflected by the electrical properties of the solution; \( I \) and \( \varepsilon_r \)).
For low surface potentials, the Gouy-Chapman potential distribution (outside the immobilized Stern layer) consists of an exponentially decaying potential ($\psi$) given by:

\[
\psi = \psi_0 (a/r) \exp[-\kappa(r-a)]
\]

\hspace{1cm} (3.6)

where $\psi_0$ is the surface potential (Stern potential), and $r$ is the distance to the center of the particle. The net potential of a charged analyte in eq. 3.6 is similarly dependent on the properties of the solution (i.e., $\kappa$) as is the magnitude of its electrokinetic charge described in eq. 3.4. The zeta potential, $\zeta$ is defined as the potential difference between the slipping plane of the diffuse layer and the bulk solution (see chapter 1.5). One of the greatest impediments towards quantitative understanding of electrophoresis is that $\zeta$ cannot be measured in a reliable way,\textsuperscript{27} that is fully independent of electrophoresis and other electrokinetic phenomena (electroosmosis and streaming potential). Currently, one must be satisfied in testing the theory of electrophoresis in a semi-quantitative way.

If it is assumed that $\zeta$ is equally sensitive to changes in $\kappa$ as $\psi$ in eq. 3.6, then the magnitude of the zeta potential and the electrokinetic charge of the analyte (eq. 3.4) are both dependent on changes in the composition of the solution (i.e., $I$ and $\varepsilon_r$). Alterations in $\zeta$ or $Q$ result in changes in the electrophoretic mobility of an analyte (eq. 3.2 or 3.3). This type of correction becomes especially significant when one is using charged micelles, cyclodextrins or other types of additives which perturb the electrical environment of the analyte by changing the ionic strength and relative permittivity of the solution. Armstrong and Rundlett\textsuperscript{28} indicated that charged additives may influence analyte electrophoretic mobilities through changes in their electric double layer and that correction methods to reference mobilities to zero additive concentration are required to take it into account. A refinement of the model to include all variables which influence observed mobilities is vital for accurate determination of binding constants and mobilities. Eq. 3.1 can be written as:
\[ \nu \delta \mu^A_{ep,T} = \frac{1}{1 + K[C]} \mu_{ep,A} + \frac{K[C]}{1 + K[C]} \mu_{ep,AC} \]  

(3.7)

where \( \nu \) is the relative viscosity correction factor, \( \delta \) is the relative dielectric correction factor (that corrects for possible changes in \( \varepsilon_r \) and \( I \) of the solution) and \( \mu_{ep,T}^A \) is the apparent measured mobility referenced to a specific temperature. The ideal state mobility, \( \nu \delta \mu_{ep,T}^A \) is normalized to zero additive concentration and is used to negate any changes in solute mobility that are not due to specific binding. The complex mobility and equilibrium constant for an analyte-additive pair can then be solved by rearranging eq. 3.7 into various linear plotting methods (eq. 2.6) or by non-linear regression. Quantitative understanding of the fundamental parameters that influence separation in CE is vital for the continuing development of novel and robust methodologies employing additives.

### 3.3 Experimental

#### 3.3.1 Apparatus

All experiments were performed on a Beckman P/ACE 5500 automated CE system using System Gold software (Beckman) with a 386 PC computer. Fused silica capillaries (Polymicro Technologies, Phoenix, AZ) with inner diameters of 50 \( \mu \)m, outer diameters of 375 \( \mu \)m, and a length of 37 cm was used. New capillaries were rinsed first with 0.1M NaOH for 3 minutes, rinsed with deionized water for 5 minutes and then washed with a 160 mM borate buffer, pH 9.0, for 10 minutes and equilibrated overnight before being used. The sample was introduced using a pressure injection for 2 seconds. All separations were carried out at 25 °C, and the UV absorption was monitored at 254 nm. A voltage of 8 kV was used throughout the experiments, maintaining an electric field of 216 V/cm. Experiments were repeated three times in order to derive accurate data. Relative viscosity measurements were
performed by a 2 second injection of a para-nitrophenol plug onto the capillary inlet, UV
detection at 254 nm using a 77 cm capillary, and measuring the time needed to push the plug
to the detector under a constant pressure of 20 psi. Triplicate measurements of the relative
viscosity were made for each concentration of HP-β-CD. Capacitance measurements were
performed on HP-β-CD solutions dissolved in de-ionized water using a 4260A Universal
Bridge (Hewlett-Packard, USA). A parallel plate capacitor was home-built using stainless
steel plates separated 0.1 cm apart by a Teflon holder. A calibration plot of measured versus
tabulated values (CRC Handbook of Chemistry and Physics, 68th Edition) of the dielectric
constant for various organic solvents was constructed and used to correct for measured
dielectric constants for the HP-β-CD solutions.

3.3.2 Chemicals and Procedure

Borax (Na₂B₄O₇·10H₂O) and the 2′-deoxyribonucleotides (dNTs): deoxyadenosine mono-
di-, and triphosphate (dAMP, dADP, dATP), deoxyguanosine mono-, di-, and triphosphate
(dGMP, dGDP, dGTP), deoxycytosine mono-, di-, and triphosphate (dCMP, dCDP and
dCTP) and deoxythymidine mono-, di-, and triphosphate (dTMP, dTDP and dTTP), were all
purchased from Sigma (St. Louis, MO, USA). Hydroxypropyl β-CD (HP-β-CD), with an
average molar substitution of 0.6, was purchased from Aldrich (Milwaukee, WI, USA).
Both a positive and negative electrospray mass spectra of HP-β-CD revealed that it consisted
of a heterogeneous mixture of various degrees of substitutions, ranging from three to ten
hydroxypropyl substituents, with a peak maximum of seven substituents per cyclodextrin.
HPLC grade methanol was purchased from Fischer Scientific (Nepean, Ont., Canada). Stock
solutions of 160 mM borate were prepared in deionized water. A stock solution of 180 mM
HP-β-CD was prepared by dissolving the appropriate amount of HP-β-CD in borate buffer.
The appropriate volumes of stock solutions were mixed with buffer to make the 160 mM borate, pH 9.0 with concentrations of HPCD ranging from 5 to 180 mM. The pH of the various HP-β-CD solutions was determined to remain constant. A stock solution of the analytes was prepared by dissolving approximately equal concentrations (1 × 10⁻³ M) of each deoxyribonucleotide in deionized water and storing in a freezer at -20 °C. This stock solution was further diluted to a concentration of approximately 5 × 10⁻⁵ M before injection into the capillary. Dimethyl-sulfoxide (DMSO) (1.2% v/v) was used as the EOF marker in the sample. Peaks were identified by spiking the sample solution with standard solutions of each deoxyribonucleotide. Before experiments were started, an Ohm’s plot was performed with the 37 cm capillary with the run buffer in order to select a voltage at which Joule heating was minimal. This was necessary to ensure the heat generated during the separation was efficiently dissipated. A voltage of 8 kV was used, and this voltage is well within the Ohm’s plot (linear) region. Numerical analysis by either linear x-reciprocal plot or nonlinear regression was performed on all data from a spreadsheet program developed by Michael Bowser using a Microsoft Excel 5.0 on a Power Macintosh 7200/90 computer.

3.4 Results and Discussion

Figure 3.1 qualitatively shows the influence of HP-β-CD on the separation of the 12 dNTs. Two pairs overlap (dTMP/dGMP and dATP/dCDP) when no additive is used. As increasing amounts of HP-β-CD is added to the run buffer, selective modulation of analyte mobility is achieved, as determined by the relative strength of complexation between each dNT and HP-β-CD molecule. As the negatively charged nucleotide complexes with the
neutral host, its mobility is dramatically decreased due to the increased size and frictional resistance of the complex (see Figure 2.1). However, superimposed upon selective mobility changes due to analyte-additive complexation is the viscosity change occurring with increasing concentrations of the additive. This effect acts equally upon all analytes, resulting in significant decreases in all measured apparent mobilities. Similarly, the electroosmotic mobility is dramatically reduced, resulting in increased migration times for most analytes at high additive concentrations, despite electrophoretic mobility reduction due to the interaction
with the additive. The changes in mobility due to viscosity become dominant at high concentrations of additive since the interactions between nucleotide and HP-β-CD are relatively weak. By observing the rate at which the migration times of the nucleotides change with additive concentration, one can qualitatively assess the magnitude of the interaction with HP-β-CD. All nucleotides with adenine bases exhibit the most significant changes in migration times, followed by small changes in migration times involving the guanine bases. However, nucleotides containing cytosine and thymidine residues exhibit little or no significant interaction with the additive, as reflected by the dramatic increases in migration times due to viscosity. These observations are consistent with previous reports on nucleotide interaction with β-CD, including the studies presented in chapter II of this thesis.

3.4.1 Non-Ideality: Refinement to the Model

Initial experiments indicated that dNT:HP-β-CD interactions in this buffer system were relatively weak, with \( K < 15 \). Thus, in order to obtain accurate values for binding constants and complex mobilities, a large concentration range of HP-β-CD (0 to 180 mM) was needed to model significant portions of the binding isotherm. Previous study of nucleotide-cyclodextrin complexation demonstrated 1:1 binding stoichiometry. The assumption that the mobility of the analyte is only modulated by the viscosity of the bulk solution (apart from direct interaction with the additive), was observed to be inaccurate in this system. This observation was revealed mainly for two reasons: 1) all analytes in this study exhibited weak or negligible interactions with the additive, and 2) a large concentration range of an additive, HP-β-CD, was used for binding studies.
Figure 3.2  A non-linear binding isotherm for dGMP interacting with HP-β-CD over a concentration range of 0 to 180 mM, using (A) viscosity, (v) corrected apparent mobilities and (B) viscosity-dielectric, (vδ) corrected apparent mobilities.

The first evidence of non-ideality was revealed by both x-reciprocal and non-linear plots of the binding isotherm using the viscosity corrected mobilities. X-reciprocal plots have been shown previously to be sensitive to non-ideality, which can be used to detect deviations in the model chosen. X-reciprocal plots indicated non-ideality by increased scattering of data and non-linearity. Figure 3.2(A) depicts a non-linear plot for dGMP over a concentration range of 0 to 180 mM HP-β-CD. The binding plots for all analytes showed a similar negative sloping curve, indicative of non-ideality of the model at high concentrations of additive. Improvements in numerical modeling can be realized when appropriate
corrections are made to the data. The effect of incorporating a dielectric and viscosity correction factor to describe weakly interacting analyte-additive interactions in CE is highlighted for dGMP in Figure 3.2(B). The rationale for this approach is developed in the following discussion.

Further evidence of non-ideality occurring in this system is depicted in Figure 3.3. Non-linear regression analysis, using viscosity corrected apparent mobilities, resulted in two types of nucleotide binding schemes; those with weak/observable and those with negligible interactions with HP-β-CD, as reflected by the magnitudes of K and $\mu_{ep,AC}$. Figure 3.3A shows predicted ideal state electrophoretic mobilities (solid lines - obtained from calculated K and $\mu_{ep,AC}$ values) versus observed ideal state mobilities (shapes) for the weakly interacting nucleotides containing adenine and guanine bases. Significant deviation between predicted and observed mobilities (with a relative error greater than 7%) is apparent at concentrations greater than 60 mM HP-β-CD for most analytes. The measured mobility of dAMP, which exhibits the highest affinity for HP-β-CD, does not deviate from predicted values below 100 mM HP-β-CD. Figure 3.3B shows clear evidence of non-ideality exhibited by the deoxynucleotides containing cytosine and thymidine bases. Assuming negligible interaction with the additive, changes in the apparent electrophoretic mobility of the analyte should be caused mainly by changes in the viscosity of the medium. If this is the case, then viscosity corrected mobilities (ideal state mobilities) theoretically should remain unchanged with additive addition. However, the measured mobilities, corrected for viscosity changes, are observed to increase dramatically with increasing HP-β-CD concentration relative to the predicted mobilities (solid straight lines). The relative error between experimental and predicted mobility values is often greater than 17%. This mobility increase cannot be attributed to molecular association with HP-β-CD, since the mobility should decrease in
Figure 3.3 Simulated ideal state mobilities, corrected for v (solid lines) of interacting analytes, (A): dAMP, dGMP, dADP, dGDP, dATP and dGTP, and weakly or non-interacting analytes, (B): dCMP, dTMP, dCDP, dTDP, dTTP and dCTP, as a function of the concentration of HP-β-CD. The different shaped markers represent average measured mobilities at: 0, 5, 10, 20, 30, 40, 50, 60, 80, 100, 120, 140,160 and 180 mM HP-β-CD. Deoxynucleotides are numbered the same as in Fig. 3.1.
magnitude upon binding with the neutral additive. This implies that some other variable is operative. These observations indicate that viscosity correction alone over-corrects for changes in observed electrophoretic mobilities, which is most apparent for very weakly interacting analytes.

3.4.2 Changes in the Dielectric Properties of HP-β-CD Solutions

In order to explain this non-ideality, one must re-examine the assumptions that are made with the model system. It is assumed that the relative permittivity and zeta potential influencing the mobility of the analyte are constant (or that the charge:size ratio of the analyte remains unchanged with additive addition). However, this presumption has not been rigorously verified. Changes in the relative permittivity (dielectric) of the bulk buffer coincide with changes to the zeta potential in the double layer of the charged analyte, and influence the electrophoretic mobility of the analyte, as is described in the theory of this chapter. Although neutral additives may not alter the system as greatly as charged agents, significant changes may become important at high concentrations of additive. The substantial mobility increase shown by the non-interacting nucleotides indicate that a reduction in the effective size and/or increase in the effective charge (zeta potential) of the analytes occur with increasing amounts of HP-β-CD addition.

In order to determine whether changes in the dielectric properties of the solution occurs with additive addition, capacitance measurements were performed on various HP-β-CD solutions (in de-ionized water in order to prevent significant ion conduction). Figure 3.4 depicts measured dielectric constant for HP-β-CD solutions varying from 0 to 180 mM. The apparent dielectric constant is observed to increase with increasing additive concentration. This result suggests that the addition of HP-β-CD increases the dielectric constant of the
Figure 3.4 Experimentally measured dielectric constant obtained through capacitance measurements as a function of HP-β-CD concentration. Concentration of HP-β-CD are: 0, 5, 10, 20, 40, 80, 120, 150 and 180 mM.

background electrolyte. It is observed that a relatively small change in dielectric constant occurs with 5 mM and 10 mM HP-β-CD solutions, which dramatically increases with concentrations greater than 20 mM. Although absolute values for dielectric constant is not the focus of this investigation, Figure 3.4 gives a clear qualitative trend of the influence of the additive HP-β-CD on the polarizability of the medium. A comparison between Figure 3.3B and Figure 3.4 demonstrate that the deviations between measured and predicted mobilities are relatively small at concentrations of 5 and 10 mM HP-β-CD, but increase significantly with amounts of HP-β-CD greater than 20 mM, reflecting the trend in measured
dielectric constants. It is important to note that external measurements of viscosity and dielectric constants may not accurately reflect these parameters in the double layer under the high electric fields present in CE. This prevents a fully quantitative understanding of the physical parameters operative in CE.

The effect of changes in solution properties and their resultant influence on the electrophoretic mobility of an analyte is still not fully understood. The addition of a neutral organic additive (HP-β-CD) to the run buffer not only increases the viscosity of the solution, but may also substantially change the value of ζ for an analyte through changes in ε, relative to the buffer containing no additive. According to eqs. 3.2 to 3.6, an increase in the relative permittivity of the solution (under a constant ionic strength) results in the expansion of the double layer (double layer thickens indicated by an increase in 1/κ). This change in the double layer has many interdependent repercussions on the physicochemical properties of the analyte: 1) an increase in the ζ (eq. 3.6), 2) an increase in the electrokinetic charge, Q (eq. 3.4), and 3) an increase in the effective size of the hydrated analyte, R_{eff} (for small molecules, since the double layer is greater in extent than the analyte itself). The net effect of these changes on the electrophoretic mobility of the analyte, according to eq. 3.2, will depend on the relative magnitudes of the increases in Q (or ζ) and R_{eff}. No observable change in electrophoretic mobility can be measured if changes in both Q and R_{eff} are of the same magnitude. However, variation in measured electrophoretic mobilities may occur when both these two parameters do not mutually negate each other. An increase in the observed electrophoretic mobilities for all the analytes suggest that an increase in their electrokinetic charge or zeta potential occurs with HP-β-CD addition. Generally this type of indirect mobility change is obscured for strongly interacting analytes, but becomes increasingly important (and apparent) when using large concentrations of additive with extremely weak
binding analytes. The exact relationship between the large increases in relative permittivity measured for HP-β-CD aqueous solutions and their values in a conductive buffer under high electric fields in CE is not well understood. Nevertheless, capacitance measurements of HP-β-CD solutions support the hypothesis of the electrophoretic mobility increase that occurs due to the increase in the permittivity of the solution with HP-β-CD addition, highlighting the need to refine the current model for analyte-additive interactions in CE to include corrections for dielectric changes.

3.4.3 The Relative Dielectric Correction Factor, $\delta$

In order to accurately determine analyte-additive interactions in terms of $K$ and $\mu_{ep, AC}$, one must correct for any changes in solute mobility that are not due to specific binding with the additive. In addition to a relative viscosity correction factor, a relative correction factor for mobility changes due to differing dielectric properties of the background electrolyte must be considered. One way to empirically estimate this factor is by selecting an appropriate marker(s) which is similar in structure to the other analytes of interest and does not interact with the additive. Changes in the mobility of the marker(s) (other than viscosity) are due to changes in the dielectric of the solution and its influence on its zeta potential. The changes in marker(s) mobility ($\mu_{ep, A}$, free mobility) relative to the marker at zero concentration of additive serves as the correction factor. After correcting for viscosity changes, the apparent relative mobility changes of the marker becomes a measure of the relative dielectric correction factor, $\delta$. It is assumed that changes occurring with the marker(s) mobility reflect those of other analytes. Needless to say, proper selection and application of appropriate markers may be challenging.

The non-ideality observed in Figure 3.3B for the deoxynucleotides possessing cytosine
Table 3.1 *Correction factors required to accurately determine analyte-additive interactions*

<table>
<thead>
<tr>
<th>[HP-β-CD] (mM)</th>
<th>$\nu^*$</th>
<th>$\delta^*$</th>
<th>$\chi^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$1.000 \pm 0.001$</td>
<td>$1.000 \pm 0.001$</td>
<td>$1.000 \pm 0.001$</td>
</tr>
<tr>
<td>5</td>
<td>$1.031 \pm 0.003$</td>
<td>$0.991 \pm 0.001$</td>
<td>$1.021 \pm 0.003$</td>
</tr>
<tr>
<td>10</td>
<td>$1.050 \pm 0.003$</td>
<td>$0.993 \pm 0.001$</td>
<td>$1.043 \pm 0.003$</td>
</tr>
<tr>
<td>20</td>
<td>$1.107 \pm 0.003$</td>
<td>$0.979 \pm 0.001$</td>
<td>$1.084 \pm 0.003$</td>
</tr>
<tr>
<td>30</td>
<td>$1.162 \pm 0.002$</td>
<td>$0.974 \pm 0.002$</td>
<td>$1.132 \pm 0.003$</td>
</tr>
<tr>
<td>40</td>
<td>$1.224 \pm 0.002$</td>
<td>$0.971 \pm 0.003$</td>
<td>$1.189 \pm 0.004$</td>
</tr>
<tr>
<td>50</td>
<td>$1.290 \pm 0.003$</td>
<td>$0.969 \pm 0.004$</td>
<td>$1.250 \pm 0.006$</td>
</tr>
<tr>
<td>60</td>
<td>$1.358 \pm 0.003$</td>
<td>$0.958 \pm 0.004$</td>
<td>$1.301 \pm 0.006$</td>
</tr>
<tr>
<td>80</td>
<td>$1.517 \pm 0.003$</td>
<td>$0.956 \pm 0.006$</td>
<td>$1.451 \pm 0.009$</td>
</tr>
<tr>
<td>100</td>
<td>$1.703 \pm 0.003$</td>
<td>$0.923 \pm 0.006$</td>
<td>$1.572 \pm 0.009$</td>
</tr>
<tr>
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<td>$0.894 \pm 0.007$</td>
<td>$1.753 \pm 0.014$</td>
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<tr>
<td>140</td>
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<td>$0.877 \pm 0.008$</td>
<td>$1.937 \pm 0.014$</td>
</tr>
<tr>
<td>160</td>
<td>$2.547 \pm 0.003$</td>
<td>$0.851 \pm 0.009$</td>
<td>$2.168 \pm 0.023$</td>
</tr>
<tr>
<td>180</td>
<td>$2.941 \pm 0.007$</td>
<td>$0.831 \pm 0.012$</td>
<td>$2.443 \pm 0.036$</td>
</tr>
</tbody>
</table>

$^*_{Relative~viscosity~correction~factor,~\nu = \eta/\eta_0}$

$^*_{Relative~dielectric~correction~factor~for~non-interacting~analyte,~\delta = \mu/\mu_1}$

$^*_{Combined~correction~factor,~\chi = \nu \cdot \delta}$
Figure 3.5 Simulated ideal state mobilities, corrected for $v$ and $\delta$ (solid lines) of interacting analytes, (A): dAMP, dGMP, dADP, dGDP, dATP and dGTP, and weakly or non-interacting analytes, (B): dCMP, dTMP, dCDP, dTDP, dTTP and dCTP, as a function of the concentration of HPCD. Shapes represent average measured mobilities at: 0, 5, 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160 and 180 mM HP-\(\beta\)-CD. Deoxynucleotides are numbered the same as in Fig. 3.1.
and thymidine bases were considered to be used as potential markers for several reasons. They were observed to be non-interacting over the concentration range studied as revealed by their binding plots, in which the changes in mobility due to additive interaction were comparable to experimental error. This resulted in plots which were scattered and highly irregular. In addition, these deoxyribonucleotides serve as excellent markers for the other interacting nucleotides because of similarity in electronic and chemical structure. It was observed that the relative dielectric correction factor of: dCDP, dCTP, dTDP and dTTP, were very similar, as reflected by their changes in mobility in Figure 3.3B. Consequently, their values were used for δ. However, it was noticed that the mono-phosphates of C and T may interact slightly with HP-β-CD as indicated by the increasing resolution between their measured mobilities at higher concentrations of additive.

Table 3.1 shows the correction factors (ν, δ and χ, where χ = vδ) needed to normalize measured mobilities to the ideal situation of zero additive concentration, so that changes in observed mobilities are only due to interactions with the additive. When both the viscosity and dielectric correction factors are used in the binding isotherms, dramatic improvement in the shapes of both the x-reciprocal (increased linearity reflected by higher correlation coefficient) and non-linear plotting methods (correct negative sloping curve) were observed - see Figure 3.2B. Improved correlation using the new correction factor was also indicated by reduced errors in the calculated parameters K and $\mu_{\text{ep, AC}}$ - see Table 3.2. Moreover, the correlation between simulated ideal state mobility plots (solid lines) and observed mobilities, using the refined correction factor for the weakly interacting nucleotides (A and G bases) and non-interacting nucleotides (T and C), is greatly improved throughout the entire concentration range of HP-β-CD, as depicted in Figure 3.5 (in comparison to Figure 3.3). The relative error between experimental and predicted mobilities is less than 1.5%. Again,
Figure 3.6 A comparison between the apparent (A), viscosity (v) corrected (B), and viscosity-dielectric (vδ) corrected (C), electrophoretic mobility of dGMP as a function of HP-β-CD concentration.

There is evidence of an extremely weak interaction between dCMP and HP-β-CD, as revealed by its corrected ideal state mobility, which slowly decreases with increasing additive concentration (Figure 3.5B).

