Comprehensive Isotachophoresis-Capillary Electrophoresis Coupled to Time-of-Flight Mass Spectrometry

Christopher Ryan Bowerbank

Brigham Young University - Provo

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COMPREHENSIVE ISOTACHOPHORESIS-CAPILLARY ELECTROPHORESIS COUPLED TO TIME-OF-FLIGHT MASS SPECTROMETRY

by

Christopher Ryan Bowerbank

A dissertation submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Chemistry and Biochemistry

Brigham Young University

April 2001
Isotachophoresis (ITP) coupled to capillary zone electrophoresis (CE) in a comprehensive manner was used to separate mixture components in both insufficient and sufficient concentrations without heart-cutting or splitting. Examples of comprehensive ITP-CE involving multiple CE injections of preconcentrated ITP zones are demonstrated. In the comprehensive arrangement, all of the sample in the first dimension (ITP) is subjected to analysis in the second dimension (CE), without significant sample loss or decrease in sample detectability resulting from removal of a portion of the sample. This is especially important for analytes at low concentrations which may form a single mixed zone instead of individual ITP zones. Direct online coupling of ITP to CE in this comprehensive arrangement involved the use of columns having different diameters with one directly inserted inside the other. A counterflow was applied when the isotachophoretic sample stack reached the bifurcation point. Large volume (10 µL) injections were made using an electrically-insulated commercial polymeric rotary valve injector for increased reproducibility compared to previous comprehensive ITP-CE
studies, with ITP and CE retention time RSD values ranging from 2-5%. An ultraviolet (UV) detector positioned at the bifurcation point was used to determine the beginning of CE injection. Application of a splitting voltage at the bifurcation point showed no affect on analyte transfer into the CE column. By using multiple injections of the ITP band(s), CE column overloading was not observed.

Online capillary isotachophoresis (ITP) and comprehensive isotachophoresis-capillary electrophoresis (ITP-CE) were also coupled with electrospray ionization (ESI)-orthogonal acceleration time-of-flight mass spectrometry (TOFMS). Separations were performed using 200 µm I.D. and 50 µm I.D. polyvinylalcohol (PVA)-coated fused silica capillaries for ITP and CE, respectively. Both ITP and ITP-CE were coupled to TOFMS for analysis of sufficient (10^{-5} M) and insufficient (10^{-