Figure 3.6 depicts electrophoretic mobility values for dGMP, highlighting the importance of incorporating a dielectric correction factor to accurately describe weak analyte-additive interactions. The ideal state mobility, plot (A) dramatically decreases with increasing additive concentration due to the combined effects of direct molecular interaction, viscosity increase and dielectric change of the buffer medium. The use of a relative viscosity
correction factor to the mobility of the analyte, plot (B) serves to negate changes in mobility due to viscosity alone. However, it is observed that at high concentrations of HP-β-CD, the mobility plot (B) of the analyte actually begins to increase above 80 mM HP-β-CD. The use of a dielectric correction factor, in conjunction with the relative viscosity correction, serves to remedy the aforementioned anomaly, plot (C). The mobility is now observed to decrease in a non-linear fashion throughout the concentration range studied. Significant deviation between mobility plots (B) and (C) is observed to occur at concentrations greater than 20 mM, similar to the trend observed for dielectric constant measurements in Figure 3.4. The weaker the interaction between analyte and additive, the more significant is the relative deviation between viscosity corrected and combined viscosity and dielectric corrected mobilities. Again, this type of non-ideality to the model only becomes apparent for weakly interacting analytes, using high concentrations of a viscous/charged additive. The net result is to model the ideal state mobility of an analyte which describes, as accurately as possible, analyte-additive interactions.

3.4.4 Binding Trends: K and $\mu_{ep, AC}$ Values

Table 3.2 lists the three fundamental parameters needed to quantitatively describe the mobility of an analyte. A comparison between K and $\mu_{ep, AC}$ values obtained through viscosity corrected and combined viscosity and mobility corrections are shown. In general, both the equilibrium constant and complex mobility for the weakly interacting analytes are overestimated when only viscosity changes are corrected. The relative error between K and $\mu_{ep, AC}$ values increases as the interaction between analyte and additive becomes weaker (ranging from 5 to 60%). The magnitude of the equilibrium constant is observed to be dependent upon the nature of the base and the degree of phosphorylation on the nucleotide.
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**Table 3.2** (continued) Determination of the fundamental parameters influencing the mobility
The equilibrium constant is observed to decrease with increasing phosphorylation for a given nucleotide base. The magnitude of nucleotide affinity towards HP-β-CD is also relatively selective, with adenine bases demonstrating the strongest interaction, followed by very weak interactions involving guanine bases and negligible interactions (unobtainable from CE experiment) for both cytosine and thymidine residues. The values of binding constants and complex mobilities reflect the qualitative changes in migration time observed for the deoxynucleotides in Figure 3.1. In addition, the relative trends in $K$ and $\mu_{ep,AC}$ values are consistent with previous data involving complexation studies with β-CD,$^{21}$ as well as data presented from chapter II of the thesis. However, one striking difference between dNT interaction between β-CD and HP-β-CD additives is the dramatic decrease in binding constants, with over a 50% reduction in affinity when using HP-β-CD. Although the derivatization of β-CD serves mainly to increase aqueous solubility, the effect on binding may be detrimental. Steric hindrance of the interior cavity and increased polarity of the cyclodextrin through the incorporation of hydroxypropyl residues, may reflect this observation.

3.4.5 Weak Interactions and Separation Optimization

A $K$ value of $\approx 2$ was determined to be the lower limit in quantifying analyte-additive interactions by CE in this system. dGTP was found to have the lowest quantifiable binding constant equal to 1.8. Molecular interactions lower than this value result in data that begin to approach experimental error. Although six of the twelve nucleotides exhibit negligible interaction with HP-β-CD, separation optimization of the mixture is still possible. Differences in $K$, $\mu_{ep,AC}$, and $\mu_{ep,A}$ are required to achieve separation. For analytes that
Figure 3.7 Separation optimization of weakly interacting deoxyribonucleotides using 15 mM HP-β-CD. Deoxynucleotides are numbered the same as Fig. 3.1.

Possess no measurable affinity for the additive, their free electrophoretic mobilities must differ. A concentration of 15 mM HP-β-CD was determined to be the minimal concentration of additive needed to fully resolve the deoxyribonucleotides (Figure 3.7). Partial resolution of dCTP and dTTP was observed since they both possess no affinity towards HP-β-CD and have very similar free electrophoretic mobilities. The use of a longer capillary or the addition of a second additive which has a greater affinity for these analytes may improve their resolution. Thus, achiral separation of even weakly interacting analytes can be easily optimized in CE through proper selection of one or more additives in the run buffer.
Figure 3.8 Correlation plots of predicted versus observed ideal state mobilities of dNTs using: (A) viscosity ($v$) and (B) viscosity-dielectric ($v\delta$) correction. Concentrations of HP-\(\beta\)-CD used for study were 15, 70, 110 and 170 mM, based on triplicate measurements.
3.4.6 Prediction of Analyte Electrophoretic Mobilities

In order to determine whether the incorporation of a dielectric correction factor improved the accuracy of the model, a comparison between viscosity corrected and combined viscosity and dielectric corrected mobilities, were measured for HP-β-CD concentrations of 15, 70, 110 and 170 mM. These concentrations were selected since they were not used originally in the model and represent a large concentration range of HP-β-CD. Figure 3.8 shows the two correlation plots of predicted (A is the viscosity corrected and B is the viscosity-dielectric corrected) versus observed ideal state electrophoretic mobilities. The equations of the line of Figure 3.8(A) and (B) were: $y = (0.766 \pm 0.041)x - (0.52 \pm 0.12), R^2 = 0.942$, and $y = (0.990 \pm 0.012)x - (0.030 \pm 0.033), R^2 = 0.996$, respectively. Clearly, a higher degree of correlation between predicted and measured mobilities is observed (reflected in the slope, y-intercept and correlation coefficient) when one corrects for both viscosity and dielectric changes. In addition, it was observed that the relative percent error between predicted and measured viscosity corrected mobilities increases with additive concentration (i.e., 15 mM versus 170 mM HP-β-CD). This observation is consistent with the measured dielectric constant changes as a function of HP-β-CD concentration and the apparent non-ideality noticed in non-linear binding and mobility plots. Thus, the use of a combined viscosity-dielectric correction factor improves the accuracy of the calculated parameters of $K$ and $\mu_{ep,AC}$, which is reflected by a higher degree of correlation with experimentally measured mobilities at any given concentration of HP-β-CD.
3.5 Conclusion

Quantitative modeling of analyte-additive interactions by CE must take into account all factors that affect the solute mobility, in addition to specific molecular association. When operating under isothermal conditions, both the viscosity and dielectric effects of additive addition to the buffer solution have to be considered. The modeling of extremely weak interactions between deoxyribonucleotides and HP-β-CD in this study revealed apparent non-idealities at high additive concentrations, when only a viscosity correction was made. Capacitance measurements of HP-β-CD solutions supported the hypothesis that changes in the dielectric environment of the solution (increase in the dielectric constant of the medium) was a probable source for this non-ideality. A relative dielectric correction factor, using non-interacting marker analytes in the system, was needed to normalize all analyte mobilities to the reference state of zero concentration of additive. Significant improvements in binding plots (non-linear and x-reciprocal), reduced error in the calculated parameters of K and $\mu_{ep, AC}$, and a higher degree of correlation between predicted and measured mobilities throughout the entire concentration range of HP-β-CD, resulted from the inclusion of a dielectric correction factor to the model. Thus, the incorporation of both a relative dielectric and viscosity correction factor to the measured apparent mobilities of analytes in CE greatly ameliorated the accuracy of the dynamic complexation model. Failure to utilize a dielectric correction factor in the model resulted in an overestimation of K and $\mu_{ep, AC}$ values. Although this type of refinement was applied with a neutral additive in association with weakly interacting analytes, correction in the model due to changes in buffer composition is suspected to be even more significant with charged micelles, ionic cyclodextrins and ion
pairing additives. Future endeavours are aimed at testing this model to other types of systems involving charged additives and analytes.

3.6 References


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(8) Penn, S. G.; Goodall, D. M. *J. Chromatogr. B* 1993, 636, 149-152.


Section B:

The Design and Application of New Additives to Enhance Separation Selectivity in CE.
Chapter IV:

A Water-Soluble Tetraethylsulphonate Derivative of 2-Methylresorcinarene (TESMR) as a Additive for CE.

"Improving separation selectivity in CE with new types of additives: Separation tuning and design."
IV: A Water-Soluble Tetraethylsulphonate Derivative of 2-Methylresorcinarene as an Additive for CE.

4.1 Introduction

Resorcinarenes\textsuperscript{1,2} are cyclic tetramers of resorcinol having a bowl-shaped polyhydroxy aromatic cavity. They are a class of synthetic macrocyclic molecules which have been the subject of research for many years.\textsuperscript{3} Various names, such as calix[4]resorcinarenes, resorcinol-derived calix[4]arenes, Högborg compounds, and octols, have been used in the literature, but the name resorcinarenes seems to be the choice in many recent studies.\textsuperscript{2} These synthetic macrocycles have been used to provide insight into the molecular recognition processes of biology through host-guest complexation. Host-guest inclusion complexes refer to molecular interactions that involve binding of "guest" molecules within the cavity, or binding site, of the "host". Since nature abounds in complex supramolecular organization and non-covalent binding, resorcinarenes represent a relatively simple, well-defined, synthetic framework to begin understanding these vital life processes.

Similar to cyclodextrins (CDs), resorcinarenes are capable of forming stable inclusion complexes with a wide variety of small molecules.\textsuperscript{4,6} However, due to the smaller size and the presence of electron-rich aromatic groups in the cavity, resorcinarenes can be expected to exhibit different selectivities than the sugar-based CD additives. Most of the types of resorcinarenes synthesized thus far are relatively hydrophobic and their complexation have been studied mainly in apolar organic solvents. Recently, Kobayashi et al.\textsuperscript{7} synthesized a highly water soluble tetraethylsulfonate derivative of 2-methyl resorcinarene (TESMR). The ability of TESMR to form complexes with a variety of alcohols, saccharides, nucleosides,
Figure 4.1 Structures of the tetraethylsulfonate derivative of 2-methylresorcinarene (TESMR).
nucleotides and amino acids in aqueous solution, was studied. The structure of TESMR is depicted in Figure 4.1.

TESMR consists of four methyl-resorcinol units covalently bound together via four ethylsulfonate linker groups. TESMR forms a unique bowl-shape cavity (all cis-configuration) in aqueous solution due to intermolecular H-bonding that is formed between adjacent hydroxy groups on each resorcinol unit. This structure is thermodynamically stable, yet it is also relatively dynamic and conformationally mobile due to the nature of the H-bonding network. TESMR generally consists of an apolar, aromatic cavity, that is surrounded by polar hydroxy groups around the rim of the cavity, and bound together by propylsulfonate "feet" that extend from the bottom of the cavity. Inclusion complexes with small guest molecules are dependent on both steric and functional (polarity) complementarity. Relatively small, hydrophobic molecules can be expected to possess higher affinity for TESMR, in comparison to large, hydrophilic guests.

The migration behaviour of analytes in CE can be modified by using additives in the buffer solution, as demonstrated by Terabe et al. and many other researchers. There have been only a few reported applications of resorcinarenes (or related calixarene derivatives) as potential additives in CE. Bächman et al. used alkyl substituted resorcinarene tetraanions in the separation of a mixture of PAHs. Poor water solubility and possible aggregate formation limited the use of these additives. Recently, Sun et al. studied the effects of a charged p-carboxyethyl calix(7)arene on the separation of a group of substituted aromatic hydrocarbons. The influence of pH, organic solvent and field strength on the separation were studied. Exploring new types of additives is vital for the continuing development of CE methods.
The aim of this study is to explore the usefulness of TESMR (Figure 4.1) as a new type of additive in CE. This resorcinarene derivative was selected as a potential additive for several reasons: 1) it has high solubility in aqueous solutions (up to 0.4 M), 2) it is too hydrophilic to form micelles or aggregates, 3) it is highly charged, inducing a large difference between the mobilities of the free analyte and the analyte-additive complex (inducing a large elution range), 4) it has different cavity properties compared with CD type additives (size and affinity), 5) it is a single component additive, in contrast to most CD derivatives, which are mostly multi-component additives, 6) a variety of functional analogues have been synthesized and well characterized, and 7) it requires only inexpensive starting materials that are readily available. Positional isomers of nitrophenol and a group of para-substituted phenols were used as test analytes to study the influence of steric and polarity properties of analytes upon complexation with the resorcinarene additive. Also, the influence of additive concentration, buffer ionic strength, pH, and organic modifier content are investigated to determine optimum separation conditions. A comparative study between the resorcinarene and a charged CD of the complexation affinity with the analytes provided insight regarding the analyte-additive interactions. The benefits of a mixed-additive CE separation system, consisting of a neutral α-CD and a charged resorcinarene, is also discussed.

4.2 Experimental

4.2.1 Chemicals

Sodium phosphate monobasic, para-, meta- and ortho-nitrophenols, HPLC grade acetonitrile (ACN), dimethylformamide (DMF), dimethylsulfoxide (DMSO) and methanol (MeOH) were purchased from Fischer Scientific (New Jersey, USA). Methyl resorcinol, 2-
bromoethyl 1-3 dioxane, para-bromo, ethyl, hydroxy, phenyl phenols and biphenol were from Aldrich (Milwaukee, WI, USA); α- and β-cyclodextrins were from Sigma (St. Louis, MO, USA). Anhydrous sodium sulphite and sodium hydroxide were purchased from BDH Chemicals (Toronto, Can.). Sulphobutyl ether β-cyclodextrin (SBE-β-CD (IV) - Advasep), with an average degree of substitution of four, was kindly provided by Cydex (Overland Park, KS, USA).

4.2.2 Syntheses and Purification of TESMR

The tetrasulfonated 2-methyl resorcinarene host (TESMR) was synthesized via an acid-catalyzed condensation of sodium 2-formylethane-1-sulfonate with 2-methylresorcinol according to the method by Kobayashi et al. A two-phase reaction of 2-(2-bromoethyl)-1-3-dioxane (10 mmol) and an aqueous solution (10 mL) of sodium sulphite (20 mmol) was stirred at 100°C for 24 hours under reflux. To the resulting homogeneous solution was added water (10 mL) and the mixture was washed with ether (20 mL x 3) to extract unreacted bromo-alkyl-dioxane. To this were successively added 20 mL ethanol, methyl resorcinol (18 mmol) and concentrated HCl (3 mL). The mixture was then stirred under nitrogen at 100°C for 24 hours under reflux. The product was evaporated and the residue was redissolved in de-ionized water. Several minor configurational isomers of TESMR produced in the reaction were removed by repeated recrystallization in methanol-water (50:50). The product was then desalted five times against de-ionized water (1 L) using a dialysis membrane with a critical molecular weight cut-off of 1000 (Spectr/Por membrane MWCO 1000) to remove inorganic salts. The water was removed and the product was dried in vacuo producing orange coloured crystals. The elemental composition of TESMR has been reported to be $C_{20}H_{14}O_{20}S_4Na_4\cdot4H_2O$. 
4.2.3 Characterization of TESMR

A number of different techniques were used to characterize TESMR. $^1$H-NMR spectra of the product were taken in D$_2$O using a 200MHz Bruker AC-200E spectrometer to confirm its purity. The $^1$H-NMR spectrum of TESMR is shown in Figure 4.2. Singlet aromatic H and methyl H signals were observed in the spectra, which was consistent with the symmetrical bowl-shaped conformation of the tetramer. $^1$H-NMR (D$_2$O, 200MHz): $\delta$ 6.95 (s, 4H, aromatic CH), $\delta$ 4.68 (t, 4H, methine CHCH$_2$), $\delta$ 2.91 (t, 8H, methylene CH$_2$SO$_2$), $\delta$ 2.51 (dt,
The molecular weight of TESMR was verified by running a positive electrospray mass spectrum (ESMS) in a methanol-aqueous acetic acid solution, as depicted in Figure 4.3. The major molecular ion observed in the spectrum is a sodium adduct peak at \( m/z \) of 1087, which confirmed the molecular weight of the macrocycle. TESMR exhibited two main absorption bands in the UV region in 40 mM phosphate buffer, pH 4.5: a stronger band in the deep UV, and a much weaker band centered at 280 nm. The absorption spectrum at this pH is shown in Figure 4.4, with absorption coefficients at 200, 280 and 305 nm of \( 9.4 \times 10^4 \), \( 7.0 \times 10^3 \) and 40 M\(^{-1}\)cm\(^{-1}\),
respectively. The purity of TESMR was also assessed by CE with UV absorbance detection at 220 nm, which showed trace impurities with the peak area well under 4% of the main component peak in phosphate buffer, pH 4.5 (see Figure 4.5A).

4.2.4 pH Stability of TESMR

It was observed that aqueous solutions of TESMR changed colour when dissolved at different buffer pH. Solutions were observed to be pale orange at pH 4 to 7, but turned dark pink at pH > 8. These observations suggest a change in chemical structure due to the titration of the acidic phenolic hydroxy groups of TESMR. The stability of TESMR was assessed using UV absorption and CE studies in 40 mM phosphate buffer at pH 4.5, 7.0 and 10.0. Its absorbance spectrum was observed to be pH dependent, with a much stronger absorption throughout the UV under basic conditions. The UV spectra at pH 4.5 and 7.0 were similar. Figure 4.4 shows a comparison in the UV profiles of TESMR at pH 4.5 and 10.0. These observations confirmed the changes observed visually. Time elapsed experiments with CE more clearly demonstrated the changes in TESMR under alkaline conditions. Equalmolar amounts of TESMR were dissolved in 40 mM phosphate buffer at pH 4.5, 7, and 10, and the samples were analyzed over a two hour period with CE. When TESMR was dissolved in phosphate buffer, pH 4.5 or 7, no change in the appearance of the electropherograms was observed within a two hour time frame. However, CE analysis demonstrated the changes that occur over time when TESMR is dissolved in phosphate, pH 10.0. Figure 4.5 depicts a time elapsed series of electropherograms for TESMR at pH 10. A gradual degradation of the main peak is accompanied by the appearance of several new peaks in the electropherogram.
Thus, visual inspection, UV spectra and CE experiments, indicate that TESMR's structure changes under basic conditions. Due to the intermolecular H-bonding network around its cavity, the phenolic hydrogens in TESMR have been reported to be relatively acidic.\(^1\)\(^2\) Since the intermolecular H-bonds are vital for "bowl-shaped" structure, it is suspected that TESMR is oxidized in alkaline conditions because of deprotonation of these H-groups. It is unclear on the nature of the TESMR structure at high pH, or the degree of degradation that is apparent in the electropherograms over time. The application of CE-ESMS may help elucidate the mechanism of TESMR degradation under alkaline conditions. Therefore, all experiments in this study were performed using an acidic phosphate buffer.
Figure 4.5 Series of electropherograms showing the lability of TESMR under alkaline conditions, pH 10.0, performed at (A) 0 min, (B) 5 min, (C) 30 min, (D) 1 hr and (E) 2 hrs.

4.2.5 Apparatus and Procedure

All experiments were performed on a Beckman P/ACE 5500 automated CE system with a diode array detector at 25°C. The run buffer contained 40 mM phosphate at pH 4.5, unless otherwise stated, with different concentrations of various additives and organic modifiers. Fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with an ID of 50 μm, an OD of 375 μm and a total length of 27 cm (20 cm to detector) were used for the experiments. New capillaries were rinsed first with 0.1M NaOH for 3 minutes, followed by a rinse with deionized water for 5 minutes and phosphate buffer for 10 minutes which was then
allowed to equilibrate overnight. Stock solutions of the analytes (1 × 10⁻² M) were dissolved in aqueous solutions that contained 20 to 50% methanol. Samples were introduced in the capillary by using a low pressure (0.5 p.s.i.) injection for 2 seconds. Analyte peaks were identified by spiking the sample solution with the appropriate stock solution of each analyte.

4.3 Results and Discussion

4.3.1 The Influence of TESMR on the Migration Behaviour of Nitrophenols

Three fundamental parameters\textsuperscript{11,12} influence the apparent electrophoretic mobility of an analyte when using additives in CE: the electrophoretic mobilities of the free analyte (\(\mu_{ep, A}\)) and the analyte-additive complex (\(\mu_{ep, AC}\)), and the capacity factor of the interaction, \(k'_{AC}\) (which is the product of the equilibrium constant and the additive concentration, \(K[C]\), for 1:1 interactions). The apparent mobility of the analyte is given by:

\[
v\delta\mu_{ep, T} = \frac{1}{1 + k'} \mu_{ep, A} + \frac{k'}{1 + k'} \mu_{ep, AC}
\]  

(4.1)

where \(v\) and \(\delta\) are the viscosity and dielectric correction factors, that convert the net mobility to an ideal state mobility, where the additive concentration approaches zero. The correction factor ensures that the change in the net analyte mobility is solely the result of the shifts in equilibria. For neutral analytes, since \(\mu_{ep, A} = 0\), eq. 4.1 is simplified to:

\[
v\delta\mu_{ep, T} = \frac{k'}{1 + k'} \mu_{ep, AC}
\]  

(4.2)

Equations 4.1 and 4.2 illustrate an important advantage in using charged additives in CE. At pH 4.5, all phenol derivatives are neutral and co-migrate with the electroosmotic flow. Any separation of the analytes with the presence of TESMR would be indicative of differential interaction with the additive. As demonstrated by the large number of charged
Figure 4.6 Structures of the neutral test analytes used in this study and a schematic of their 1:1 dynamic complexation with TESMR, showing the parameters influencing the analyte's mobility.
micelles (i.e., MEKC), charged additives can effectively modulate the mobility of both charged and neutral analytes through differential interaction reflected by differences in $k'$ ($K[C]$) and $\mu_{ep,AC}$. Neutral additives do not influence the mobility of neutral analytes because both $\mu_{ep,A}$ and $\mu_{ep,AC}$ are zero. According to eq. 4.2, the use of a highly charged additive can increase the mobility of neutral or minimally charged analytes, since the net mobility is proportional to the complex mobility. It is for these reasons that charged cyclodextrins are increasingly being used for highly effective chiral separations in CE.\textsuperscript{13-17}

TESMR was observed to have a large negative electrophoretic mobility of $-3.91 \times 10^{-4}$ cm$^2$V$^{-1}$s$^{-1}$ at pH 4.5, compared to a relatively low electroosmotic flow of $1.51 \times 10^{-4}$ cm$^2$V$^{-1}$s$^{-1}$. This suggests that TESMR is highly charged even under the acidic conditions used throughout the investigation. Figure 4.6 illustrates the principle of the effect of charged additives in CE separations, with the neutral analytes used in this study. The analyte-additive complex migrates towards the cathode against the EOF, resulting in increased analyte migration times. The net mobility of an analyte is determined by the fraction of the complexed analyte and is a weighted average of the complex mobility, as shown in eq. 4.2. The fraction is determined by the capacity factor, which is determined by both the equilibrium constant and the concentration of the additive. The direction of the net mobility of the analyte can be reversed if the TESMR concentration is sufficiently high.

Figure 4.7 shows the effect of TESMR on the separation of nitrophenol isomers. $p$-Nitrophenol is partially separated from the other two in the absence of TESMR because of its relatively stronger acidity, but the other two co-elute with the EOF marker, as shown in electropherogram A. As little as 1 mM TESMR is required to fully resolve all three positional isomers, as shown in electropherogram B. The relative migration order of the
Figure 4.7 The effect of TESMR on the separation of three neutral positional isomers using (A) 0 and (B) 1.0 mM TESMR. Conditions: Buffer 40 mM phosphate, pH 4.5; capillary length 37 cm; voltage 28 kV; UV @ 305 nm. Peaks: 1 p-nitrophenol, 2 m-nitrophenol and 3 o-nitrophenol.

nitrophenol isomers indicate that the affinity of TESMR is strongest for o-nitrophenol and the weakest for p-nitrophenol, because of the spatial constraints dictated by the size of the cavity.

4.3.2 Analyte Peak Asymmetry

As shown in Figure 4.7, significant peak tailing was observed for the analytes when TESMR was added to the buffer. Both the magnitude of the tailing and the migration times
increased with the increasing resorcinarene. Peak tailing is a serious problem when using these potent additives. This phenomenon has been studied by a number of groups when highly charged additives, such as sulphobutyl β-CD (SBE-β-CD) are used. A possible explanation is that lipophilic analytes may be mass transfer resistant due to the high charge density in the vicinity of highly charged macrocyclic molecules (i.e., slow kinetics). However, separations at higher temperatures (up to 40°C, which should have higher reaction rates) did not show significant peak shape improvement.

Peak asymmetry in CE has been generally attributed to two main sources: electrokinetic dispersion and surface adsorption. Electrokinetic dispersion occurs when there is an unstable moving boundary in the sample plug, caused by perturbations in the electric field distributed along the capillary, that are due to conductivity or pH differences between sample and run buffer zones. Peak tailing is prominent when the conductivity of an analyte in the sample zone is much greater than the conductivity of the co-ion in the buffer zone. Peak asymmetry in this case can be minimized when using charged additives, by reducing the analyte and/or additive concentrations. Since the concentration of TESMR used in this study is rather small, electrokinetic dispersion is unlikely to be the main cause of the observed peak asymmetry.

Analyte adsorption on the inner capillary wall is generally attributed to electrostatic and/or hydrophobic forces. Under normal conditions, since TESMR and the capillary wall are both negatively charged, it is unlikely that the resorcinarene would be adsorbed electrostatically. However, at pH 4.5, the capillary wall is only partially ionized, which is reflected by a relatively slow EOF. When no TESMR is added to the run buffer, the analytes are close to neutral at pH 4.5, and they all co-elute with the EOF marker. There is no peak
tailing observed. When the mobility of TESMR is measured by injecting a plug into the capillary at this pH, no peak tailing is observed either. These observation suggest that neither the nitrophenols, nor TESMR alone is adsorbed by the capillary wall. However when the nitrophenols are injected into the capillary in the presence of TESMR in the run buffer under acidic conditions, severe tailing is observed. This suggests that, with the presence of highly charged additives such as TESMR, hydrophobic interactions between the analytes and the silanol groups on the capillary wall were enhanced, resulting in severely skewed peaks. The presence of highly charged ions in the solution induce stronger solvent-solvent interactions, and thus weaker solvent-solute interactions and stronger solute-solute interactions or hydrophobic interactions (i.e., between capillary wall and analyte). This argument is further supported by the fact that increasing the analyte concentration led to increased peak asymmetry.

Experiments were conducted to observe the influences of buffer ionic strength, pH, and organic modifier on peak tailing. Various concentrations of phosphate buffer (40, 80, 120 and 160 mM) at pH 4.5 were used with 2 mM TESMR, and the peaks shapes of the nitrophenol isomers were examined. No significant improvement in peak shape was observed with increased ionic strength. Increasing resolution due to the slower EOF was observed at the cost of greater peak tailing, higher currents, and more Joule heating. The pH of the run buffer was observed to have a noticeable effect on peak shape. The use of an increasingly basic pH buffer resulted in much sharper peaks over a wide range of TESMR concentrations. Despite its observed lability under alkaline conditions, TESMR was still observed to interact significantly with nitrophenols. When the pH was incrementally changed from 4.5 to 9.0, significant improvements in peak shape was found to occur at pH >
7.6. The nitrophenols and the capillary wall are sufficiently ionized at higher pH, resulting in reduced analyte-capillary wall interactions. Peak tailing has been consistently observed when charged cyclodextrins were used in acidic buffer conditions with neutral analytes.\textsuperscript{8-12} The main drawbacks of using basic buffers include the increase in background noise due to the strong absorption of TESMR throughout the UV region and the gradual degradation of TESMR that has been observed to occur over time.

In cases where acidic conditions have to be used for optimum separation, organic modifiers can be used to increase the solvent strength, therefore, minimize the interactions between the analytes and the capillary wall, thereby improving peak shape. Four types of organic modifiers are used in this study: MeOH, ACN, DMF and DMSO. As shown in Figure 4.8, each of the organic modifiers has a different effect on the separation and peak asymmetry. The addition of the polar protic solvent, i.e., methanol, to the run buffer has minimal effects on peak shape, but results in a small reduction in resolution. However, polar aprotic solvents were observed to have significant improvements in peak shape, albeit with a much larger reduction in resolution. When DMSO is used, although the resolution is reduced in comparison to DMF and ACN, it gives the greatest improvement in peak shape when higher concentrations of TESMR are used. Therefore, DMSO was chosen as the most effective organic modifier. It is apparent that the reduced analyte-wall interaction is achieved at the cost of reduced analyte-additive interaction, because of the increased solvent strength (stronger solvent-solute interaction). Peak tailing was minimized to a great extent when using only a small percentage of DMSO (2 to 5\%) in the run buffer. Previous arguments to explain peak tailing in terms of conductivity differences (electrokinetic dispersion) do not seem to be relevant with these observations. Excellent resolution and peak symmetry of the
Figure 4.8 Influence of organic modifiers on the resolution and peak asymmetry of nitrophenols using 5% (A) MeOH, (B) ACN, (C) DMF and (D) DMSO, and 1.5 mM TESMR. All other conditions are the same as Figure 4.7.

nitrophenol isomers were achieved with 2.5% DMSO in the run buffer, using 6 mM TESMR, see Figure 4.9(A). One of the other advantages of using organic modifiers is that the reduced conductivity of the run buffer allows the application of higher voltages for more efficient separations. The main disadvantage of using organic modifier with charged additives in CE separations is that a higher concentration of additive is often required to achieve the same resolution. However, due to the high solubility of the resorcinarene in aqueous solution, this is not a serious limitation.
4.3.3 Resorcinarenes vs Charged Cyclodextrins

The binding affinities between the nitrophenol isomers and two different negatively charged macrocyclic host molecules: TESMR and SBE-β-CD, were compared. Similar to TESMR, the use of SBE-β-CD to the run buffer resulted in dramatic peak tailing. A relatively large percentage of DMSO (30%) was required to minimize peak asymmetry. It is not clear why a higher concentration of DMSO is required in this case. SBE-β-CD is a multi-component additive with an average degree of substitution of four sulphobutyl groups (ranging from one to ten), whereas TESMR is a pure, single component additive. Increased analyte band broadening may be caused by the heterogeneity of SBE-β-CD, and the wide range of complex mobilities formed upon analyte complexation. In contrast, analyte binding with the homogeneous TESMR additive results in the formation of a single analyte-additive complex, with a discrete complex mobility. Thus, well characterized and reproducibly synthesized single component complexing agents are the preferred type of additive to be used in CE applications to minimize band dispersion.

Figure 4.9 shows a comparison of the effects of two charged additives for the separation of the nitrophenol isomers. SBE-β-CD was observed to have the greatest affinity for p-nitrophenol, an intermediate interaction with m-nitrophenol and the weakest binding to o-nitrophenol. This trend is similar to what has been demonstrated with other cyclodextrins. On the other hand, TESMR has the opposite selectivity, resulting in a reversed migration order of the nitrophenol isomers. The unique affinities displayed by each additive reflect the specific shape, size and polarity of their cavity and the resultant stability of the analyte additive complex with the nitrophenol isomers.
Figure 4.9 Comparison of the binding affinities of nitrophenol isomers using two different negatively charged additives: (A) 6 mM TESMR (2.5% DMSO) and (B) 2 mM SBE-β-CD (30% DMSO). Analyte peaks are numbered the same as Figure 4.7. Separation voltage was 20 kV for (A) and 25 kV for (B) in order to compare relative migration times.

4.3.4 Analyte Size, Polarity and Migration Order

Figure 4.10 shows the electropherograms for the separation of six para-substituted phenols with TESMR and SBE-β-CD additives. Because all analytes are close to neutral at the pH of the run buffer, the separation is due to the differential interaction of the analytes with each charged additive. Although the use of TESMR and SBE-β-CD results in opposite
migration orders for the positional isomers, they demonstrated similar trends in binding affinity for the group of para-substituted phenols. Phenyl-substituted phenol displayed the strongest interaction with both additives, whereas the hydroxy-substituted phenol (1,4-benzene-diol) showed the weakest interaction. The addition of a hydroxy functionality on the phenyl residue (biphenol), resulted in a weaker interaction with the additives in comparison to that of phenyl-phenol. The remaining three substituted phenols: ethyl-, 
bromo- and nitro-phenols, displayed similar intermediate affinities with both additives. The similarity in the size and polarity of these analytes reflect their binding interactions with both additives. Lower concentrations of SBE-β-CD were required to separate the mixture of para-substituted phenols relative to TESMR. However, SBE-β-CD was observed to be unable to discriminate between p-ethylphenol and p-bromophenol under the conditions used in this study. Since each macrocyclic molecule displayed similar trends in binding affinity for the para-substituted phenols, the driving force for complexation should be hydrophobic in nature for both additives. Hence, steric and functional (polarity) complementarity between the analyte and the additive molecules seems to play an important role in the separation system.

4.3.5 Mixture of Charged and Neutral Additives: Separation Design

Although a variety of conditions can be modified in CE to improve the separation (e.g., ionic strength, pH, organic modifier, temperature, etc.), the use of mixed additives can be one of the most predictable way to rapidly optimize separation conditions. A systematic approach in the application of multiple additives has been previously examined\(^\text{11}\) and the effects of a mixture of charged and neutral additives on analyte migration behaviour have also been discussed.\(^\text{24}\) When TESMR (highly negatively charged) and α-CD (neutral) are used simultaneously, the apparent mobility of an analyte, assuming 1:1 interactions with each additive, can be described by:

\[
v \delta \mu _{ep,T} ^A = \frac{1}{1 + k'_{AC} + k'_{AD}} \mu _{ep,AC} + \frac{k'_{AC}}{1 + k'_{AC} + k'_{AD}} \mu _{ep,AC} + \frac{k'_{AD}}{1 + k'_{AC} + k'_{AD}} \mu _{ep,AD} \quad (4.3)
\]

where \(\mu _{ep,AC}, \mu _{ep,AD}, k'_{AC}\) and \(k'_{AD}\) are the analyte-additive complex mobilities and capacity factors for the analyte-resorcinarene (AC) and analyte-cyclodextrin (AD) complexes,
Figure 4.11 Schematic of the use of a complementary dual additive system, TESMR and α-CD, to enhance the resolution (increase separation window) of specific analytes in a mixture.

respectively. Since neutral analytes and a neutral cyclodextrin (α-CD) additive are used in this study (\(\mu_{ep,A}\) and \(\mu_{ep,AD}\) are 0), eq. 4.3 can be simplified to:

\[
\nu_0 \delta \mu_{ep,T} = \frac{k'_{AC}}{1 + k'_{AC} + k'_{AD}} \mu_{ep,AC} \tag{4.4}
\]

In this case, the apparent mobility of an analyte can be effectively modulated by the use of two additives through differences in three fundamental physico-chemical properties: \(k'_{AC}\),
The migration behaviour of an analyte can be better controlled by the appropriate selection of additives to maximize differences in binding affinities and/or complex mobilities. It was in this spirit that TESMR and α-CD were selected as a dual additive system for the separation of nitrophenol isomers. A previous study\cite23 of the complexation of α-CD with nitrophenol isomers showed that it exhibited the highest affinity for the para isomer and the lowest affinity for the ortho isomer (similar to SBE-β-CD), which is opposite to the binding trends observed with TESMR. In addition, the complex mobilities are drastically different for each analyte-additive complex since resorcinarene is highly negatively charged and α-CD is neutral. Thus, both additives have complementary selectivities for the nitrophenol isomers in terms of binding affinities and complex mobilities. Figure 4.11 illustrates the concept of a complementary dual additive system, using TESMR and α-CD, to enhance the resolution of a separation.

Figure 4.12 depicts a series of electropherograms involving the nitrophenol isomers, when TESMR alone, and TESMR and α-CD were added to the run buffer. Table 4.1 lists the mobility values of the three nitrophenols and their resolution measured from the electropherograms, including when no additives are added. Both p- and m-nitrophenols show significant binding to the neutral α-CD and migrate with a shorter migration time (reduced mobility), resulting in decreased resolution when TESMR is present as compared to when TESMR is used alone. In contrast, because α-CD displays the weakest interaction with o-nitrophenol and TESMR the strongest, increased resolution of the ortho isomer from the other two is achieved. Although the analyte mixture was rather simple in this study, one can envision the potential of modifying the mobilities of specific analytes in a complex mixture through the appropriate use of multiple additive systems.
Table 4.1 The effect of TESMR and α-CD on the separation of nitrophenol isomers

<table>
<thead>
<tr>
<th>[TESMR] (mM)</th>
<th>[α-CD] (mM)</th>
<th>$\mu_{\text{ep, para}}$ ($\times 10^5$ cm$^2$V$^{-1}$s$^{-1}$)</th>
<th>$\mu_{\text{ep, meta}}$ ($\times 10^5$ cm$^2$V$^{-1}$s$^{-1}$)</th>
<th>$\mu_{\text{ep, ortho}}$ ($\times 10^5$ cm$^2$V$^{-1}$s$^{-1}$)</th>
<th>Rs (p, m)</th>
<th>Rs (m, o)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>-0.11</td>
<td>0</td>
<td>0</td>
<td>0.31</td>
<td>0</td>
</tr>
<tr>
<td>(A) 6</td>
<td>0</td>
<td>-1.62</td>
<td>-2.62</td>
<td>-3.74</td>
<td>3.41</td>
<td>4.37</td>
</tr>
<tr>
<td>(B) 6</td>
<td>8</td>
<td>-0.98</td>
<td>-1.36</td>
<td>-3.42</td>
<td>3.21</td>
<td>7.08</td>
</tr>
</tbody>
</table>

Figure 4.12 Application of a dual additive system for the resolution of specific analytes (ortho isomer - arrow) from a mixture where (A) 6 mM TESMR / 0 mM α-CD and (B) 6 mM TESMR / 8 mM α-CD. Conditions and analyte peak numbering are the same as Fig 4.7.
4.4 Conclusion

The potential of employing a novel type of macrocyclic resorcinarene additive to enhance CE separations was investigated. The highly soluble TESMR can be used to discriminate between various positional isomers and different types of substituted phenols. Analyte peak tailing was significant under acidic conditions when using highly negatively charged additives. The addition of polar aprotic solvents to the run buffer was found to be the most effective remedy to minimize peak asymmetry. TESMR and SBE-β-CD were shown to have opposite selectivity with a group of nitrophenol isomers in terms of steric constraints dictated by the analytes shape and size. However, both additives exhibited similar trends in binding affinity with respect to analyte functionality, as demonstrated with a group of para-substituted phenols. The benefits of utilizing mixed additive systems for the fine-tuning of resolution in CE was explored using a neutral α-CD and a negatively charged TESMR additive pair. The applications of new types of additives serves to increase the versatility of CE in chemicals separations.

4.5 References

(2) Timmerman, P.; Berboom, W.; Reinhoudt, D. N. Tetrahedron 1996, 52, 2663-2704.


Section C:

Practical Assay Development in CE:
Collaborative Projects with
Academia and Industry.
Chapter V:

Analysis of $\gamma$-Carboxyglutamic Acid Content of Protein, Urine and Plasma by Capillary Electrophoresis Laser-Induced Fluorescence.

"Design of selective and sensitive separation systems for biomedical research."
V: Analysis of γ-Carboxyglutamic Acid Content of Protein, Urine and Plasma by Capillary Electrophoresis Laser-Induced Fluorescence

5.1 Introduction

Gamma-carboxyglutamic acid (Gla) is a non-essential amino acid with a dicarboxylic acid side chain that was first identified in 1974 from prothrombin. Gla is synthesized in vivo from glutamic acid (Glu) through a post-translational, vitamin K-dependent carboxylase pathway in the hepatic microsomes, and also in non-hepatic tissue, such as kidney, spleen, lung and bone. Isolation of abnormal prothrombin from patients and cows treated with vitamin K antagonists indicated the importance of Gla in metal binding and biological activity of the protein. Sufficient Gla residues in prothrombin are required to bind Ca\(^{2+}\) and phospholipid in order to induce conformational changes that accelerate proteolytic activation and normal blood coagulation. Among the amino acids, Gla possesses a unique affinity for Ca\(^{2+}\) due to the chelation of the metal by its bidentate, dicarboxylic functionality. In prothrombin, ten Gla residues in the amino-terminal region (termed the Gla domain) bind calcium, leading to a calcium-dependent conformational change of the protein. The conformational change of prothrombin upon calcium binding is required for interaction with phospholipid membrane surfaces during prothrombin activation in the prothrombinase complex. Various other Gla containing proteins have since been discovered, including the blood coagulation factors (factor X, factor IX and factor XII), coagulation inhibitors (proteins C, S and Z), osteocalcin / bone Gla protein (BGP) and matrix Gla protein (MGP), atherocalcin / arterial Gla protein (AGP), renal Gla protein (RGP), and testicular Gla protein (TGP). The quantification of Gla from wild type vitamin K-dependent proteins is vital to ensure complete carboxylation of the protein, which may be correlated to its observed
Figure 5.1 Synthesis of Gla (V) and osteocalcin. Gla residues are synthesized from Glu in precursor protein in osteoblasts. Gla synthesis is dependent on vitamin K (reduced form) and CO2. Warfarin inhibits the synthesis of the Gla residues and prevents calcium-bound Gla dependent conformational change in osteocalcin that promotes its binding to hydroxyapatite in bone. Warfarin or vitamin K deficiency results in an under- or non-carboxylated osteocalcin.
biological activity. Figure 5.1 depicts the synthesis of Gla in the vitamin-K dependent polypeptide, osteocalcin, highlighting the importance of this amino acid in protein conformation. The Gla residues in osteocalcin induce protein conformational change upon calcium binding, thereby promoting its binding to hydroxyapatite in bone. In contrast, under- or non-carboxylated osteocalcin, caused by warfarin treatment or vitamin-K deficiency, does not bind normally to bone. Osteocalcin accumulation in bone has been reported to be important for proper mineral deposition and bone resorption.4

Gla also exists as free amino acid or in short peptides in urine,7 serum,8 and plasma,9 to serve as the final catabolic pathway of vitamin K-dependent proteins. The monitoring of urine or plasma Gla content can be useful for the diagnosis of certain pathological conditions of vitamin K-dependent processes involving blood coagulation, bone metabolism and ectopic calcification. Gla analysis from biological fluid has been a useful biochemical marker, in conjunction with other serum proteins, in the monitoring of various diseases such as osteoporosis,10 deep-vein thrombosis and liver disease,9 primary hyperparathyroidism,11 diabetes mellitus,12 dermatomyositis and scleroderma.13 A significant decrease in urinary Gla has also been observed during warfarin treatment.9,14

No single method has been widely adopted for the analysis of Gla from hydrolyzed protein and biological fluid. At present, Gla analyses are carried out by a variety of techniques, including standard amino acid analysis with ninhydrin detection,15,16 thin layer chromatography,17 tritium-exchange labeling,18 anion-exchange and reverse-phase chromatography with fluorescence detection,19-23 gas chromatography,24 and mass spectrometry.25 Previous chromatographic methods have required milligrams of purified protein in order to carry out Gla analysis.19,20,23 Moreover, time consuming desalting
The development of a rapid micro-separation technique that requires sub-microgram amounts of protein would be highly desirable in the biological field. The original identification of Gla was aided by electrophoresis, which demonstrated the high negative mobility exhibited by peptides containing this unique amino acid. In this respect, CE, when coupled with laser-induced fluorescence detection, should be a useful method for the analysis of Gla because of its small sample volume requirements, high sensitivity and its ability to separate charged molecules. To our knowledge, there has been only one reported CE methodology for Gla analysis from protein. Hiskey et al. proposed a qualitative method to compare the differences in Gla content of peptide fragments upon partial decarboxylation using CE with UV detection. This work describes the development of a highly specific and sensitive Gla assay using CE-LIF. The method required a microgram or less of protein, or microlitres of biological fluid. Samples were then analyzed directly after fluorescent derivatization without desalting. The assay was applied to the analysis of Gla from three vitamin K-dependent proteins, as well as the measurement of free Gla from both urine and plasma samples.

5.2 Experimental

5.2.1 Chemicals

The aqueous separation buffer consisted of 100 mM Tris (Trizma electrophoresis reagent grade, Sigma Chemical Co., St. Louis, MO) at pH 8.3. The pH of the separation buffer was adjusted by using 1 M HCl (BDH Chemicals, Toronto, Ont., Canada). Standard amino acid solutions of L-aspartic acid, L-glutamic acid, L-γ-carboxyglutamic acid and fluorescein isothiocyanate (FITC) isomer I (98%) were all purchased from Sigma. HPLC grade acetone,
acetonitrile, pyridine and perchloric acid (69 to 72%) were obtained from Fischer Scientific (Nepean, Ont., Canada). Sodium hydroxide and sodium bicarbonate were purchased from BDH Chemicals. Human wild type plasma prothrombin and human blood coagulation factor X were purchased from Haematologic Technologies, Inc. (Vermont, USA). Bovine osteocalcin and ferric iron binding protein were generously donated by Dr. Rick Jenny from Haematologic Technologies, Inc. and Dr. Michael Murphy of the Biochemistry and Molecular Biology Department of the University of British Columbia, respectively.

5.2.2 Apparatus

Separations were performed on a Beckman P/ACE 5500 automated capillary electrophoresis system equipped with a argon ion laser (Beckman Instruments Inc., Mississauga, Ont., Canada). Uncoated capillaries (Polymicro Technologies, Phoenix, AZ) were used with inner diameters of 75 μm, outer diameters of 375 μm and lengths of 57 cm. New capillaries were first rinsed with 1.0 M NaOH (5 minutes at 20 psi), followed by rinsing with the separation buffer (10 minutes at 20 psi). The capillary was then left to equilibrate overnight in the separation buffer prior to use. Each separation was preceded by a 2.0 minute 20 psi rinse with 0.1 M NaOH, followed by a 4 minute rinse with the separation buffer. The samples were then introduced using a 3 second low pressure injection at 0.5 psi (approximately 18 nL volume injected) and the separation was carried out for 20 minutes at 30 kV (normal polarity) under a temperature of 25 °C. Fluorescence was induced with the 488 nm line of a 4 mW argon ion laser which was coupled to the CE instrument via a fiber optic cable. Emission was monitored at 520 nm with a Beckman fluorescence detector. Data were collected and processed using System Gold.
5.2.3 Protein Hydrolysis

Both acidic and basic hydrolyses were performed on all protein samples. Protein solutions were prepared in 20 mM Tris, 150 mM NaCl, pH 7.5 at a concentration of 1 mg/mL. Hydrolysis was carried out using 1.0 μL (1.0 μg) of protein sample in 30 μL of 2.5 M KOH for alkaline hydrolysis and 6.0 M HCl for acid hydrolysis. The mixture was transferred into a 1.5 by 100 mm capillary tube (KIMAX-51, VWR-Canlab, Ont., Canada) and the ends of the tube were heat-sealed over a Bunsen burner flame. The tubes were then submerged completely in the wells of a heating block filled with industrial oil and the hydrolysis was carried out at 110 °C for 16 hours. The sample was then transferred from the capillary tube into an eppendorf tube, which was centrifuged at 13,000 rpm for 5 minutes prior to derivatization.

5.2.4 Urine and plasma samples

Human urine samples were collected from a healthy volunteer immediately after rising in the morning. Urine was stored frozen at -20 °C and subsequently thawed, centrifuged at 13,000 rpm for 10 minutes and used directly for fluorescent labeling. Pooled plasma was obtained from 30 normal donors and stored frozen. Plasma was first deproteinized with 50% (v/v) acetonitrile or filtered with a millipore eppendorf tube with a 5000 MWCO filter at 3000 x g for 30 minutes prior to derivatization.
5.2.5 Fluorescent labeling

A stock solution of 50 mM FITC was prepared in HPLC grade acetone:pyridine (80:20, v/v). Standard amino acid solutions (0.1 M) were prepared in 0.2 M sodium bicarbonate buffer at a pH 9.0. The FITC concentration was greater than a ten-fold excess relative to the concentrations of standard amino acids and protein hydrolysates in order to ensure complete reaction. The protein hydrolysates were neutralized with aqueous, ice-cold 7% perchloric acid prior to derivatization. The neutralized mixture was then left on ice for 15 minutes and the insoluble potassium perchlorate was removed by centrifugation at 13,000 rpm for 10 minutes. The supernatant was transferred to a new eppendorf tube (16 μL aliquot) which was diluted with 0.2 M bicarbonate buffer, pH 9.0 to a total volume of 28 μL. Similarly, fluorescent labeling of pre-treated urine and plasma samples (as described in the previous section) was carried out with a 16μL aliquot of each in an eppendorf tube, and was diluted with bicarbonate buffer to a volume of 28 μL. The derivatization reaction was done through the addition of 2 μL of 50 mM FITC to the neutralized protein hydrolysates, urine and plasma samples (total volume of 30 μL). Formation of the fluorescein thiocarbamyl derivative of the amino acid (FTC-AA) was allowed to react for 24 hours in the dark. The mixture was then diluted with de-ionized water prior to CE-LIF analysis. A time course experiment of protein hydrolysates revealed that the peak area for FTC-Gla rapidly increased during the first 12 hours and then slowly increased within a 24 hour period. FTC-Gla did not degrade for at least 3 days, indicating that the fluorescently labeled amino acid is quite stable under these conditions. Figure 5.2 summarizes the procedure used to analyze Gla from either hydrolyzed protein, urine or plasma samples.
Figure 5.2 Flow chart depicting the general procedure used to analyze Gla from hydrolyzed protein, urine and plasma.

Figure 5.3 Structures of the fluorescein thiocarbamide (FTC) derivatives of the three different acidic amino acids.
5.2.6 Calibration Plot

A standard calibration curve for FTC-Gla was obtained in the range of $5.0 \times 10^{-8}$ to $2.5 \times 10^{-10}$ M. The calibration plot was obtained using an excess of $2.5 \times 10^{-3}$ M Gla with a limiting amount of $1.0 \times 10^{-5}$ M FITC. This calibration procedure was used in order to calculate the concentration of intact FTC-Gla. Duplicate reactions were carried out for 8 hours in the dark. The concentration of FTC-Gla was calculated by comparing the peak area of FITC at the start of reaction (known concentration) with the peak area of FTC-Gla generated after completion of the reaction. The standard calibration plot for gave a linear relation within a two hundred fold concentration range between measured peak area ($y$) and Gla concentration ($x$, in nM), $y = (17.07 \pm 0.05)x - (0.9 \pm 1.2)$ and $R^2 = 0.9999$.

5.3 Results and Discussion

5.3.1 Analysis of Gla of Protein Hydrolysates

Identification of Gla in proteins was originally hampered by conventional amino acid analyzers since it is readily decarboxylated under acidic conditions to produce Glu. Hence, base hydrolysis of protein is required to quantify Gla residues. The number of Gla residues per mole of protein can then be calculated by determining the concentration ratio of Gla:protein for each sample.

The CE system was designed based on the acidic properties of the amino acids of interest. Figure 5.3 depicts the structures of the three fluorescein thiocarbamide labeled acid amino acids: FTC-Glu, FTC-Asp and FTC-Gla. Separation was optimized using 100 mM Tris buffer, pH 8.3, which provided excellent separation of the acidic amino acids within a 20 minute analysis time. Figures 5.4 and 5.5 show the separation of the labeled amino acids.
**Figure 5.4** Electropherogram obtained from the *base* hydrolysis of human prothrombin by CE-LIF. Peak identification: 1 FTC-Glu, 2 FTC-Asp, and 3 FTC-Gla.

**Figure 5.5** Electropherogram obtained from the *acid* hydrolysis of human prothrombin by CE-LIF. Peak identification: 1 FTC-Glu and 2 FTC-Asp.
obtained from the alkaline and acid hydrolysates of human prothrombin, a 72 kDa blood coagulant protein. The separation is specifically designed for the analysis of the acidic amino acid derivatives because of their high negative electrophoretic mobility at the pH of the run buffer, in the presence of excess underivatized FITC and other FTC-labeled amino acids contained in the hydrolysate mixture. Moreover, FTC-Gla is readily separated from FTC-Glu and FTC-Asp, because of its larger negative electrophoretic mobility from the extra carboxyl functionality. Identification of Gla was confirmed by spiking with Gla standard and the analysis of non-Gla containing protein (ferric iron binding protein) that showed no corresponding peak to that observed with prothrombin. In addition, acid hydrolysis of prothrombin resulted in the disappearance of the Gla peak (via decarboxylation) and a corresponding increase in the Glu signal relative to Asp as shown in Figure 5.5. The identity of the peak migrating just before 13 minutes in Figure 5.5B is not known. This peak only exists in the acid hydrolysates and does not seem to be associated with the three amino acids studied.

The fluorescent yield for FTC-Gla was about 76% to that of FTC-Glu. The relative lower sensitivity for FTC-Gla, in comparison to FTC-Glu, is reflected in the slopes of the calibration plots. This value was similar to the Gla/Glu fluorescent yield obtained with OPA derivatization reported previously. The lower response factor for Gla relative to Glu and Asp has also been observed by other research groups. The repeatability of the Gla assay gave a RSD often less than 2.0%, whereas the reproducibility of separately prepared samples averaged 5.0% for proteins. The limit of detection (S/N = 3) for Gla using this method was about $5.0 \times 10^{-11}$ M.
Electropherograms were also obtained from the hydrolysates of human coagulation factor X (Figure 5.6) and bovine osteocalcin (Figure 5.7). Relative to human prothrombin, factor X is a 55 kDa blood coagulant protein that contains 11 Gla residues and bovine osteocalcin is a 5.6 kDa bone polypeptide reported to have 3 Gla residues per protein. Qualitatively, each protein shows a different Glu:Asp:Gla composition by comparing their relative peak areas. To quantify the number of Gla residues per protein, the concentration ratio of Gla to protein is determined from the measured peak area of FTC-Gla and its corresponding concentration prior to dilution, and the known concentration of the protein used for hydrolysis. However, experimentally calculated Gla numbers were observed to be significantly lower compared to literature values. According to the differences in experimentally measured and literature values of Gla content for prothrombin, a total recovery for Gla of approximately 26% was obtained from this method. Since the total number of Gla residues is known for prothrombin, this recovery value is used as a correction factor to calculate the actual Gla numbers obtained from the proteins. A correction factor of 3.8 was used to obtain the moles of Gla per mole of protein. It is assumed that factors affecting recovery of Gla are similar for each type of protein. This recovery is reasonable since there are cumulative losses incurred during hydrolysis, neutralization, fluorescent derivatization and detection.

Table 5.1 summarizes the results obtained from Gla analysis of hydrolyzed proteins. Experimental values of Gla content correspond well with estimates obtained from amino acid sequencing data. There is a minor discrepancy between the number of Gla residues calculated for bovine osteocalcin, 2.1, compared to the reported value of 3. However, this is not unreasonable since it has been observed that Gla residues in human osteocalcin are often heterogeneous, varying in number from 2-3 Gla residues per protein. Therefore, this
Figure 5.6 Electropherogram obtained from the *base* hydrolysis of human factor X by CE-LIF. Peak identifications are the same as Figure 5.4.

Figure 5.7 Electropherogram obtained from the *base* hydrolysis of bovine osteocalcin by CE-LIF. Peak identifications are the same as Figure 5.4.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Gla content</th>
<th>Gla content</th>
<th>Gla content</th>
<th>Gla content</th>
<th>Gla content</th>
<th>Gla content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine osteocalcin</td>
<td>3.1 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>3.6 ± 0.3</td>
<td>9.7 ± 0.9</td>
<td>17.8</td>
<td>X</td>
</tr>
<tr>
<td>Human factor X</td>
<td>11</td>
<td>(11.0 ± 0.8)</td>
<td>(20.0 ± 1.1)</td>
<td>(5.28 ± 0.29)</td>
<td>1.82</td>
<td>X</td>
</tr>
<tr>
<td>Human prothrombin</td>
<td>10</td>
<td>(10.0 ± 0.7)</td>
<td>(13.9 ± 0.95)</td>
<td>(3.66 ± 0.25)</td>
<td>1.39</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1: Gla content of base hydroxylated protein by CE-LIF
method provides a relatively simple way to verify the extent of carboxylation of Glu residues to Gla from various naturally-occurring or recombinant sources of protein.

5.3.2 Analysis of Free Gla in Urine and Plasma

The analysis of free Gla contained in urine was directly performed without sample pretreatment. Figure 5.8 depicts the electropherogram obtained from urine. Gla was readily resolved and detected from other components contained in urine. Gla identity was confirmed again by spiking with standard and by observing the disappearance of the peak with acidic hydrolysis. Triplicate measurements of FTC-Gla in urine gave a concentration of \((9.2 \pm 0.2) \times 10^{-6}\) M. Previous urinary Gla measurements have been generally normalized relative to the amount of creatinine excreted.\(^{12}\) To make this result meaningful, the concentration of Gla has to be normalized relative to the amount of creatinine that is measured independently.

The analysis of free Gla in pooled plasma was also performed. Plasma was first deproteinized using acetonitrile or ultrafiltration prior to derivatization. It was observed that the selection of deproteinization method influenced the detection of Gla in plasma. The use of acetonitrile for deproteinization resulted in no detectable Gla signal, whereas ultrafiltration (Figure 5.9) allowed for the detection of trace amounts of Gla that corresponded to a concentration of \((4.9 \pm 0.3) \times 10^{-8}\) M. These results suggest that there is nearly a two hundred-fold lower concentration of Gla in plasma compared to urine. The electropherogram obtained from plasma is also significantly more complex than that derived from urine. The proper selection of deproteinization may effect the recovery of Gla and/or its subsequent labeling reactivity with FITC. The effect of deproteinization procedure on Gla analysis from plasma has been observed by other research groups.\(^{8,22}\) It was suggested that the absence of Gla after deproteinization using acetonitrile is caused by co-precipitation of Gla with protein
Figure 5.8 Electropherogram depicting the analysis of free Gla content from human urine. Peak identification is the same as Figure 5.4.

Figure 5.9 Electropherogram depicting the analysis of free Gla content from ultrafiltrated human plasma. Peak identification is the same as Figure 5.4.
in the sample matrix. Therefore, ultrafiltration should be used for deproteinization in this type of analyses. Hanss et al.\textsuperscript{9} reported free plasma Gla level for healthy subjects is about $(14.6 \pm 0.3) \times 10^{-8}$ M. Assuming the recovery obtained from plasma is similar to that found for protein hydrolysates, the results obtained for pooled plasma are comparable with literature values. However, the inherent variability of free Gla content in both urine and plasma among individuals requires a systematic clinical study, incorporating a complementary analysis of relevant serum proteins, in order to make it a useful diagnostic tool for certain blood and bone related diseases.

5.4 Conclusion

CE is one of the most powerful separation technique presently available, because it utilizes both electric field and equilibrium in the separation process. Simple yet extremely powerful separation systems can be designed according the specific properties of the analytes. The determination of Gla content contained in human prothrombin, human coagulation factor X and bovine osteocalcin analyzed via a one-step alkaline hydrolysis with FITC labeling by CE-LIF was demonstrated. The separation was highly selective for the acidic amino acids with excellent mass and concentration sensitivity. The small sample requirement of CE-LIF permits the characterization of sub-micrograms of protein with commercial instrumentation. Free Gla in urine was analyzed directly with minimal sample pretreatment, whereas plasma required deproteinization by ultrafiltration prior to analysis. Gla analysis by CE-LIF provides a convenient way to verify the extent of the post-translational carboxylation of both wild and recombinant sources of Gla containing proteins. Analysis of mutated variants of vitamin-K dependent proteins (under-carboxylated) by CE-LIF may aide in the understanding of the role of specific Gla residues in the biological
activity of the protein. Clinical analysis of free Gla contained in urine or plasma may be useful for the diagnosis of specific diseases involving biological processes mediated by the vitamin K-dependent proteins.

5.5 References


Chapter VI:

Quantitative Assay for Epinephrine in Dental Anesthetic Solutions by Capillary Electrophoresis.

"Design of selective and sensitive CE separation systems for industry."
VI: Quantitative Assay for Epinephrine in Dental Anesthetic Solutions by Capillary Electrophoresis

6.1 Introduction

Local anesthetic solutions have recently gained widespread acceptance among emergency healthcare personnel. The ease of use, minimal toxicity and reduced costs are commonly cited advantages of local anesthetic solutions over alternative reagents. Epinephrine, a natural catecholamine, is often a minor component in commonly administered local anesthetic solutions. It primarily acts as a vasoconstrictor to confine the anesthetic to a limited area. Air oxidation is a major problem for epinephrine in pharmaceutical products. The addition of antioxidants, such as sodium metabisulphite, to anesthetic formulations serve to minimize epinephrine oxidation. However, regular testing of commercially available anesthetic solutions is essential to ensure the presence of the minimally required epinephrine concentration stated on the label.

Previous techniques for the characterization and quantitation of epinephrine in anesthetic solutions have relied primarily upon cation exchange and reverse phase HPLC methods. There are only a few reported CE methodologies for epinephrine analysis in formulated products. Peterson and Trowbridge developed a quantitative assay by CE for the determination of the enantiomeric ratio of epinephrine in a pharmaceutical formulation. However, to our knowledge there are no CE methods for epinephrine analysis in anesthetic solutions. Indeed, CE is only beginning to be used as a robust and reliable analytical technique in the pharmaceutical industry. It is well suited for such analyses because of its small sample volume requirements, reduced overall costs and minimal environmental impact.

The purpose of this investigation is to develop a quantitative assay for epinephrine of fifteen different types of dental anesthetic solutions with minimal sample pretreatment. The
developed method was validated by determining the accuracy, precision, linearity, specificity and ruggedness of the assay. Selective focusing of epinephrine allows for superior concentration detection limits with a commercial UV detector.

6.2 Experimental

6.2.1 Buffer Preparation and Chemicals

The aqueous background electrolyte used for CE separation consisted of 160 mM borate (Borax, Sigma Chemical Co., St. Louis, MO) and 1 mM ethylenediaminetetraacetic acid (EDTA, BDH Chemicals, Toronto, Ontario, Canada). The pH of the background electrolyte was adjusted to 10.1 by using 0.1 M NaOH (BDH Chemicals). Lauryl sulfate (sodium dodecyl sulfate, SDS) and epinephrine (epinephrine (-) bitartrate) were purchased from Sigma. Samples that required dilutions were dissolved in either the running buffer, or a solution containing 220 mM glucose (Raylo Chemical Co., Alberta, Canada) and 30 mM para-hydroxybenzoic acid (Eastman Organic Chemicals, New York, USA). Dental anesthetic solutions were provided by Astra Pharma Canada. The dental anesthetic solutions contained lidocaine (Xylocaine™, 9 different products, 5-20 mg/mL), bupivacaine (Sensorcaine™, 3 different products, 2.5-5 mg/mL), carticaine (Astracaine™, 2 different products, 40 mg/mL), or prilocaine (Citanest™, 1 product, 40 mg/mL) as the local anesthetic. Solutions also contained various additives and impurities: sodium metabisulphite, sodium chloride, methylparaben, 2,6-xylidine, o-toluidine and hydroxybenzoic acid. The concentration of epinephrine in dental anesthetic solutions (5-20 μg/mL) is nearly a thousand times lower than the local anesthetic (2.5-40 mg/mL).
6.2.2 Capillary Electrophoresis System

Separations were performed on a P/ACE 5500 automated CE system (Beckman Instruments Inc., Mississauga, Ontario, Canada). Fused silica capillaries (Polymicro Technologies, Phoenix, AZ) with inner diameters of 75 μm, outer diameters of 375 μm and lengths of 57 cm were used. New capillaries were first rinsed with 1.0 M NaOH for 5 minutes under high pressure (20psi), followed by rinsing with the separation buffer for 10 minutes. The capillary was then left to equilibrate overnight in the separation electrolyte prior to use. Each separation was preceded by a 1.5 minute high pressure rinse with 0.1 M NaOH, followed by a 4 minute rinse with the separation electrolyte. The samples were then introduced using a 25 second low pressure injection (0.5 psi) and the separation was carried out for 15 minutes at 15 kV under a temperature of 25 °C. Absorbance was monitored at 280 nm and data was collected and processed using Beckman P/ACE station version 1.0 software.

6.3 Results and Discussion

6.3.1 Separation Optimization

The design of CE separation systems can be facilitated through understanding of the fundamental parameters influencing the mobility of an analyte, in conjunction with knowledge of its specific chemical properties. Figure 6.1 shows the structures of the various components used in the dental anesthetic formulations. Borate was selected as the background electrolyte because of its ability to selectively complex with analytes having vicinal diols. Epinephrine, a catecholamine, should be the only analyte in the anesthetic solution capable of complexing with borate. In this case, borate acts as the buffer and as a selective complexing agent (additive). The separation of epinephrine from the neutral solutes is optimized using 160 mM borate, pH 10.1. The high ionic strength and pH of the buffer
Figure 6.1 Schematic of the various components contained in the dental anesthetic solutions.
enhances borate complexation with epinephrine. It was observed that the addition of 1 mM EDTA to the run buffer resulted in better reproducibility and sharper peaks in epinephrine analysis. Figure 6.2 depicts two electropherograms obtained from commercially available multi-use and single-use (Xylocaine) dental anesthetic solutions. The anesthetic solutions were directly injected into the capillary with no sample pretreatment. Epinephrine is separated from the excess of neutral components (lidocaine, methylparaben etc.) which elute with the electroosmotic flow. Excellent concentration sensitivity for epinephrine was observed due to the large sample volumes injected.

As depicted in Figure 6.2, a very high column efficiency (N = 180 000) was obtained for epinephrine, even though a relatively large sample plug (5.7% of the total capillary length or 150 nL) was used. In contrast, lidocaine migrates as a broad plug. Normally, the separation in CE is severely compromised when the plug length is greater than 1 % of the capillary (typically less than 10 nL) because of band broadening. Sample matrix plays a vital role in CE separation performance. The anesthetic solutions were observed to be relatively acidic with a pH ranging from 3 to 4. A possible explanation of epinephrine focusing in this system is the development of a dynamic pH junction that occurs between the injected acidic sample zone and background basic buffer zone. Epinephrine is a zwitterion that possesses opposing mobilities under acidic (positively charged) and basic (negatively charged) conditions. Hence, epinephrine would migrate rapidly to the front edge of the sample plug with a positive electrophoretic mobility until it comes into contact with the background electrolyte where it would migrate with a negative electrophoretic mobility. Epinephrine is focused because of the difference in its migration behavior in the low pH sample solution and the high pH background electrolyte. The hydroxide ions in the background electrolyte migrate through the sample plug and raise the pH of the plug, and the focused epinephrine then
Figure 6.2 Two electropherograms showing the separation of epinephrine (peak 1) from excess solute components by directly injecting (A) multi-use and (B) single-use Xylocaine dental anesthetic solutions that contain 5 and 10 μg/mL of epinephrine, respectively.
migrates through the rest of the capillary with a negative electrophoretic mobility. This focusing method does not need ampholytes, and should be generally applicable to all zwiterionic analytes such as amino acids and peptides, as long as the pH difference between the sample solution and the background electrolyte is adjusted according to the isoelectric point of the analyte of interest. Aebersold and Morrison\(^9\) utilized a pH junction in the focusing of peptides, by altering the pH of the sample solution relative to the background buffer. Epinephrine focusing in this case arises naturally (or rather, fortunately) due to the acidic conditions of the anesthetic formulation and the basic background electrolyte. An important aspect of this preconcentration technique is that focusing can still occur with the presence of high concentrations of salt (155 mM NaCl). Most other sample stacking techniques require a plug of low conductivity solution.\(^{10-14}\) On-column preconcentration techniques using transient isotachophoresis\(^{15-17}\) and sample stacking by acetonitrile-salt mixtures\(^{18,19}\) also permit the use of larger injection plugs to enhance concentration sensitivity in CE in the presence of high salt in the sample. Further work is presented in Section D of the thesis to better understand this novel type of focusing phenomenon, including ways to apply it to other specific analytes of interest.

### 6.3.2 Dental Anesthetic Solubility

Despite the encouraging results obtained with epinephrine analysis from Xylocaine solutions, the direct injection of three other dental anesthetic formulations (Astracaine, Sensorcaine and Citanest) with the aqueous borate buffer resulted in sample precipitation in the capillary. Efforts were made to improve the solubility of these hydrophobic anesthetics in the alkaline conditions of the original buffer system. The addition of acetonitrile or methanol to the borate buffer prevented sample precipitation, but resulted in the loss of
Figure 6.3 Electropherogram showing the separation of epinephrine (1) from excess prilocaine in *Citanest* dental anesthetic solution that contains 5μg/mL epinephrine. Sample was diluted by 50% with run buffer prior to injection.

Figure 6.4 Electropherogram showing the separation of epinephrine (1) from excess bupivacaine in *Sensorcaine* dental anesthetic solution that contains 5μg/mL epinephrine. Sample was diluted 75% with glucose-hydroxybenzoic acid solution prior to injection.
Figure 6.5 Electropherogram depicting the separation of epinephrine (1) from excess carticaine of Astracaine dental anesthetic solutions containing 5 μg/mL epinephrine, using 50 mM SDS in the run buffer.

It was observed that dilution of the dental anesthetic solutions prior to injection prevented sample precipitation, while retaining excellent separation. Figures 6.3 and 6.4 show the electropherograms obtained from Citanest and Sensorcaine. Citanest was diluted with aqueous borate buffer. The high insolubility of Sensorcaine in the borate buffer required dilution with a viscous and acidic buffer solution made from glucose and hydroxybenzoic acid. This solution was used since it resembles the original matrix of the dental anesthetic solution. Astracaine solutions that were diluted with the preceding solution
were soluble in the run buffer, but a large background signal overlapped with epinephrine elution. The use of 50 mM SDS in the borate buffer removed the background noise, resulting in a good separation of epinephrine from other components in the solution (Figure 6.5). Dilution of Astracaine solutions was also carried out with the 50 mM SDS borate buffer. Table 6.1 summarizes the sample preparations for the dental anesthetic solutions used in this report.

6.3.3 Method Validation

Appropriate method validation information concerning the use of a new analytical technique for the analysis of pharmaceuticals is required by regulatory authorities. The precision of the method was evaluated in terms of repeatability (same day) and reproducibility (inter-day and inter-analyst). Six replicate injections of three standard epinephrine solutions: 2 μg/mL, 10 μg/mL and 32 μg/mL, were used for the repeatability study. The repeatability for epinephrine standards, based on average peak areas, gave an RSD of 2.3%. The main factor responsible for the low precision is the long injection times that were used for the analyses. Six replicate injections of four different anesthetic solutions done on two different days by one analyst (inter-day) and by two analysts (inter-analysts), a total of 48 runs each, were used for the reproducibility study. Both inter-day and inter-analyst reproducibility studies gave an average RSD of 2.0%.

The linearity of the detector response vs. concentration was tested by a calibration curve constructed from three sets of standard solutions of epinephrine at seven different concentrations, ranging from 2.00 to 32.0 μg/mL. Linear regression of the calibration curve gave a linear equation, with a correlation coefficient (R²) of 0.9994. The limit of detection (LOD, S/N = 3) for epinephrine determination by this CE method was 90 ng/mL (or 5 × 10⁻⁷
<table>
<thead>
<tr>
<th>Dental Anesthetic Solution</th>
<th>Sample Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylocaine</td>
<td>No dilution required</td>
</tr>
<tr>
<td>Citanest</td>
<td>Two-fold dilution with running buffer</td>
</tr>
<tr>
<td>Sensorcaine</td>
<td>Three-fold dilution with hydroxybenzoic acid buffer</td>
</tr>
<tr>
<td>Astracaine</td>
<td>Two-fold dilution with running buffer</td>
</tr>
</tbody>
</table>
Figure 6.6 Comparison of the electropherograms of (A) adenochrome and (B) adenochrome spiked with epinephrine (1) that highlights the selectivity of the separation.
M). The LOD was estimated based on Knoll's method. The concentration sensitivity for epinephrine can be further extended by using larger injection volumes, in conjunction with longer and wider capillaries.

Accuracy and recovery of the method was determined by analyzing four different anesthetic solutions fortified with epinephrine at 40, 100 and 160% of the label claim. The average percent recovery was 95.3%, with a correlation coefficient ($R^2$) of 0.9987.

Method specificity was determined by two lines of evidence: the spiking of pure components in the anesthetic solutions and observing the resultant electropherogram and by comparing the electropherograms of epinephrine and adenochrome (oxidized epinephrine products) to ensure no interference in epinephrine quantitation. The spiking of various components in the anesthetic solutions confirmed the separation of epinephrine from the matrix. In addition, the use of a diode array detector confirmed that the absorbance spectrum of the peak in the electropherogram matched that of pure epinephrine. As shown in Figure 6.6, the analysis of adenochrome demonstrated that the oxidized products of epinephrine did not interfere with epinephrine quantitation. Adenochrome consisted of a mixture a highly charged solutes which migrated after epinephrine. Table 6.2 summarizes the validation protocol for epinephrine using the CE method.

6.4 Conclusion

A robust method for the quantification of epinephrine from fifteen dental anesthetic solutions by CE is developed. Appropriate dilution of six of the fifteen anesthetic formulations was necessary to prevent precipitation, whereas the remaining solutions were injected directed into the capillary. The use of the anionic surfactant SDS to the optimized run buffer was required to improve the separation of astracaine solutions. A novel focusing
Table 6.2 Validation protocol for epinephrine analysis using CE

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeatability (n=18)</td>
<td>RSD = 2.3%</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>RSD = 1.9%</td>
</tr>
<tr>
<td>Inter-day (n=48)</td>
<td>RSD = 2.0%</td>
</tr>
<tr>
<td>Inter-analyst (n=48)</td>
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<tr>
<td>Linearity  y = peak area</td>
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</tr>
<tr>
<td></td>
<td>R² = 0.9994</td>
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<tr>
<td>LOD (S/N = 3)</td>
<td>90 ng/mL</td>
</tr>
<tr>
<td>Accuracy and recovery (n=12)</td>
<td>Average recovery = 95.3%</td>
</tr>
<tr>
<td>Correlation plot  y = theoretical conc. (µg/mL)</td>
<td>y = 1.017x + 0.2766</td>
</tr>
<tr>
<td></td>
<td>R² = 0.9987</td>
</tr>
<tr>
<td>Specificity</td>
<td>(1) by spiking of epinephrine</td>
</tr>
<tr>
<td></td>
<td>(2) by analysis of adenochrome</td>
</tr>
</tbody>
</table>
phenomenon is observed to arise naturally for epinephrine in the presence of high concentrations of salt and other components contained in the sample solutions. Selective epinephrine focusing is believed to arise naturally due to the development of a dynamic pH junction between the sample and the background electrolyte. This allowed the injection of large amounts of sample, resulting in improved concentration sensitivity for epinephrine analysis by CE with UV detection. The limit of detection for epinephrine is $5 \times 10^{-7}$ M when using long injection plugs with a commercial UV absorbance detector. The CE assay for epinephrine was supported by method validation which demonstrated excellent precision, accuracy, linearity and specificity.

6.5 References


Section D:

Selective Analyte Focusing Using a Discontinuous Electrolyte System in Capillary Electrophoresis.
Chapter VII:

Selective Focusing of Catecholamines and Weakly Acidic Analytes in CE Using a Dynamic pH Junction.

"Design of a powerful separation system in CE: Combining the virtues of excellent selectivity and sensitivity."
7.1 Introduction

CE is currently one of the most powerful separation technologies available to the analytical chemist. The effective heat dissipation of narrow bore capillaries permits the application of high electric voltages, thereby increasing the efficiency and reducing the analysis time of a separation. Moreover, both electric field and equilibria can be employed to induce the separation of diverse analytes ranging from metal ions, to neutral, charged, or chiral molecules, to large biological macromolecules. However, CE suffers from poor concentration sensitivity due to small injection volumes and its reduced optical detector pathlength, in comparison to most chromatographic methods. Emerging microfabricated separation devices present even greater limitations on analyte concentration sensitivity. Despite the relative success of laser-induced fluorescence and electrochemical detection to improve the concentration sensitivity, the most universal and widely applicable detection mode is UV absorbance. Two ways have been used to improve the sensitivity of CE with UV detection: to enlarge the detector pathlength or to increase the amount of sample injected into the capillary. The effective optical pathlength for detection in CE can be extended to a limited extent with a Z-cell format or other capillary geometries, but at the cost of reduced capillary efficiency. Sample enrichment via an off-line solid-phase extraction method may also be used to improve concentration sensitivity prior to separation by CE. However, it is generally difficult to automate with existing commercial instruments. Ideally, the most facile way to improve concentration sensitivity in CE is to increase the amount of sample that can be injected directly into the capillary. Usually the sample volume may not exceed 1% of the
total capillary volume (typically less than 10 nL) because of the deleterious effects of band broadening due to sample overloading. Hence, there is a need to develop practical methods to increase the amount of sample that can be loaded into the capillary, with minimal affect on separation performance.

Several on-line preconcentration techniques have been developed for increasing the amount of sample that may be injected into the capillary. All sample concentration strategies exploit differences in the physicochemical properties between the sample matrix and the background electrolyte. Such a discontinuous buffer system is vital to promote analyte focusing through differences in its conductivity, salt content, buffer pH, organic content or additive concentration. Specific analyte molecules that possess different electrophoretic mobilities within these two regions may focus into a sharp zone during the separation process. Sample stacking is one of the most common approaches to improve concentration sensitivity in CE. When the sample matrix has a significantly lower conductivity than that of the background electrolyte, an amplified electric field is distributed across the sample zone. Analytes within the sample zone are then accelerated with a local amplified electrophoretic mobility and stack at the boundary of the background electrolyte zone, where its mobility is significantly reduced. Sample stacking has been applied to charged and recently, neutral analytes using either hydrodynamic or electrokinetic injection. Large volume sample stacking and field amplified sample injection permit the injection of extremely large amounts of sample into the capillary, resulting in greater than a 100-fold increase in sensitivity. However, one major prerequisite for most sample stacking methods is that the analyte resides in a low salt environment relative to the background electrolyte. Thus, samples of biological or environmental origin often require desalting prior to analysis. Better understanding of sample matrix effects on analyte focusing is needed to enable the design of effective sensitivity enhancement strategies in CE.
On-line preconcentration techniques in CE that are suitable to samples in high salt are of particular interest to the analytical chemist. To this effect, sample stacking of analytes with acetonitrile, in the presence of high salt, has been reported by Shihabi.\textsuperscript{16-18} This technique is particularly well suited for the analysis of small molecules in plasma, since acetonitrile is also used for deproteinization of serum proteins prior to CE analysis. A pH mediated stacking of analytes in high salt has also been used to improve concentration sensitivity in CE.\textsuperscript{19,20} Titration of the sample with acid or base after injection induces a localized amplified field in which analytes are stacked. Enhancement in the amount of sample injected into the capillary has also been carried out by mechanisms other than sample stacking. Transient isotachophoresis is an on-line preconcentration technique that can focus specific analytes of interest in the presence of high salt.\textsuperscript{21-25} The focusing of sample components is induced when specific analytes have mobilities intermediate to that of a leading and terminating electrolyte, which are contained in the sample and background electrolyte. The ability to specifically tune both the resolution and sensitivity in CE, based on fundamental chemical properties of individual analytes, is needed to make CE a realistic alternative to help solve the increasing complexity of sample mixtures.

The catecholamines, such as epinephrine, norepinephrine and dopamine, are important neurotransmitters and neurohormones of the central and sympathetic nervous systems.\textsuperscript{26} They have diverse and wide ranging effects on the human body and provide central control of various autonomic and hormonal functions. Moreover, several neurological disorders, including Parkinson's disease, schizophrenia, anxiety disorders and memory impairment, have been associated with improper catecholamine regulation.\textsuperscript{27} The monitoring of catecholamines by CE often requires sensitive laser-induced fluorescence\textsuperscript{28-30} or electrochemical detection\textsuperscript{31-33} in order to quantitate low concentrations of these neurotransmitters normally found in tissue. It would be useful to further extend the
concentration sensitivity of catecholamines with UV absorbance detection, through simply increasing the volume of sample injected into the capillary.

A unique focusing phenomenon has been previously reported by our group for epinephrine analysis of dental anesthetic solutions by CE. Large sample volumes (150 nL) of the dental anesthetic solutions were directly injected into the capillary, resulting in pronounced epinephrine focusing in the presence of high salt. However, the mechanism of the focusing was not clear. The aim of this investigation was to mimic epinephrine focusing observed in dental anesthetic solutions, in order to better understand the influence of specific sample matrix components involved with this technique. The effect of salt, organic additives, buffer and pH on epinephrine focusing was examined. It was observed that pH and borate complexation were the most important factors contributing to epinephrine focusing. The development of a dynamic pH junction between buffered sample and background electrolyte zones was hypothesized to be vital for selective epinephrine focusing, even in the presence of high salt. This focusing technique was also applied to norepinephrine, dopamine, tyrosine and phenol. To date, there has been only one reported use of a pH junction to enhance concentration sensitivity in CE. Aebersold and Morisson used a dynamic pH junction to improve the detection of zwitter-ionic peptides. In this paper, it was demonstrated that the use of a dynamic pH junction can induce the focusing of not only zwitter-ionic analytes, but also any weakly acidic species, that possesses different mobilities in the sample and background electrolyte zones. Dynamic pH junctions have already been used to optimize the separation of weakly acidic and basic analytes in CE. In this case, a dynamic pH junction was used to provide a simple way to selectively focus analytes of interest, based on their unique chemical properties.
7.2 Experimental

7.2.1 Chemicals

The aqueous separation buffer consisted of 160 mM borate (Borax, Sigma Chemical Co., St. Louis, MO) and 1 mM ethylenediaminetetraacetic acid (EDTA, BDH Chemicals, Toronto, Ont., Canada). The pH of the separation buffer was adjusted by using 1.0 M NaOH (BDH Chemicals) or 2.0 M HCl (Fischer Scientific, Nepean, Ont., Canada) within a range of pH 8.5 to 11.0. HPLC grade acetonitrile was purchased from Fischer Scientific. Epinephrine (epinephrine (-) bitartrate), norepinephrine (arterenol (-) bitartrate), dopamine (3-hydroxytyramine hydrochloride), DOPA (D, L-3, 4-dihydroxyphenylalanine), and tyrosine were all purchased from Sigma. Catechol, phenol, sodium metabisulfite and sodium chloride were obtained from BDH Chemicals. All standard solutions of the analytes used in this study were dissolved in aqueous 160 mM borate, 155 mM NaCl, 3 mM sodium metabisulfite, and 1 mM EDTA, pH 8.5, unless otherwise stated. Injection studies performed initially on epinephrine standard solutions also contained 220 mM glucose (Raylo Chemical Co., Alberta, Canada) and 30 mM para-hydroxybenzoic acid (Eastman Organic Chemicals, New York, USA). These solutions were made in order to mimic the composition of dental anesthetic solutions that contained high concentrations of the local anesthetic (20 to 180 mM) and various additives, such as sodium chloride, sodium metabisulfite, methylparaben and hydroxybenzoic acid. The dental anesthetic solutions were acidic, with a pH of about 3.5.

7.2.2 Apparatus and Procedure

Separations were performed on a Beckman P/ACE 5000 automated capillary electrophoresis system (Beckman Instruments Inc., Mississauga, Ont., Canada). Uncoated capillaries (Polymicro Technologies, Phoenix, AZ) were used with inner diameters of 75 μm,
outer diameters of 375 μm and lengths of 57 cm, 47 cm or 37 cm. New capillaries were first rinsed with 1.0 M NaOH (5 minutes, 20 psi), followed by rinsing with the separation buffer (10 minutes, 20 psi). The capillary was then left to equilibrate overnight in the separation buffer prior to use. Each separation was preceded by a 1.5 minute, 20 psi rinse with 0.1 M NaOH, followed by a 4 minute rinse with the separation buffer. The samples were then introduced using a low pressure 0.5 psi injection (varied from 2 to 99 seconds) and the separation was carried out for 15 minutes at 15 kV (normal polarity) under a temperature of 25 °C. The average flow rate of the low pressure (0.5 psi) injection was determined to be 7.87 cm/min using a 57 cm capillary. Absorbance detection was made with a Beckman P/ACE UV-Vis detector at 280 nm. Data were collected and processed using System Gold software (Beckman).

7.3 Results and Discussion

7.3.1 Band Broadening vs. Band Narrowing

In CE, band broadening or peak variance, $\sigma^2$ is the result of a number of different affects that contribute additively to the total spreading of the peak during separation, $\sigma_T^2$;

$$\sigma_T^2 = \sigma_D^2 + \sigma_I^2 + \sigma_J^2 + \sigma_E^2 + \sigma_A^2 + \sigma_W^2 + \sigma_O^2$$

(7.1)

where, the terms on the right side of eq. 7.1 represent variances caused by diffusion ($\sigma_D^2$), injection ($\sigma_I^2$), Joule heating ($\sigma_J^2$), electrophoretic dispersion ($\sigma_E^2$), adsorption ($\sigma_A^2$), width of detector zone ($\sigma_W^2$) and other effects ($\sigma_O^2$), respectively. Under ideal conditions, the major contribution to band broadening in CE is longitudinal diffusion. However, the variance caused by injection may dominate all other band broadening processes, including diffusion, if the volume injected is not limited to about 10 nL, or less than 1% of the total capillary length. Injection volumes much greater than this result in sample overloading and significant band
broadening. Excessive analyte dispersion results in decreased sensitivity and limited peak resolution. Thus, the use of large sample volumes to improve concentration sensitivity in CE is only possible if there exists a focusing mechanism to counter the effects of injection length on the total band variance.

In order to quantitatively determine whether focusing is induced in a sample, the injection and detector bandwidths can be measured and compared. In CE, peak widths are time dependent due to the differential migration rates of different species past the detector. This is not the case in other column separations, such as HPLC, where all species move past the detector at the same rate. Hence, bandwidth and peak width are not equivalent terms. Bandwidths are the lengths of the analyte zones in the capillary, and do not depend on the migration rates of the analyte. The detector bandwidth, which is the width of the analyte band when it is migrating through the detector window, is calculated from an electropherogram using the measured peak width and the speed of the analyte past the detector. The injection bandwidth, which is the width of the injected sample plug, may be calculated from the measured flow rate of a low pressure injection and the total injection time.

After injection, the analytes in the sample plug are normally subject to dispersive forces during electromigration prior to detection. Therefore, the bandwidth at the detector, \( w_{\text{det}} \) can be expected to be larger than the injection bandwidth, \( w_{\text{inj}} \) due to diffusion and other band broadening processes described in eq. 7.1. One may assess the degree of focusing by calculating the detector to injection bandwidth ratio (DIBR). Two scenarios may occur with the sample plug: band broadening and band narrowing, which are defined by \( \frac{w_{\text{det}}}{w_{\text{inj}}} > 1 \) and \( \frac{w_{\text{det}}}{w_{\text{inj}}} \leq 1 \), respectively. Band broadening is normally encountered in conventional CZE.
Figure 7.1 Schematic of the effect of on-line focusing of a dilute sample in CE using a (I) continuous and (II) discontinuous electrolyte system. Injection of a large volume of sample into the capillary in a continuous electrolyte system (I) results in normal band dispersion and broad, poorly resolved peaks, whereas a discontinuous electrolyte system (II) may permit analyte focusing into narrow zones (reduced volumes) prior to zone separation. This improves detector sensitivity, while maintaining resolution of the peaks.
experiments using a continuous buffer system, whereas band narrowing may occur with isotachophoretic and isoelectric focusing modes of CE that utilize a discontinuous buffer system. Analyte focusing is indicative when the measured DIBR is less than one. In other words, the effect of dispersion is countered by a greater focusing effect that reduces the size of the sample plug, permitting the use of larger sample injection volumes for improved concentration sensitivity. Figure 7.1 depicts the effect of a large volume injection of a dilute mixture in CE when either normal band broadening or band narrowing is operative. Since it is customary to dissolve the sample in a matrix that closely resembles that of the separation buffer (to minimize electrodispersion), a continuous electrolyte system is most often used in capillary zone electrophoresis. However, there are certain conditions when changing the nature of the sample matrix relative to the background buffer can be advantageous for sensitivity enhancement (i.e., discontinuous electrolyte system). It is apparent that focusing of analytes results from the compression of the initial large sample zone into narrow bands prior to detection. This not only improves detector sensitivity, but also enhances separation resolution due to peak sharpness. After focusing, analytes separate normally by zone electrophoresis since the electrolyte discontinuity in the capillary is gradually dissipated with replacement of the sample with the background electrolyte at the inlet. In contrast, normal band dispersion using a continuous electrolyte system results in broad peaks that are poorly resolved. Band focusing is the result of the differential electrophoretic mobility of the same analyte in two different electrolyte zones (discontinuous) distributed within the capillary, whereas band separation is due to differential electrophoretic mobility of different analytes in a continuous electrolyte system. The mobility of an analyte can be modified by specific properties of the sample matrix relative to the background electrolyte used in the separation.
Figure 7.2 Chemical structures of the catecholamines and their related analogues used in the investigation of selective analyte focusing using a dynamic pH junction: 1-epinephrine, 2-norepinephrine, 3-dopamine, 4-DOPA, 5-tyrosine, 6-catechol, 7-phenol.

7.3.2 Sample Matrix Effects on Epinephrine Focusing

The initial observation of epinephrine focusing from the direct injection of large volumes (150 nL or 6% of total capillary length) of dental anesthetic solutions as demonstrated in chapter VI (see Figure 6.2), was surprising since it occurred under normally unfavorable conditions. First, the injection of high salt samples that possess a matrix different from the run buffer is generally detrimental to separation performance in CE because of electrophoretic dispersion. Secondly, the injection of sample volumes greater than 1% of the capillary most often leads to deleterious analyte band broadening due to the large injection bandwidth. The main purpose of this work was to investigate the factors responsible for
epinephrine focusing in the dental anesthetic matrix. In order to assess the specific analyte properties required for focusing under these conditions, a systematic study of the catecholamines and several other related chemicals was investigated. The structures of the test analytes used in this study are depicted in Figure 7.2.

A preliminary injection study of various epinephrine standard solutions was conducted to identify which sample matrix components were important for analyte focusing in this system. The influence of salt and various types of organic additives were studied. Figure 7.3 depicts a series of electropherograms in which epinephrine standard solutions were injected for 25 seconds (I) and 50 seconds (II). These injection times correspond to volumes of about 150 nL and 300 nL of sample, respectively. Each standard solution contained $2.7 \times 10^{-5}$ M of epinephrine, 3 mM sodium metabisulfite and 1 mM EDTA, but differed with respect to other additives added to the sample matrix. All the separations were conducted using 160 mM borate, 1 mM EDTA, pH 10.2 as the running buffer.

Epinephrine solutions containing no added sodium chloride [Fig. 7.3(A)] and 155 mM sodium chloride [Fig. 7.3(B)], both exhibited serious band broadening, as was normally expected with such large injection plug lengths. Despite having a lower conductivity than the running buffer, Figure 7.3(A) demonstrates that epinephrine dissolved in a low ionic strength matrix still did not focus efficiently using such large volumes. Figure 7.3(C) shows the effect of adding acetonitrile and salt on analyte stacking as reported previously by Shihabi. It was observed that significant focusing of epinephrine did occur with acetonitrile and salt addition to the sample matrix. However, the exact mechanism of analyte focusing through this procedure is still uncertain. Changes in the apparent pH, viscosity and/or conductivity of the sample matrix with organic solvent-salt addition may be operative. As an attempt to mimic the viscous conditions of the anesthetic formulation, the effect of adding 220 mM
Figure 7.3 The influence of specific matrix components on epinephrine focusing using a (I) 25 and (II) 50 second injection. All sample solutions contain 27 μM epinephrine, 3.0 mM sodium metabisulphite and 1.0 mM EDTA (A), in addition to 155 mM NaCl (B), 155 mM NaCl in 60% acetonitrile (C), 155 mM NaCl and 220 mM glucose (D), 155 mM NaCl, 220 mM glucose and 30 mM hydroxybenzoic acid (E). Conditions: aqueous 160 mM borate buffer, 1 mM EDTA, pH 10.2; voltage, 15 kV; current, 130 μA; capillary length 57 cm. Note that the time axes for the electropherograms were shifted horizontally for clarity sake.
glucose (a substitute for the local anesthetic) as an additive to the sample, in the presence of salt was also examined. Figure 7.3(D) demonstrates that the addition of a viscous organic additive, such as glucose, with salt was observed to induce notable epinephrine focusing. It is apparent that the induction of sample focusing is not unique to acetonitrile alone. However, when hydroxybenzoic acid (a minor component contained in anesthetic solutions) was added with glucose and salt, pronounced epinephrine focusing was observed, as shown in Figure 7.3(E). In addition, it was determined that hydroxybenzoic acid in the sample matrix alone (without glucose) was vital for epinephrine focusing, since it produced similar band narrowing as in Figure 7.3E. Also, when the pH of this sample solution (pH 2.80) was varied to a pH of 10.2 (equivalent to the run buffer), no focusing was observed for epinephrine. Moreover, although all the standard epinephrine solutions that contained sodium metabisulfite and EDTA were acidic (pH 3.0 to 3.5), it was noticed that only the dental anesthetic solutions and hydroxybenzoic acid-salt solutions were relatively buffered, in comparison to the other standard solutions used in the study. Thus, it was surmised that the difference in pH between the sample and the separation buffer was the most important factor affecting epinephrine focusing. Furthermore, epinephrine focusing occurred primarily when both sample and separation electrolyte were relatively buffered.

7.3.3 Epinephrine Focusing Via a Dynamic pH Junction

The effect of buffer pH on epinephrine focusing was next examined. Figure 7.4 depicts a series of electropherograms in which the sample is dissolved in borate buffer with a pH ranging from 8.5 to 10.2, while the pH of the background buffer is fixed. Figure 7.4(I) shows the influence of sample pH on epinephrine focusing, when the background buffer of 160 mM
borate, 1 mM EDTA is pH 10.2. When the pH of the sample and the background electrolyte is the same [Figure 7.4(I)A, pH 10.2], epinephrine migrates as a long, diffuse plug. However, as the pH of the sample is decreased incrementally, epinephrine gradually focuses into a sharp band. A difference of only approximately 1.7 pH units is required to achieve significant band narrowing. Optimal epinephrine focusing was determined to occur with a pH difference of 1.9, when using a borate buffer at pH 10.4, and a sample pH 8.5. Similarly, Figure 7.4(II) shows almost a mirror image of Figure 7.4(I), in that the separation buffer is fixed at pH 8.5. Again, when both the sample and background electrolyte have the same pH, no epinephrine focusing is observed. A gradual increase of the sample pH relative to the background buffer results in pronounced epinephrine focusing, that occurs when the pH of the sample is about 1.7 units greater than that of the run buffer. Figure 7.4(I) is referred to as a normal pH junction, since it reflects the focusing observed previously with dental anesthetic solutions, when the pH of the sample is lower than that of the buffer, whereas Figure 7.4(II) is a reversed pH junction for epinephrine. The presence of high salt in the sample did not deteriorate the focusing. The use of 40 mM phosphate, pH 4.5 as a buffer in the sample solution also resulted in epinephrine focusing. Hence, one may use mixed buffer systems to effectively control the pH of the electrolyte over a wide range.

In order to quantitatively assess the magnitude of epinephrine focusing, the values for the injection and detector bandwidths were compared. Table 7.1 shows the measured injection and detector bandwidths, as well as the detector to injection bandwidth ratio, at the various sample pH values for epinephrine from the electropherograms depicted in Figure 7.3(I). When both the sample and background zones have the same pH, Fig. 7.3(I)A, the detector bandwidth for epinephrine increases over 3-fold from the initial injection bandwidth of 3.3
Figure 7.4 Electropherograms demonstrating the use of a dynamic pH junction on epinephrine focusing via a (I) normal pH junction and (II) reversed pH junction. The background buffer used is 160 mM borate, 1mM EDTA at a pH of (I) 10.2 and (II) 8.5. All sample solutions contained 27μM epinephrine, 160 mM borate, 155 mM NaCl, 3 mM sodium metabisulfite and 1 mM EDTA. The pH of the sample is varied incrementally from 10.2 (A), 9.5 (B), 9.0 (C), 8.5 (D), 8.5 (E), 9.0 (F), 9.5 (G) and 10.2 (H). Conditions are the same as Figure 7.3.
Table 7.1 Bandwith values of epinephrine focusing when using a dynamic pH junction.

<table>
<thead>
<tr>
<th>Sample pH</th>
<th>Injection Bandwidth $w_{\text{inj}}$ (cm)</th>
<th>Detector Bandwidth $w_{\text{det}}$ (cm)</th>
<th>$W_{\text{det}}/W_{\text{inj}}$ (DIBR)</th>
<th>Net Effect of Bandwidth Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.2</td>
<td>3.3</td>
<td>10.2</td>
<td>3.1</td>
<td>Broadening</td>
</tr>
<tr>
<td>9.5</td>
<td>3.3</td>
<td>6.4</td>
<td>1.9</td>
<td>Broadening</td>
</tr>
<tr>
<td>9.0</td>
<td>3.3</td>
<td>1.7</td>
<td>0.5</td>
<td>Narrowing</td>
</tr>
<tr>
<td>8.5</td>
<td>3.3</td>
<td>0.7</td>
<td>0.2</td>
<td>Narrowing</td>
</tr>
</tbody>
</table>

The background electrolyte in all runs consisted of 160 mM borate, pH 10.2, as depicted in Figure 7.3 (I).
cm. Significant band broadening had occurred since the DIBR is much greater than one, with a value of 3.1. Thus, the combined effects of long injection length, longitudinal diffusion and electrophoretic dispersion (sample has a high salt content), along with other possible sources of band dispersion, result in an extremely broad analyte zone (10.2 cm) migrating past the detector window. However, as the pH of the sample is gradually decreased, band narrowing of the epinephrine zone occurs, reflected by a DIBR of less than one. For instance, when the sample pH is changed to 8.5 (Fig. 7.3 (I)D), the DIBR is 0.22. Thus, the initial injection zone is narrowed by nearly 5-fold, whereas the expected detector bandwidth (if the sample pH is 10.2) is reduced almost 15-fold, to a bandwidth of only 0.7 cm.

7.3.4 Electrophoretic Mobility Differences Under a Dynamic pH Junction

As shown in Figure 7.2, epinephrine is a zwitter-ion possessing both a basic secondary amine and an acidic di-hydroxy functionality. Borate can selectively complex with vicinal diols\(^{40}\) to further decrease the acidity of the catecholamine. The electrophoretic mobility of epinephrine, using 160 mM borate, 1 mM EDTA, pH 8.5 as the run buffer, is extremely low at \(-2.12 \times 10^{-5} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}\), migrating close to the EOF (Figure 7.5A). Thus, significant borate complexation occurs under this pH to provide a large enough negative charge to counterbalance the positive charge generated by the secondary ammonium group. However, epinephrine has a significantly larger negative electrophoretic mobility of \(-1.41 \times 10^{-4} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}\) at pH 10.4 (Fig. 7.5B). This pH approaches the pK\(_a\) of the secondary amine (pK\(_a\) = 10.5),\(^{41}\) thereby reducing the positive charge contributed by the amine, and increasing the overall negative electrophoretic mobility of epinephrine. Also, borate complexation tends to increase at higher pH values. Thus, epinephrine focusing may be caused by the dramatic changes in its electrophoretic mobility within the sample and background electrolyte zones,
Figure 7.5 Series of electropherograms depicting the changes in mobility of epinephrine using a continuous (A) pH 8.5, (B) pH 10.4, and a discontinuous electrolyte system (C) dynamic pH junction: sample pH 8.5 and background pH 10.4. Electropherograms (A) and (B) used a 2 second injection of 100 μM epinephrine, whereas (C) used a 25 second injection of 10 μM epinephrine. All samples also contained 160 mM borate, 3 mM sodium metabisulphite and 1 mM EDTA. Conditions are the same as Fig. 7.3.

caused by differences in borate complexation and pH in these two zones. Figure 7.5(C) shows the net effect of using a pH junction between sample and background electrolyte zones, that permits longer injection volumes, increased concentration sensitivity and enhanced separation efficiencies. It is apparent that epinephrine migrates with a reduced average electrophoretic mobility (shorter migration time) because a portion of the capillary is filled with the low pH buffer of the sample. Also, the apparent mobility of epinephrine using a dynamic pH junction is an average of the mobility fractions of the neutral (zwitter-ion) and negatively charged species. The normal LOD (S/N = 3) for epinephrine by UV detection at
280 nm, using a 2 second injection time (12 nL), was found to be about $1.0 \times 10^{-5}$ M. However, the application of this pH junction, using a 99 second injection time (540 nL or about 23% of the total capillary length), extended its LOD by 250-fold to about $4.0 \times 10^{-8}$ M. Despite the very long sample plug lengths injected, separation efficiencies were excellent with theoretical plates greater than $4.0 \times 10^5$. It was observed that the use of large injection volumes with deep UV detection at 200 and 214 nm resulted in significant background noise, which negated the advantages of the higher molar absorptivity for epinephrine at these wavelengths. It is apparent that epinephrine focusing via a pH junction is a powerful band narrowing technique, since the bandwidth of Figure 7.5(C) (0.6 cm) is narrower than Figure 7.5(B) (1.0 cm), despite over a 12-fold increase in the sample volume injected. Consequently, normal diffusional band broadening processes are countered by a greater focusing effect, highlighted by a DIBR of 2.56 (broadening) and 0.18 (narrowing) for Figures 7.5(B) and (C), respectively. The pH junction that is generated between sample and buffer zones has a limited lifetime, since the background buffer gradually re-establishes the pH of the capillary, which is reflected by eventual stabilization of the current. Hence, this type of focusing effect is referred to as a dynamic pH junction.

### 7.3.5 Selective Focusing of Catecholamines Using a Dynamic pH Junction

The effect of analyte focusing using a pH junction on the two other related catecholamines, norepinephrine and dopamine, was next examined. Figure 7.6 shows the measured theoretical plates (reflecting the degree of band narrowing) of the three catecholamines, as the pH of the background electrolyte is varied incrementally from pH 8.5 to 11.0, while the sample is fixed at pH 8.5. It was observed that each catecholamine focused at a unique pH. In addition, the optimal pH required for catecholamine focusing generally
Figure 7.6 Plot of the measured plate number (N) versus pH of the running buffer for the three catecholamines: 1, epinephrine; 2, norepinephrine; 3, dopamine. Selective focusing of the analytes via a dynamic pH junction occurs at unique pH values. All samples contained 10 μM catecholamine in 160 mM borate, 155 mM NaCl, 3 mM sodium metabisulphite and 1 mM EDTA, pH 8.5. Conditions: Aqueous 160 mM borate buffer, 1 mM EDTA, pH is varied from 8.5 to 11.0; voltage, 15 kV; capillary length, 57 cm; injection, 25 seconds.

displayed a sharp transition zone within a narrow pH range. The optimal pH needed to focus epinephrine, norepinephrine and dopamine in this system were 10.4, 10.0 and 10.6, respectively, as reflected by the maxima of the plots in Figure 7.6. The selective focusing induced by a dynamic pH junction reflects the specific chemical properties of the analytes. Minor differences in chemical structure among the catecholamines, as shown in Figure 7.2, is responsible for their specific focusing. These chemical properties effect their relative acidity and basicity, which is reflected in different effective electrophoretic mobilities at various pH.
Although each catecholamine optimally focuses at a different pH, a single pH junction can be used to simultaneously separate and focus these analytes. Figure 7.7 shows the effect of a dynamic pH junction with the injection of about 300 nL of sample. This corresponds to an injection bandwidth of 6.6 cm. Despite the large volume of sample injected, excellent peak resolution of the catecholamines is retained. This is mainly due to the tremendous analyte band narrowing, resulting in efficiencies of about $3.3 \times 10^6$ for dopamine. Figure 7.8 visually depicts the calculated detector bandwidths compared with the initial injection bandwidth for each catecholamine from Figure 7.7. The expected detector bandwidth of the catecholamines (if no pH junction is used) is also shown to emphasize the potency of this focusing technique. All three analytes undergo band narrowing since their DIBR is much less than one. In addition, each catecholamine focuses to a different extent, highlighted by their different detector bandwidths of 0.77 cm, 0.91 cm and 0.19 cm for epinephrine, norepinephrine and dopamine, respectively. In contrast, the expected detector bandwidth is about 11 cm, when using an injection length of 6.6 cm. These different detector bandwidths demonstrate that a dynamic pH junction is a specific focusing technique dependent upon the chemical properties of the analytes. Plots such as Figure 7.6 can be used to rationally design optimal focusing conditions for specific analytes of interest. A similar strategy is commonly used for optimizing CE separation conditions by plotting the measured electrophoretic mobilities of various analytes under different electrolyte conditions, such as pH.

7.3.6 Application of a Dynamic pH Junction to Focus Weakly Acidic Analytes

In order to assess what specific analyte chemical properties are required for focusing, a comparative study of the effects of a pH junction on four related catecholamine analogues were examined. Dopamine, DOPA, tyrosine and catechol were selected as test analytes,
**Figure 7.7** Electropherogram demonstrating the simultaneous separation and focusing of three catecholamines using an optimized pH junction: 3.0 μM epinephrine (1), norepinephrine (2), and 2.0 μM dopamine (3). Catecholamine samples also contained 160 mM borate, 155 mM NaCl, 3 mM sodium metabisulphite and 1 mM EDTA, pH 8.5. Conditions: Aqueous 160 mM borate, 1 mM EDTA, pH 10.6; voltage, 15 kV; capillary length, 57 cm; injection, 50 seconds.

**Figure 7.8** A series of bargraphs comparing the measured bandwidths that highlight the efficacy of analyte focusing via a dynamic pH junction from Figure 7.7: (A) injection bandwidth, (B) expected detector bandwidth (no pH junction), (C) epinephrine detector bandwidth, (D) norepinephrine detector bandwidth, and (E) dopamine detector bandwidth.
whose structures are shown in Figure 7.2. The body synthesizes dopamine from the decarboxylation of DOPA, which is in turn derived from the hydroxylation of the amino acid tyrosine. Catechol is selected as a control to determine the effect of the basic ethylamine portion of dopamine. Figure 7.9 shows a series of electropherograms of the analytes at various background buffer pHs ranging from 8.5 to 11.0. Initially, when the pH of the sample and background electrolyte is the same [Fig. 7.9 (A)], broad sample plugs are observed for all four analytes when using an injection volume of 150 nL. However, tyrosine was observed to focus gradually as soon as the pH of the buffer is greater than 9.0. Dopamine did not completely focus until a higher pH of 10.6, as was observed previously. Optimal separation and focusing of both tyrosine and dopamine is achieved with pH difference of 2.5 units [Fig. 7.9(H)]. In contrast, both catechol and DOPA are observed not to focus significantly within the pH range studied. It is important to note that both catechol and DOPA are negatively charged at pH 8.5, whereas dopamine and tyrosine are close to neutral. In order to determine whether the zwitter-ionic (amine and carboxylate) or the phenolic portion of tyrosine is necessary for focusing, phenol was also used as an analyte. Similar to tyrosine, phenol was observed to optimally focus at a pH of 10.7, with extremely high theoretical plates of \(1.4 \times 10^6\). The LOD for phenol and tyrosine is less than \(5.0 \times 10^{-7}\) M, when employing an optimized pH junction using this method.

Figure 7.10 shows the measured electrophoretic mobilities of the analytes studied in this report. Certain trends relating to the effect of a dynamic pH junction on analyte focusing can be deduced from this plot. It is apparent that epinephrine, norepinephrine, dopamine and tyrosine have zero or extremely small negative mobilities at pH 8.5. However, their negative mobility rapidly increased with higher pH. On the other hand, catechol and DOPA both have substantial negative mobilities at pH 8.5. In fact, the mobility for catechol was observed to
Figure 7.9 A series of electropherograms exploring the role of chemical functionality on selective focusing via a dynamic pH junction, using four dopamine analogues: 1-dopamine; 2-tyrosine; 3-DOPA; 4-catechol. Conditions: Buffer, aqueous 160 mM borate, 1 mM EDTA, pH is varied from (A) 8.5, (B) 9.0, (C) 9.5, (D) 10.0, (E) 10.2, (F) 10.5, (G) 10.7 and (H) 11.0; voltage, 12 kV; capillary length, 47 cm; injection, 20 seconds.

remain unchanged with increasing pH, implying that it is fully ionized above pH 9.0. The mobility for DOPA was observed to change slowly with increasing pH, as a result of the deprotonization of the primary amino functionality. However, the extent of the mobility change was much less than that observed for the catecholamines and tyrosine. Hence, focusing is not unique to zwitter-ionic catecholamines or amino acids, but to any weakly acidic analyte that has a low mobility in one buffer matrix and a significantly larger mobility in the other buffer zone. It is unclear what critical mobility difference is required to induce focusing of specific analytes. Generally, the optimal pH required for analyte focusing occurs in the pH region where the mobility changes most rapidly. This region corresponds to a pH
Figure 7.10 A plot of the measured analyte electrophoretic mobility as a function of pH. All samples contained 160 mM borate, 155 mM NaCl, 3 mM sodium metabisulphite, 1 mM EDTA, pH 8.5. Conditions: Buffer, aqueous 160 mM borate, 1 mM EDTA, pH is varied from 8.5 to 11.0; voltage, 10 kV; capillary length, 37 cm; injection, 2 seconds. Analyte peak numbering: 1-epinephrine, 2-norepinephrine, 3-dopamine, 4-DOPA, 5-tyrosine and 6-catechol.

interval that just follows the steepest portion of the mobility plot, such as Figure 7.10. For instance, dopamine exhibits the greatest mobility change (slope of the mobility curve) between the pH range of 10.4 and 10.6, as shown in Figure 7.10. Beyond pH 10.6, the mobility of dopamine does not change substantially. Optimal focusing of dopamine was observed to occur at pH 10.6 (see Fig. 7.6). Similarly, epinephrine, norepinephrine and tyrosine display optimal focusing at unique pH values, that correspond to regions in Figure 7.10 where their mobilities begin to level off, after a rapid mobility increase.
Figure 7.11 Electropherograms highlighting the use of a dynamic pH junction to selectively focus phenol (1) and not catechol (2): when (A) both the sample and background electrolyte are pH 8.5, (B) pH 10.7 and (C) dynamic pH junction, where the sample is pH 8.5 and the background buffer is pH 10.7. Concentration of analytes are 200 μM in electropherograms (A) and (B), and 20 μM in electropherogram (C). Conditions: Buffer, aqueous 160 mM borate; voltage 15 kV; capillary length, 57 cm; injection time, (A) and (B) 3 s and (C) 30 s.

7.3.7 Separation and Focusing Design in CE Using a Dynamic pH Junction

The ability to rationally design a separation to provide selective and sensitive analysis of specific analytes, based on their fundamental physicochemical properties, is the highly coveted "Holy Grail" of separation science. It was in this spirit that phenol and catechol were selected as test analytes to highlight ways to design specific separation and focusing strategies using a dynamic pH junction, based on knowledge of a species chemical properties. Differences in their relative acidities and complexation abilities permit their separation by zone electrophoresis, as well as inducing selective focusing using a dynamic pH junction, as
Figure 7.12 Proposed mechanism involved with selective analyte focusing of weakly acidic analytes, such as phenol and catechol, using a pH junction: (A) a large sample plug that is buffered at a low pH is first injected into the capillary that is filled with a high pH buffer, (B) rapid focusing of phenol occurs as a dynamic pH junction is established within the sample zone, whereas catechol continues to migrate as a wide sample plug and (C) separation of the analytes by normal CZE. The pH of the sample and background can be specifically tuned to focus analytes of interest.

depicted in Figures 7.11. Phenol (pKᵦ = 10.1) is observed to have a negligible mobility at pH 8.5 (neutral), whereas its mobility increased to $1.78 \times 10^{-4}$ \( \text{cm}^2 \text{V}^{-1} \text{s}^{-1} \) at pH 10.7. In contrast, catechol has a large negative mobility of about $2.41 \times 10^{-4}$ \( \text{cm}^2 \text{V}^{-1} \text{s}^{-1} \) at pH 8.5 and pH 10.7 (fully ionized at both pH values) because of its selective complexation with borate in the run buffer. Figure 7.12 illustrates a proposed mechanism of selective analyte focusing using a dynamic pH junction. After injection of a large volume of the sample in the capillary, rapid focusing of phenol occurs. The initial static pH junction does not persist with application of
the voltage. A dynamic pH junction may be established within the sample zone as it is
titrated by the migrating borate and hydroxide anions in the background buffer. This
dynamic pH junction sweeps through the sample zone over a finite time interval. Selective
focusing of phenol occurs since it acquires a mobility (negatively charged) as the dynamic
pH junction sweeps across the sample zone, resulting in a rapid compression of the analyte
zone. Gradual dissipation of the pH junction occurs over time as a continuous background
electrolyte is restored throughout the capillary, allowing the separation to occur by normal
zone electrophoresis. However, catechol which has no significant mobility change
throughout the dynamic pH junction, does not focus and continues migrating and diffusing
throughout the separation. High concentrations of salt do not perturb the focusing. It is
important to note that the use of a pH junction also noticeably reduced the apparent migration
time of phenol because of its large mobility differences in the two electrolyte zones. The
exact nature of the buffer discontinuity (i.e., dynamic pH junction) generated through the
capillary during the separation is still unclear. A discontinuous electrolyte system that uses
anionic micelles in the background electrolyte to sweep across the sample zone has been
recently demonstrated to effectively focus large volumes of neutral analytes. A general
focusing strategy that uses a variety of different additives, ranging from metal ions to large
biological ligands, may be used in a discontinuous electrolyte format to focus analytes of
interest. Hence, selective focusing can be tuned based on mobility differences of analyte(s)
through appropriate selection of pH and/or additive (complexing agent) in the two electrolyte
zones. However, since a large portion of the capillary was originally filled with sample, the
effective length of the capillary involved with separation is reduced. Thus, there is a
compromise between concentration sensitivity enhancement and separation, indicating that
there is maximum limit to the volume of sample that can be injected.
7.4 Conclusion

Selective focusing of epinephrine, norepinephrine, dopamine, tyrosine and phenol was postulated to occur via a dynamic pH junction between sample and background electrolyte zones in the presence of high salt. The use of dynamic pH junctions can be easily incorporated into conventional CE separations through appropriate changes in the composition of the sample matrix relative to the running buffer. This technique was found to be useful for zwitter-ionic and weakly acidic analytes that displayed large mobility differences in the sample and buffer matrices selected. Changes in the pH or the addition of complexing agents to the run buffer may be used to selectively modify an analyte's mobility. Band narrowing of analyte zones was shown to occur through measurement of the detector to injection bandwidth ratio. Significant improvements in concentration sensitivity and separation efficiencies can be attained through this focusing technique. Knowledge of the chemical properties of analytes can be applied towards rational design of concentration sensitivity enhancement strategies. The use of a dynamic pH junction may also be applied to further extend the sensitivity of CE using LIF and EC detection. A discontinuous electrolyte system, such as a dynamic pH junction, may also incorporate the effect of various types of additives, such as metal chelating agents, cyclodextrins or micelles to induce analyte focusing through selective mobility changes in two electrolyte zones. Further work is needed to better understand this focusing technique, including its general application to other types of analytes.

7.5 References


Chapter VIII:

On-Line Focusing of Nucleotides Using a Discontinuous Electrolyte System.

"Application of focusing strategies to improve CE sensitivity for nucleotide pool analysis and the assessment of antiviral therapeutic drugs."
VIII: On-Line Focusing of Nucleotides Using A Discontinuous Electrolyte System by CE.

8.1 Introduction

Selective focusing using a discontinuous electrolyte system has recently been applied to the zwitter-ion, epinephrine and other catecholamines, as well as weakly acidic analytes, which is presented in chapter VII in this thesis. Concentration sensitivities and separation efficiencies greater than $4.0 \times 10^{-8}$ M and $1.0 \times 10^{6}$ theoretical plates respectively, were amenable using an optimized pH junction with UV detection. A better understanding of this focusing technique and its application to specific analytes of interest is currently needed to help guide researchers on effective strategies to improve concentration sensitivity in CE. The ability to specifically tune both the separation and sensitivity in CE, based on fundamental chemical properties of individual analytes, is needed to make CE a realistic alternative to help solve the increasing complexity of sample mixtures.

Nucleic acid analysis is an essential aspect in many areas of biochemical, medicinal, forensic and clinical research, as well as emerging pharmaceutical industries and biotechnology, including the Human Genome Project. Intracellular nucleotide profiles are vital in studies of cell metabolism and their changes associated with a variety of disease processes. Drugs based on nucleoside analogues have been effective in treating various viral infections and cancers. Despite the high efficiency of CE based separations of nucleotides, the concentration sensitivity is often much greater than 1 $\mu$M with UV detection. This is often inadequate sensitivity for many applications. Hence, on-line focusing methods, which are suitable to real biological matrices, would be highly beneficial for nucleic acid research using CE with conventional UV detection. As a continuation of the work developed
in chapters VI and VII of this thesis, the aim of this investigation was to study the influence of a discontinuous electrolyte system on the focusing of (deoxy)ribonucleosides and their phosphorylated derivatives, including the antiviral nucleoside analogues, penciclovir and carbovir. The influence of base structure, type of sugar and degree of phosphorylation of the nucleic acids were examined. The most important sample properties which induced nucleotide focusing were pH and borate complexation. Focusing was postulated to occur by changes to the analyte's mobility (velocity) through differences to the pH and borate content (complexing agent) in the sample and background electrolyte. This focusing method was found to be applicable to nucleotide pool analysis from cell extracts containing high salt without prior desalting or off-line preconcentration. Large volumes of sample (upwards of 300 nL) were injected directly into the capillary, while retaining the high efficient separations typical of CE. In addition, the focusing procedure is easy to perform since it does not require coating of the capillary or the use of polarity switching, as is often necessary for large volume sample stacking.\(^8\)\(^{12}\)

8.2 Experimental

8.2.1 Chemicals and Procedure

The aqueous separation buffer consisted of 160 mM borate (Borax, Sigma Chemical Co., St. Louis, MO). The pH of the separation buffer was adjusted by using 1.0 M NaOH (BDH Chemicals) or 2.0 M HCl (Fischer Scientific, Nepean, Ont., Canada) within a range of pH 7.0 to 11.0. Sodium acetate was purchased from Sigma. Standard 2’-deoxyribonucleosides and their phosphorylated analogues: deoxyadenosine mono-, di-, and triphosphate (dA, dAMP, dADP, dATP), deoxyguanosine mono-, di-, and triphosphate (dG, dGMP, dGDP, dGTP), deoxycytosine mono-, di-, and triphosphate (dC, dCMP, dCDP and dCTP) and
deoxythymidine mono-, di-, and triphosphate (dT, dTMP, dTDP and dTTP), and the corresponding 2'-ribonucleotides: adenosine mono-, di-, and triphosphate (A, AMP, ADP, ATP), guanosine mono-, di-, and triphosphate (G, GMP, GDP, GTP), cytosine mono-, di-, and triphosphate (C, CMP, CDP and CTP) and uridine mono-, di-, and triphosphate (U, UMP, UDP and UTP) were all purchased from Sigma Chemical Co. Pencyclovir (PCV) and carbovir (CBV) samples were generously donated by SmithKline Beecham and Glaxo Wellcome Pharmaceuticals, respectively. Sodium chloride was obtained from BDH Chemicals. Stock solutions of analytes were prepared by dissolving approximately equal concentrations (1 × 10⁻² M) of each nucleotide in deionized water and storing in a freezer at -20 °C. These stock solution were then further diluted with the appropriate sample matrix prior to injection into the capillary. Peaks were identified by spiking the sample solution with standard solutions of each nucleic acid.

8.2.2 Preparation of Mouse Lymphoma Cell Extract

A mouse lymphoma L1210 cell suspension [50 mL, 1.0 × 10⁶ cells/ mL] was pelleted and the supernatant discarded. The cell pellet was rinsed with phosphate buffered saline (PBS) and then extracted with a 1 mL hypotonic solution: 50 % ethanol, 10 % PBS, 2 mM EDTA. The extract was evaporated to dryness and then resuspended in 100 μL of deionized water. The cell extract was then injected directly or diluted a further 100-fold in 160 mM borate, 150 mM NaCl, pH 7 electrolyte prior to CE analysis.

8.2.3 Apparatus and Procedure

Separations were performed on a Beckman P/ACE 5000 automated capillary
electrophoresis system (Beckman Instruments Inc., Mississauga, Ont., Canada). Uncoated capillaries (Polymicro Technologies, Phoenix, AZ) were used with inner diameters of 75 μm, outer diameters of 375 μm and lengths of 57 cm or 37 cm. New capillaries were first rinsed with 1.0 M NaOH (5 minutes, 20 psi), followed by rinsing with the separation buffer (10 minutes, 20 psi). The capillary was then left to equilibrate overnight in the separation buffer prior to use. Each separation was preceded by a 1.5 minute rinse with 0.1 M NaOH, followed by a 4 minute rinse with the separation buffer. The sample was introduced using a low pressure 0.5 psi injection ranging from 1 to 50 seconds. The average flow rate of the low pressure injection was determined to be 7.87 and 12.35 cm/min using a 57 and 37 cm capillary, respectively. All separations were carried out at 25 °C, and absorbance detection was made with a Beckman P/ACE UV-Vis detector at 254 nm. Data were collected and processed using System Gold software (Beckman).

8.3 Results and Discussion

8.3.1 The Effect of Base Type on Deoxynucleoside Focusing

In CE, it is customary to dilute samples in a similar buffer matrix used for the separation. However, it is often advantageous to dilute samples in matrices of different composition in order to induce sample focusing. As an extension to the previous work developed in chapter VII for the focusing of catecholamines and weakly acidic analytes, the effects of a discontinuous electrolyte system was tested with nucleic acids. Since the bases, G and U (T) possess a weakly acidic lactam functionality, pKₐ = 9.4 and 9.7 respectively, dNs containing these nucleobases may selectively focus under an appropriate pH junction. Figure 8.1 depicts the effect of a dynamic pH junction between sample and background buffers (borate) on the focusing of dG, when using a large sample injection volume of approximately 180 nL. The
Figure 8.1 Series of electropherograms demonstrating the use of a dynamic pH junction to induce dG focusing. All sample solutions contained 20 μM dG in 160 mM borate, 150 mM NaCl, pH 8.0. The pH of the background electrolyte is varied from 8.0 (A), 8.5 (B), 9.0 (C), 9.2 (D), and 9.5 (E). Conditions: Buffer, aqueous 160 mM borate; voltage, 15 kV; capillary length 57 cm; injection, 30 s.

The sample pH is fixed at 8 throughout the experiments, while the pH of the background buffer is varied incrementally from pH 8 to 9.7. The sample matrix is relatively conductive, since it also contains additional salt (150 mM NaCl).

Deoxyguanosine is neutral at pH 8, but becomes partially ionized at pH > 8.5. Normally, such large sample volume injections result in broad analyte peaks, as highlighted in electropherograms (A) and (B) of Figure 8.1. However, a pH difference of only 1.5 units is sufficient to focus dG into a sharp zone, with a separation efficiency of greater than $3 \times 10^5$ theoretical plate numbers. Optimum focusing of dG was observed to occur at pH 9.7, with a gradual loss of focusing at pH values greater than 10. It is important to note that dG focusing
occurs even under high salt conditions, in contrast to most sample stacking modes. In this case, differences in the electrophoretic mobility of the analyte are not due to conductivity differences or amplified electric fields distributed between the sample and background electrolytes, but is dependent upon changes in the ionization of the analyte within these two buffer regions in the capillary. The pH junction used in the capillary is transient (dynamic) in nature, since it is titrated by the background buffer, which gradually dissipates the buffer discontinuity. For instance, the electrophoretic mobility of dG is zero in the sample region, but drastically changes to \(-1.41 \times 10^{-4} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}\) in the background buffer zone (pH 9.5).

Thus, focusing of analytes via a dynamic pH junction is a selective process dependent upon specific analyte properties, such as the presence of acidic or basic functionalities.

In order to quantitatively assess the magnitude of dG focusing in Figure 8.1, the values for the injection and detector bandwidths were compared. Table 8.1 shows the measured injection and detector bandwidths, as well as the detector to injection bandwidth ratio (DIBR), at the various background buffer pH values for dG from the electropherograms depicted in Figure 8.1. When both the sample and background zones have the same pH, Fig. 8.1 (A), the detector bandwidth for dG increases by 30% from the initial injection bandwidth of 3.9 cm. Significant band broadening had incurred since the DIBR is much greater than one, with a value of 1.27. Even greater band broadening occurs when using a pH 9.5 buffer in both sample and background electrolyte. The combined effects of long injection length, longitudinal diffusion and electrophoretic dispersion (sample has a high salt content), along with other possible sources of band dispersion, result in an extremely broad analyte zone (6.4 cm) migrating past the detector window, reflected by a DIBR of 1.61. However, as the pH of the background is gradually increased, band narrowing of the dG zone occurs, reflected by a DIBR of less than one. For instance, when the background pH is changed to 9.5, as depicted
Table 8.1  Bandwith values of \( dG \) focusing when using a dynamic \( pH \) junction.

<table>
<thead>
<tr>
<th>Buffer pH</th>
<th>Injection Bandwidth ( w_{inj} ) (cm)</th>
<th>Detector Bandwidth ( w_{det} ) (cm)</th>
<th>( \frac{w_{det}}{w_{inj}} ) (DIBR)</th>
<th>Net Effect of Bandwidth Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>3.9</td>
<td>5.0</td>
<td>1.3</td>
<td>Broadening</td>
</tr>
<tr>
<td>8.5</td>
<td>3.9</td>
<td>4.1</td>
<td>1.0</td>
<td>Broadening</td>
</tr>
<tr>
<td>9.0</td>
<td>3.9</td>
<td>2.4</td>
<td>0.6</td>
<td>Narrowing</td>
</tr>
<tr>
<td>9.2</td>
<td>3.9</td>
<td>1.1</td>
<td>0.3</td>
<td>Narrowing</td>
</tr>
<tr>
<td>9.5</td>
<td>3.9</td>
<td>0.5</td>
<td>0.1</td>
<td>Narrowing</td>
</tr>
<tr>
<td>9.5*</td>
<td>3.9</td>
<td>6.4</td>
<td>1.6</td>
<td>Broadening</td>
</tr>
</tbody>
</table>

Sample electrolyte in all runs consisted of 160 mM borate, 150 mM salt, \( pH \) 8.0, as depicted in Figure 8.1.

*Sample electrolyte consisted of 160 mM borate, 150 mM sodium chloride, \( pH \) 9.5.
in Fig. 8.1(E), the DIBR is 0.14. Thus, the initial injection zone is narrowed by nearly 7-fold, whereas the detector bandwidth (if the sample pH is 9.5) is reduced almost 12-fold, to a bandwidth of only 0.54 cm.

Figure 8.2 demonstrates the specificity of this on-line focusing technique for various deoxynucleosides using an optimized dynamic pH junction. dNs having the bases, A and C, are neutral in both the sample and background buffers, and thus, co-migrate as broad peaks with the EOF. In contrast, dNs having weakly acidic bases, G, T and U focus into sharp zones with extremely high efficiency ($N = 7 \times 10^5$), while maintaining good baseline resolution. Excellent concentration sensitivity of less than $1 \times 10^{-7}$ M is easily attainable, while using injection volumes greater than 300 nL for dN analysis with UV detection. In addition, this method works for both the cyclic dG and the acyclic guanosine analogue, PCV which is used as an antiviral drug for the treatment of the Human Herpes Simplex Virus (HHSV). Hence, the specific nature of the base is a critical aspect of this focusing technique.

Figure 8.3 visually depicts the calculated detector bandwidths compared with the initial injection bandwidth for each dN from Figure 8.2. Nucleosides containing weakly acidic bases: dT, dG, dU and PCV undergo focusing with a measured detector bandwidth of 0.42, 0.40, 0.30 and 0.29 cm, respectively. All four analytes undergo band narrowing since their DIBR is much less than one. For instance, PCV has a DIBR of 0.044. This represents nearly a 23-fold reduction in size of the initial injection plug of 6.6 cm. In contrast, dA and dC do not possess weakly acidic bases and are electrically neutral in both sample and background buffer zones, co-migrating with the EOF. Thus, dA and dC undergo normal band broadening, resulting in a detector bandwidth of 7.6 cm and a DIBR of 1.16. These different detector bandwidths demonstrate that a dynamic pH junction is a specific focusing technique.
**Figure 8.2** Electropherogram showing the effect of nucleobase on the separation and selective focusing of trace amounts of deoxyribonucleosides (dNs). All sample solutions contained 0.2 μM of dN in 160 mM borate, 150 mM NaCl, pH 8.0. Conditions: Buffer, aqueous 160 mM borate, pH 9.7; voltage, 15 kV; capillary length, 57 cm; injection, 50 s. Analyte peak numbering corresponds to: 1-dA, 2-dC, 3-dT, 4-dG, 5-dU and 6-PCV.

**Figure 8.3** A series of bargraphs comparing the measured bandwidths that highlight the efficacy of analyte focusing via a dynamic pH junction from Figure 8.2: (A) injection bandwidth, (B) dA/dC detector bandwidth, (C) dT detector bandwidth, (D) dG detector bandwidth, (E) dU detector bandwidth and (F) PCV detector bandwidth.
dependent upon the chemical properties of the analytes.

8.3.2 The Effect of Sugar Type on Nucleoside Focusing

The effects of sugar structure on nucleic acid focusing was next tested by comparing both dNs and Ns. Since borate selectively complexes with vicinal diols on ribose to form a tetrahydroxy borate anion complex, ribonucleosides generally have larger negative electrophoretic mobilities than their corresponding deoxyribonucleoside analogues in borate buffer. It was observed that all the ribonucleosides are partially ionized using a borate buffer at pH 8. Hence, the use of the same optimized pH junction developed for the dNs, resulted in significant band broadening of the nucleosides, reflected by a DIBR of about 1.23 and an efficiency less than $6 \times 10^3$ theoretical plates. The reduction of the sample pH from 7.5 to 7.0 improved the focusing of the Ns (minimize borate complexation), but resulted in a loss of its buffering capacity. Hence, an acidic sodium acetate buffer at pH 4 was used, in order to ensure that the Ns did not form anionic complexes with borate in the sample. Figure 8.4 depicts the effect of using a discontinuous buffer system with a pH junction, on both N and dN focusing. Similar to previous experiments, all samples also contained high salt when using large sample injection volumes. Nucleic acid focusing is dependent on both the type of sugar and base. Although dA and dC are neutral and migrate as broad peaks with the EOF, A and C are both negatively charged and migrate as focused peaks, because of selective borate complexation in the background buffer with the vicinal diol residues of the ribose unit, as shown in Fig. 8.4 (A) and (B). In contrast, dG, dU, G and U all migrate as well separated and focused peaks, since they are ionized in the background buffer due to the relative acidities of the base and/or sugar residues, as demonstrated in Fig 8.4(C) and (D). Despite using a large injection bandwidth (3.9 cm) and residing in the capillary for over twenty
**Figure 8.4** Series of electropherograms demonstrating the effects of nucleobase and nucleosugar on the separation and selective focusing of deoxyribonucleosides (dNs) and ribonucleosides (Ns). All sample solutions contained 2.0 μM of dN/N in 200 mM acetate buffer, 150 mM NaCl, pH 4.0. Conditions were the same as Fig. 8.1. Experiments were performed on each pair of dN and N.

**Figure 8.5** Simultaneous separation and focusing of a nucleoside mixture. Conditions are the same as Fig. 8.4. Analyte peak numbering corresponds to: 1-dA, 2-dC, 3-dT, 4-dG, 5-dU, 6-PCV, 7-C, 8-A, 9-G and 10-U.
minutes, analyte band dispersion is countered by a greater focusing effect for even the late migrating analytes, such as U. This is reflected by a detector bandwidth of only 0.64 cm, DIBR of 0.16 and plate numbers greater than $1.3 \times 10^5$. Figure 8.5 depicts the simultaneous separation and focusing of a nucleoside mixture. Thus, focusing of nucleosides using a discontinuous electrolyte system is dependent upon the specific chemical properties of both the base and sugar. Moreover, this type of focusing is not limited to a single buffer type, but may be carried out through the use of an appropriate mixed buffer system, in order to selectively modify the mobility of analytes over a wider pH range.

### 8.3.3 The Effect of Degree of Phosphorylation on Nucleotide Focusing

Despite the ability to focus weakly acidic nucleosides, it was originally surmised that nucleotides, containing strongly acidic phosphate groups, may not focus via the same process. The application of the sodium acetate pH junction previously used for the Ns generated a single sharp peak for the mono-, di-, and triphosphates of adenosine. This indicated that the di- and triphosphorylated nucleotides were labile under the acidic conditions used in the sample buffer and co-migrate with AMP. The high energy phosphate bonds of the di- and trisubstituted nucleotides are generally more sensitive to acid hydrolysis than the corresponding monophosphate or nucleoside.\(^{16}\) However, it was observed that the use of the borate-salt (pH 7) electrolyte in the sample induced nucleotide focusing, as was observed previously for their corresponding nucleosides. Figure 8.6 demonstrates the concentration sensitivity enhancement that is possible with this method by comparing the separation and sample enrichment of a group of adenosine nucleotides using a typical injection, 10 nL (A) and a large volume injection, 300 nL (B). Nearly a 50-fold improvement
Figure 8.6 Electropherograms comparing the effects of a conventional injection (10 nL) with a continuous (A), and a large volume injection (300 nL) using a discontinuous electrolyte system (B). About a 50-fold enhancement in sensitivity is demonstrated with the nucleotides using a concentration of 10 μM (A) and 0.2 μM (B) respectively. Sample solutions contained 160 mM borate, 150 mM NaCl, pH 9.7 (A) or pH 7.0 (B). Conditions: Buffer, aqueous 160 mM borate, pH 9.7; voltage 12 kV; capillary length, 37 cm; injection 1 s (A) and 30 s (B). Analyte peak numbering: 1-dA, 2-A, 3-dAMP, 4-dADP, 5-dATP, 6-AMP, 7-ADP, 8-ATP.

Figure 8.7 Electropherograms contrasting the effect of large volume injections (30 seconds) using (A) a continuous (sample, pH 9.7), and (B) a discontinuous (sample, pH 7) electrolyte system. Conditions and analyte peak numbering are the same as Fig. 8.6.
in sensitivity is possible with this technique, although the amount of sample injected only increased by 30-fold. Hence, sensitivity is enhanced by the focusing of large volumes of analytes into extremely narrow zones, sharper than those obtained from conventional small volume injections in CE. Moreover, separation performance in terms of selectivity, resolution and column efficiency is minimally affected. Band narrowing of nucleotides has occurred since the detector bandwidth for ATP is only 0.61 cm, compared to an injection bandwidth of about 6.2 cm. Thus, over a 10-fold reduction in the original sample injection plug is caused by the on-line focusing of the nucleotides, with a DIBR of about 0.10. However, normal band broadening occurs when using a small injection plug of 0.20 cm [Fig. 8.6 (A)], resulting in a similar detector bandwidth of 0.61 cm calculated from Fig. 8.6(B), but a DIBR of 3.0.

Figure 8.7 highlights the effect of using a discontinuous electrolyte system in the focusing of the same nucleotides. When the nucleotides are diluted in the same sample matrix as the background buffer (continuous electrolyte system), deleterious band broadening is observed when using such large injection volumes. However, when the same analytes are diluted in a reduced pH borate electrolyte, the nucleotides are fully separated and migrate as sharp peaks. The reproducibility of this on-line preconcentration technique had a RSD of 1.0% and 2.5% for the migration times and peak areas of the nucleotides respectively. Linearity of the method was excellent over a 100-fold concentration range, with $R^2 = 0.9995$, and a LOD of under $4.0 \times 10^{-8}$ M for AMP using UV detection. The sensitivity can be further extended by using larger injection volumes, in conjunction with longer and wider capillaries.

Since borate has poor buffering capacity at pH 7, it was suspected that nucleotide focusing may not require a buffered pH junction, as was used previously for their
Figure 8.8 Series of electropherograms illustrating the effect of salt concentration in sample matrix on nucleotide stacking. All sample solutions contained 1.0 μM of the group of adenosine nucleotides with varying concentrations of NaCl: (A) 0 mM, (B) 5 mM, (C) 25 mM, (D) 75 mM, (E) 150 mM and (F) 300 mM. Conditions and analytes are the same as Fig 8.6.

corresponding nucleosides. In order to determine which factors were vital for nucleotide focusing, the effect of salt, borate electrolyte and borate-salt electrolyte were compared. It was observed that all three sample matrices induced similar nucleotide focusing. Since the pH of borate was modified with aqueous HCl to lower the pH below 9, borate electrolyte solutions already contained significant Cl⁻ concentration prior to salt addition. Hence, it was concluded that the presence of salt in a neutral or weakly acidic solution was an important factor inducing nucleotide focusing. Sufficient salt concentration in the sample is required to maintain a similar conductivity as the background borate buffer. Figure 8.8 confirms the relative importance of salt in the sample matrix. All solutions had a slightly acidic pH of
around 5.6. The effect of nucleotide focusing was compared at various salt concentrations. Optimum focusing of nucleotides occurs with 150 mM NaCl in the sample [Fig. 8.8 (E)]. Nucleosides exhibited focusing with salt in the sample similar to the buffered sample solution used previously. However, NaCl is not unique in its ability to induce the focusing of the nucleotides. A comparison of nucleotide focusing with other simple ionic salts, such as NaBr, KCl, and KBr, showed minimal differences when using the same concentration as NaCl. These observations indicated that optimal focusing of nucleotides is highly dependent upon the ionic strength of the sample solution, as opposed to the specific nature of the cation or anion. More importantly, the sample solutions do not contain borate in order to ensure that the mobility of the nucleotides is lower than in the background buffer due to borate complexation. Because all sample matrices have a relatively high conductivity, similar to that of the background electrolyte, sample stacking via an amplified electric field is not a plausible mechanism to describe this phenomenon. There have been previous reports of the beneficial use of salt in the sample to promote analyte focusing. Transient isotachophoresis (TITP) has been a proposed mechanism explaining analyte focusing that occurs with high concentrations of salt in the sample. Indeed, a few researchers have observed sample self-stacking occurring naturally from the specific sample matrix analyzed.\textsuperscript{17-19} However, the focusing observed for the nucleotides in this system is hypothesized to be caused by the differential mobility of the analytes in a discontinuous electrolyte system. The inherent simplicity and effectiveness of this focusing procedure makes it a facile way to enhance sensitivity in CE. The use of real-time imaging of analytes within portions of the capillary with CCD technology may aide in better understanding the focusing mechanism of such discontinuous electrolyte systems.
8.3.4 Mechanism of Nucleotide Focusing Using a Discontinuous Electrolyte System

It is apparent from Figures 8.6, 8.7 and 8.8 that focusing occurred even for the deoxyadenosine nucleotides using a discontinuous electrolyte system. Although dA (and dC) were observed not to focus because of the lack of changes in the state of ionization due to pH differences (neutral bases) or borate complexation (deoxyribose sugar) in the sample and background electrolyte zones, dAMP, dADP and dATP all undergo band narrowing. Thus, changes in the mobility of the deoxyadenosine nucleotides are solely the result of the presence of the phosphate groups. Since, the phosphates are relatively strong acids16 (dAMP being the strongest, pKₐ ≈ 0), focusing is probably not the result of changes in the analyte's ionization due to pH differences in the sample and background buffer. Similar to ribose sugars, borate may selectively complex with adjacent vicinal hydroxy groups on phosphate. It has been observed that the greater degree of phosphorylation (larger number of hydroxy sites), the weaker is the relative acidity of each additional phosphate.20 This is demonstrated by the similar electrophoretic mobilities for nucleotides with di- and tri-substituted phosphates. However, the acidity of these phosphate groups may be further lowered through selective borate complexation. Hence, dADP and dATP can be expected to focus more readily than dAMP (peak is slightly skewed), because of additional hydroxy sites for borate complexation, as is observed in Figures 8.6 and 8.7. On the other hand, the riboadenosine nucleotides undergo focusing due to borate complexation with the vicinal hydroxy groups on both the ribose and the phosphate. It is unclear on the exact mechanism of borate interaction with the phosphate groups on the nucleotides. Evidence of differential nucleotide mobility within the sample and background electrolyte system is revealed by substantial decreases in their apparent migration time in comparison to a continuous buffer system, as depicted in Figure 8.6. However, part of the decrease is also the result of the increased injection volume.
A. Effect of Type of Base

Weakly Acidic Lactam Groups

\[
\begin{align*}
\text{B = Guanine, G} & \quad \text{Uridine, U} & \quad \text{Thymine, T} \\
\end{align*}
\]

Non-Acidic / Neutral Groups

\[
\begin{align*}
\text{Adenine, A} & \quad \text{Cytosine, C} \\
\end{align*}
\]

B. Effect of Type of Sugar

No Borate Complexation

\[
\begin{align*}
\text{B} & \quad \text{P} \\
\text{X} = & \quad 2'-\text{Deoxyribose Group (dN)} \\
\end{align*}
\]

Borate Complexation

Ribose Group (N)

C. Effect of Degree of Phosphorylation

Borate Complexation

\[
\begin{align*}
P = & \quad \text{Mono-, Di-, Tri-Substituted Phosphate (Nucleotides - dNTs / NTs)} \\
\end{align*}
\]

Figure 8.9 Hypothesized mechanism to describe nucleic acid focusing using a discontinuous electrolyte system. Analyte mobility differences are caused by: (A) ionization of the weakly acidic bases, and (B) selective borate complexation with the hydroxy groups on the ribose unit, and (C) phosphate residues. The type of borate interaction with the phosphates on the nucleotides is unclear. Differences in pH and borate concentration between sample and background electrolytes may induce nucleic acid focusing. Thus, focusing by this mechanism is not observed for dA and dC because of no mobility changes in the two electrolyte zones.
A comparison study of the focusing of the nucleotides in a phosphate buffer (53 mM, pH 9.7) was conducted in order to ascertain the relative importance of borate in the background buffer. Nucleotide samples were similarly diluted in 150 mM salt solution. No focusing of the nucleotides and nucleosides was observed using phosphate as the background buffer. However, focusing of the deoxynucleosides, dG and dU (dT) still occurred in this system. This suggests that the focusing of weakly acidic nucleosides is solely the result of a dynamic pH junction (using either borate or phosphate as the background buffer), whereas both nucleosides and nucleotides required borate for focusing due to differential borate complexation, as well as the effect of a dynamic pH junction. Further study is required to better understand this phenomenon. Figure 8.9 summarizes the mechanism of analyte mobility changes influencing nucleotide focusing based on observations in this study.

Figure 8.10 visually depicts the effects of selective nucleic acid mobility changes (dA versus G) inside the capillary in terms of observed band narrowing and normal band dispersion when using a discontinuous electrolyte system. After injection of a large volume of dilute sample, rapid focusing of G occurs because of mobility changes in the two different electrolyte zones of the sample and background buffer. Both G and dA are neutral in the sample zone, but only G becomes negatively charged because of deprotonation of the weakly acidic lactam group of guanine and selective borate complexation with the ribose sugar unit of G.

The original pH junction between the sample and background electrolyte zones does not persist during the separation. A dynamic pH junction may be established through the sample zone as it is titrated with migrating borate and hydroxide anions from the background buffer with the application of the voltage, as depicted in Figure 8.10(B). This type of mechanism is similar to isoelectric focusing methodologies, except that the pH junction is not a continuous pH gradient supplied by ampholytes, but is dynamic in nature, existing only for a short period.
Figure 8.10 Proposed mechanism involved with selective analyte focusing of nucleic acids, such as dA and G, using a discontinuous electrolyte system: (A) a large sample plug that is at a low pH salt solution is first injected into the capillary that is filled with a high pH buffer (pH junction), (B) rapid focusing of G occurs as a dynamic pH junction is established within the sample zone, whereas dA continues to migrate as a wide sample plug and (C) separation of the analytes by normal CZE. Changes in the mobility of G occurs in the background buffer because of titration of the weakly acidic guanine base and selective borate complexation with the ribose unit, whereas dA is electrically neutral in both sample and background zones.

of time within the sample zone. As the dynamic pH junction sweeps across the sample zone, G molecules acquire a large negative mobility and rapidly compress the initial wide analyte zone. After focusing, the buffer discontinuity is gradually dissipated (reflected by stabilization of the current) and the analytes are separated by conventional zone electrophoresis. However, since A is neutral in both electrolyte zones, it does not focus under the dynamic pH junction and migrates as a broad and diffuse band past the detector. It
is interesting to compare Figures 8.10 and 7.12. Catechol does not focus significantly since it is negatively charged, whereas A is neutral, within both the sample and background electrolyte zones. In either case, selective focusing is the result of mobility changes caused by the presence of specific functional groups of the analyte that are weakly acidic or undergo selective complexation in the background electrolyte relative to the sample zone.

8.3.5 Application of Discontinuous Electrolyte System for Analysis of Cell Extracts

Figure 8.11 depicts the separation and focusing of 24 different deoxyribonucleotide and ribonucleotide standards using a discontinuous electrolyte system. Excellent concentration sensitivity ($= 10^{-7}$ M) and good separation performance is achieved even with the injection of large volumes of sample. In addition, the focusing technique is simple to perform, using conventional instrumentation without complicated method procedures. Figure 8.12 demonstrates the application of this focusing technique for the analysis of the anti-viral nucleotides analogues of CBV. Similar to ATP, excellent linearity was demonstrated for CBV-TP using this method, with a $R^2$ of 0.9994 over a 100-fold concentration range.

Analysis of the nucleotide pool from mouse lymphoma cells was next studied to determine whether this focusing method is applicable to real biological samples. Because of the poor concentration sensitivity of CE, nucleotide analysis from cell extracts often requires tedious off-line preconcentration methods. In addition, the high salt content of cell extracts generally requires desalting procedures prior to CE separation. In order to facilitate nucleotide pool analysis and to improve assay reproducibility, the content of the cell extract was analyzed directly. Figure 8.13 compares the nucleotide pool of a mouse lymphoma from the direct injection of a typical small volume and a large volume injection of the cell extract. Over a 10-fold improvement in sensitivity is achieved with this nucleotide focusing method.
Figure 8.11 Electropherogram showing the separation and focusing of 24 different NTs and dNTs from a standard mixture using a discontinuous buffer system. Samples contained 0.2 μM of each nucleotide in 160 mM borate, 150 mM NaCl, pH 7.0. Analyte peak numbering corresponds to: 1-dAMP, 2-dCMP, 3-dADP, 4-dATP, 5-dTMP, 6-dCDP, 7-dCTP, 8-AMP, 9-CMP, 10-dGMP, 11-CDP, 12-CTP, 13-ADP, 14-ATP, 15-dGDP, 16-dGTP, 17-GMP, 18-dTDP, 19-dTTP, 20-GDP, 21-GTP, 22-UMP, 23-UDP and 24-UTP. Conditions were the same as Fig 8.6.

Figure 8.12 Electropherogram demonstrating the application of large volume focusing of trace amounts of the antiviral nucleoside and nucleotide analogues of carbovir using conventional UV detection. Sample solutions contained 0.1 μM of nucleotides in 160 mM borate, 150 mM NaCl, pH 7.0. Conditions: Buffer, aqueous 160 mM borate, pH 9.7; voltage 12 kV; capillary length, 37 cm; injection 50 s. Analyte peak numbering corresponds to: 1-CBV, 2-CBV-MP, 3-CBV-DP and 4-CBV-TP.
Figure 8.13 Electropherograms comparing the nucleotide profile from a mouse lymphoma using: (A) direct injection (4 s) and (B) a large volume injection (40 s) of the cell extract. Conditions were the same as Fig 8.6.

with minimal change to the nucleotide profile and separation performance. Biological matrices are ideal samples for this focusing technique since they naturally contain large amounts of salt. The analysis of changes to the nucleotide pool from cell lines is an important component in many areas of clinical sciences, including the study of the effects of chemotherapy on various diseases. Hence, on-line focusing of nucleotides is applicable for biological matrices through the use of an appropriate discontinuous buffer system.

8.4 Conclusion

On-line focusing of nucleic acids in the presence of salt was achieved using a discontinuous electrolyte system in CE. The extent of band narrowing of large sample plugs
was assessed by comparing the bandwidth at detection and injection, reflected by the magnitude of DIBR. Large volume injections of sample (upwards of 300 nL) resulted in enhanced concentration sensitivity for nucleotides using conventional UV detection, with a LOD of about $4.0 \times 10^{-8}$ M for ATP. In addition, separation performance in terms of band broadening, resolution and selectivity were minimally affected. Analyte focusing was found to be caused by analyte mobility differences in the sample electrolyte and background buffer zones. Changes in the mobility of nucleic acids were affected by differences in pH and borate complexation within these two zones. Analyte focusing via a dynamic pH junction was determined to be vital for nucleosides possessing weakly acidic nucleobases, such as G, U and T. Differential borate complexation was deemed crucial for nucleoside and nucleotide focusing due to the formation of anionic borate complexes with vicinal diol groups on ribose and/or phosphates. This type of on-line focusing was also applied to the analysis of nucleotides from cell extracts. Over a 50-fold improvement in sensitivity and the elimination of time consuming preconcentration and desalting procedures for biological samples can be realized using this method. Analysis of the anti-viral nucleoside analogues, such as PCV and CBV, was also amenable by this technique. A general focusing strategy that utilizes discontinuous electrolyte systems in CE has not yet been fully examined. Differences in the conductivity, pH, concentration of micelle or other complexing agents in the sample and background electrolyte zones during electromigration, may be used to effectively compress analyte zones. Better understanding of specific ways to improve both the selectivity and sensitivity, based on fundamental properties of analytes, serves to ameliorate the usefulness of CE in chemical separations.
8.5 References


(10) Geldart, S. E.; Brown, P. R. *Amer. Lab.* 1997, December, 48-51.


"The path to immortality is hard,
and only a few find it. The rest await
the Great day when the wheels of the universe
shall be stopped and the immortal sparks
shall escape from the sheaths of substance.

Woe unto those who wait, for they must return again,
unconscious and unknowing, to the seed-ground
of the stars, and await a new beginning."

- The Divine Pymander of Hermes Trismegistus,
from "Alchemy: The Art of Knowing"
Chapter IX:

Summary,
Separation Design &
Epilogue.
IX: Summary, Separation Design and Epilogue

9.1 Summary

"The aim of science is to discover and illuminate truth, and that, I take it, is the aim of literature, whether biography or history or fiction. It seems to me, then, that there can be no separate literature of science."

- Rachel Carson

This thesis has attempted to further develop, refine, understand and apply various principles of separation science to the recently established micro-separation technique of capillary electrophoresis. Rational design of separation systems in CE has been the major theme throughout this thesis. A combination of theoretical and applied approaches to scientific research has been undertaken to best achieve this goal. This thesis has presented four interdependent areas in CE that have been crucial for separation design: theory, selectivity, assay development, and sensitivity.

Any mature field in science requires significant theoretical background to provide insights and to help support further progress and discoveries. In this respect, CE has been primarily an empirical science. A deep understanding of the basic parameters governing CE separation to help solve real problems has been lacking. The increasing prevalence of additives in CE separations requires improved understanding of their most effective uses. Section A (Chapters II & III) of this thesis has contributed to the development, refinement and application of a dynamic complexation model to describe the mobility of an analyte based on three fundamental parameters: the electrophoretic mobilities of the free analyte and analyte-additive complex, and the equilibrium constant. Corrections for changes in the mobility of an analyte not due to specific additive complexation was required to improve the accuracy of the model. A relative viscosity and dielectric correction factor were determined to be important for modeling the mobility of an analyte in CE, especially for weakly
interacting species and high concentrations of additive in the run buffer. The dynamic complexation capillary electrophoresis (DCCE) model can be used to quantitatively measure thermodynamic parameters, such as the equilibrium (association/formation) constants. In addition, under a constant temperature and specific buffer conditions (i.e., buffer type, pH, ionic strength), the mobility of an analyte can be predicted at any additive(s) concentration, capillary length and voltage (within linear region of Ohm plot). This can be applied towards more rapid and effective separation optimization strategies. In addition, DCCE provides a understanding of the fundamental separation parameters which can aide in the design of separation systems in CE. The use of computer modeling (prior to actual experiments) to assess different types of analyte-additive interactions can also prove effective, in conjunction with DCCE, in separation design. Further work is required to test the validity of the DCCE model to describe the mobility of weakly interacting analytes when using charged additives, such as ionic surfactants and cyclodextrins. These additives can be expected to drastically alter the dielectric properties of the buffer, requiring significant correction (i.e., relative dielectric correction factor) to the measured analyte mobility. More importantly, like any theory, repeated testing of the DCCE model under a variety of conditions is required to ensure its continued validity. Also, practical ways to apply the model to help guide researchers towards more effective separation methodologies is ultimately necessary to prove the usefulness of the theory to the average separation practitioner.

One of the virtues of CE is that the selectivity of the separation can be easily varied by simply adding different types of additives to the run buffer. This is vital for the general separation of chiral and achiral analytes, including neutral species. In contrast to chromatography, where one is usually limited to a single type of stationary phase (separation column), CE may use unlimited combinations of additives (CE equivalent of stationary phase) in relatively small amounts (typically microlitre volumes). The use of complementary
additives simultaneously can result in extremely versatile and powerful separations. In section B (Chapter IV) of this thesis, TESMR, an aromatic-based macrocycle, was synthesized, characterized, and applied as a new type of additive in CE separations. The usefulness of TESMR was assessed by comparing its selectivity with an ionic cyclodextrin, in the separation of a group of neutral phenol derivatives. Relative to the charged cyclodextrin, TESMR displayed both different and similar selectivities (i.e., binding affinities) with the phenol analogues in terms of analyte size and shape (geometric isomers) and polarity (functional groups), respectively. By applying the understanding of separation theory in CE from Section A of this thesis, a complementary dual additive system, using a negatively charged TESMR and neutral cyclodextrin, was used to demonstrate the ability to modify the mobility of a specific analyte of interest in a complex mixture. Further work is needed to test the suitability of TESMR as an additive in CE to separate other types of important analytes, such as amino acids, nucleic acids, and polycyclic aromatic hydrocarbons. The high background UV absorbance of TESMR imposes some limitations for its use in CE analysis, especially for the direct detection of weakly absorbing species. However, this property may likewise be used to detect such species (e.g., sugars) via indirect UV detection. Since the primary structure of TESMR can be easily modified through selective organic chemical reactions, one can vary its selectivity by synthesizing a variety of functional analogues. The use of an array of modified resorcinarenes, in conjunction with other types of additives, may result in limitless ways for researchers to tune separation selectivity in CE. Indeed, one may envision the possibility to unify the still disjointed fields of organic/inorganic, analytical and theoretical/physical/computational fields of chemistry to form an integrative approach towards separation design. The concept of such an approach is presented in the end of the summary.
Industry and governmental agencies are often hesitant to adopt new technologies, until their merit has been proved by research over a long period of time. Capillary electrophoresis is currently in such a scenario, despite over eighteen years of development since its first application. This is partially due to the only recent manufacture of automated CE instruments commercially. In addition, the reliability of such instruments for long-term quantitative assays of real samples has yet to be studied thoroughly. Section C (Chapters V & VI) of this thesis presented the development and assessment of two quantitative assays by CE. Each assay applied the understanding gained from the previous work involved with CE theory, the use of additives and general separation design. The first assay consisted of an inter-departmental (chemistry and biochemistry) collaborative project involving the analysis of Gla from hydrolyzed protein, urine and plasma using CE with LIF detection. The separation was designed according to specific properties of the analyte. The FTC-labeled acidic amino acids: Glu, Asp, and Gla, were separated because of their high negative electrophoretic mobility in the run buffer, in comparison to the other amino acids. Excellent selectivity, sensitivity, linearity, accuracy and precision was demonstrated by the Gla assay. Future work may include reducing the time required (24 hours) for fluorescent labeling by varying the reaction conditions, or by selecting a different fluorescent tag. This would be important for rapid clinical studies using CE-LIF (free Gla analysis of urine or plasma) of patients having blood or bone related diseases involving the vitamin-K dependent proteins. However, the total time for Gla analysis may still not be entirely reduced for Gla analysis of proteins, since it requires a long period of time (18 hours) to ensure complete hydrolysis. Application of the discontinuous electrolyte system for selective Gla focusing, as developed in Section D, may also be helpful to improve assay sensitivity, allowing one more flexibility in reducing the time required for fluorescent labeling. The second assay consisted of an academic-industrial collaborative project involving the analysis of epinephrine from fifteen
different commercial dental anesthetic solutions. Similarly, the understanding gained from previous work, aided in the design of an effective separation methodology. Due to the specific chemical properties of the analytes in the anesthetic formulation, epinephrine was separated from the other components in the mixture by using borate as a specific complexation additive (as well as buffer). UV absorbance detection was not sufficiently sensitive to detect the low concentration of epinephrine present in the anesthetic solutions when using the small injection volumes typical of CE. Fortunately, an interesting focusing phenomenon was observed to occur naturally for epinephrine when large amounts of the anesthetic solution were directly injected into the capillary. This enhanced concentration sensitivity for epinephrine since a large volume of the dilute sample was focused into a narrow zone prior to detection. A dynamic pH junction between sample and background electrolyte zones was postulated to be responsible for selective analyte focusing. Method validation of the epinephrine assay for regulatory agencies was excellent in terms of precision, accuracy, linearity, and selectivity. Overall, the epinephrine assay by CE proved to be a robust and reliable method.

In life, the most celebrated advantages of a scientific, political, or philosophical system, frequently overshadow its inherent deficiencies. Similarly, the tremendous separation efficiency attainable in electrophoresis from the use narrow bore fused silica capillaries comes at a price to the analytical chemist, namely reduced concentration sensitivity. The majority of routine analyses utilize UV absorbance as the primary mode of detection. However, the concentration sensitivity in CE with UV detection is often much greater than micromolar concentration levels, which is often insufficient for trace analyses. Simple, yet effective ways to overcome this shortfall in sensitivity in CE is required. Section D (Chapters VII & VIII) involved a follow-up investigation of the focusing mechanism originally observed for epinephrine analysis in the previous section. It was determined that a
dynamic pH junction was most the most likely cause of selective epinephrine focusing. This conclusion was confirmed by mimicking the focusing effect observed in the real anesthetic solutions by varying the pH of standard epinephrine sample solutions in borate buffer. A pH difference of only 1.5 units between sample and background buffers was required to induce pronounced epinephrine focusing. A dynamic pH junction was also used to focus two other related catecholamines: norepinephrine and dopamine, in addition to weakly acidic species, such as tyrosine and phenol. The general principle of a discontinuous electrolyte system to selectively focus analytes of interest was also applied to nucleosides and nucleotides. In this case, different buffer types, or unbuffered salt solutions may be used to induce analyte focusing. This on-line focusing technique was amenable to samples of high salt and real biological matrices, such as cell extracts. It was surmised that focusing is the result of significant analyte electrophoretic mobility changes that occur as a dynamic pH junction sweeps across the sample zone (i.e., migrating borate and hydroxide anions from background buffer) upon application of the voltage. The net effect is selective focusing of analytes which possess weakly ionizable functional groups or moieties capable of complexation with additives (such as borate). Differences in the pH and borate complexation between the sample and background zones were observed to be responsible for analyte mobility changes. Solutes in the sample which did not undergo changes in electrophoretic mobility (neutral or fully ionized analytes) within the dynamic pH junction were observed to not focus (e.g., DOPA, catechol, dA, and dC). Further work is required to better understand this focusing mechanism and ways to apply it to different types of analytes including, acidic, basic, zwitter-ionic and neutral species. Insight into the dynamics of the pH junction may be realized through visualizing the focusing effect in real-time using a pH sensitive dye in the sample or background buffer, using a bare silica capillary that is coupled to a CCD camera. This may provide a non-invasive picture of the events involved with induction of the
dynamic pH junction within the sample zone. This on-line focusing technique still needs to
tested for weakly basic and neutral analytes. A general focusing strategy that makes use of
discontinuous electrolyte systems has not yet been developed for CE. Differences in the
conductivity, pH or concentration of complexing agents in the sample and background
electrolyte zones may be employed to induce selective analyte focusing. Samples as diverse
as pharmaceuticals (basic opiates) and environmental (neutral aromatics) species may benefit
from improved concentration sensitivity when using this method in CE. This technique may
also be applied to further improve the sensitivity of LIF and electrochemical detection in CE.

9.2 Separation Design

Guideposts or maps are commonly used to help orient people amidst chaotic and complex
surroundings. A general separation "map", depicted in Figure 9.1 is presented here based on
work and insight gained throughout this thesis to help guide researchers towards rational
separation designs in CE. A fully integrative approach to separation is proposed to separate
complex samples, incorporating the diverse fields of organic/inorganic (synthesis), analytical
(analysis), and theoretical/physical/computational (modeling/theory) chemistry.

First, the goals of the separation need to be clarified, in terms of required selectivity and
sensitivity. There are two main types of separation: 1) a selective analysis of one or a few
components, and 2) a general separation of a wide variety of analytes in a mixture (sample
profile). Each type of separation dictates the other steps required in the separation design.
Also, the sensitivity that is needed to detect analyte(s) of interest during the separation is also
addressed. In addition to the level of sensitivity, two general types of detector formats can be
used: selective and universal. In CE, several different types of detectors that possess various
levels of sensitivity are commonly available: 1) selective/high sensitivity: LIF,
Electrochemical detection, 2) universal/high sensitivity: ESMS detection, and 3) universal/low sensitivity: UV absorbance, PDA detection. Selective separation of trace amounts of analyte benefit from the use of selective/high sensitivity detector formats, whereas separations involving high concentrations of analytes in a sample profile profit from universal/low sensitivity detectors. It is important to note that the sensitivity of UV and PDA detectors may be further extended by on-line focusing strategies that may be implemented later during optimization of the buffer conditions.

Once the general requirements of the separation have been assessed, initial knowledge of some of the physicochemical properties of the analyte(s), such as size/shape, charge state (neutral, zwitter-ionic, positively, negatively charged), polarity, solubility, and the presence of functional groups or chromophores, greatly facilitates separation design. This in turn aides in the selection of the most effective buffer system for separation. Important properties to consider for the buffer include: buffer type, pH (useful pH range), ionic strength, miscible organic solvents, low conductivity, and low background absorbance or fluorescence. In addition, knowledge of some of the properties of the sample matrix such as: salt content, pH, presence of particulates (requiring filtering) and its compatibility (solubility) with buffer, can help guide proper selection of buffer conditions used for separation, as well as possible strategies for on-line focusing techniques. Under a constant temperature, the electrophoretic mobility of the free analyte, $\mu_{ep,A}$, is an intrinsic property of a species, dependent on the properties of the buffer. Hence, the primary role of the buffer in CE is to provide reproducible conditions to maximize $\mu_{ep,A}$ differences in all the species of a mixture for separation. Trials of different buffers can be done to optimize the buffer conditions. Nevertheless, optimization of the buffer conditions alone may not be sufficient to fully resolve all solutes in a complex mixture. The use of additives in the run buffer may be
required. Selective on-line focusing of analytes may be applied to boost the sensitivity of trace amounts of analytes for detection using UV absorbance. Appropriate selection of sample and background electrolyte matrices is made based upon the specific properties of the analyte(s) of interest.

The appropriate use of additive(s) in the run buffer can further enhance the selectivity in CE, since both electric field (electrophoresis) and equilibria (chromatography) influence separation. In this case, separation is based on differences in three fundamental properties: $\mu_{ep, A}$, $\mu_{ep, AC}$ and $K$. The types of additives that may be used is indeed enormous, ranging from metal ions to complex biological molecules. Generally, one may use a "complementary" principle to help select the most appropriate additive. Steric and charge (polarity) complementarity between the analyte(s) and additive (or receptor site of additive) is required for significant non-covalent interaction. For instance, charged additives with relatively small, hydrophobic cavities (e.g., charged CDs, TESMR) are best suited to separate small, neutral, hydrophobic analytes, whereas a neutral, polar surfactant (e.g., Brij 35) may best be used to separate large, charged, polar analyte(s) in CE. Computer molecular modeling of analyte-additive interactions can be used before experimentation to provide insight into the most appropriate additive. Initial screening of a variety of different additive types, or a series of additive analogues, can be done rapidly in order to eliminate unlikely candidates. In addition, one may use computer modeling to tailor design new additives of interest for specific analytes. The results from this work can then be used by organic chemists to synthesize and characterize the newly designed additive. Selective organic chemistry can also modify the structure of existing additives, by incorporating charged functionalities, or ring structures. Computer modeling, in conjunction with organic synthesis, can facilitate the proper selection and use of additives for specific CE separations.
Once the buffer conditions and additive(s) have been selected, trial experiments at several different additive concentrations can then be done. Dynamic complexation capillary electrophoresis can be used to quantitatively model (by computer) the mobility of all analytes as a function of additive concentration. Calculation of the three fundamental parameters influencing the separation can then be used to predict optimum separation conditions by selecting the additive(s) concentration which produces the least amount of overlap between analytes. One may also model the separation at various capillary lengths and voltages (within the linear region of the Ohm plot) to provide a separation with the greatest resolution under a minimum amount of time. Inadequate resolution of a separation may require fine tuning (e.g., adding organic solvents), or re-assessment of buffer conditions, including the type of additive, or the possibility of using multiple additives. Once the separation is optimized, the assay is validated in terms of required parameters such as: precision, accuracy, linearity, LOD, selectivity, robustness and reliability. This type of approach may lead to more rapid and effective separation optimization strategies in CE. Furthermore, the use of various chemical approaches to research (i.e., synthesis, analysis, theory, computational) serves to deepen the understanding of separation mechanisms and their practical applications to separation design. Figure 9.1 illustrates the rationale that may be used to systematically optimize separations in CE. It is important to realize that this framework is simply a general strategy and guide post, which may require further modification dependent on the specific separation of interest.
Figure 9.1 Schematic of the separation design framework illustrating the rationale used for systematic optimization of a separation through an integrative approach.
9.3 Epilogue

"We make an idol of truth itself; for truth apart from charity (love) is not God, but his image and idol, which we must neither love nor worship."

- B. Pascal

Throughout this thesis, I have often experienced a mixture of insight and confusion, inspiration and disenchantment, direction and bewilderment, struggling to manifest creative ideas and concepts into the hard objective realism of scientific research. There were many times when I believed I fully understood a phenomenon, only to observe a contradictory result that shattered the theory to pieces. Learning to appreciate and acknowledge even the unexpected and nonsensical data required a great deal of humility and trust, since these observations often led me to discover new and interesting phenomenon. Ideas often required time to slowly develop, as did the experimental work to demonstrate them repeatedly. Despite the necessity for perseverance and confidence in attitude towards scientific research, it is also prudent to realize moments to change perspective or direction, while letting go of inflexible conceptions and mental misconstructions. Nature abounds in fascinating and wondrous phenomena that still baffle many scientists around the globe. The natural sciences are just one way that man can understand aspects of truth in nature. Since the sciences are a vehicle used by man, they are still subject to the same subjective illusions, desires and other (un)conscientious psychological forces that dwell within him. As Pascal has warned us, excessive credence in the ultimate sanctity of any one theory as divine truth (an atheist's form of religious fervour), without retaining a sense of wonder, respect or some form of human dignity (charity), can easily lead one astray to the confines of blind idolatry; indeed it is very unscientific. The potential misuse of some forms scientific discoveries and their technologies in the world may reflect this tendency. I believe that I have gained a deeper
respect for the complexity and wonder of natural phenomena during the time I have laboured under this thesis. Moreover, I realize that the more one tries to understand nature, the more one may yet understand oneself.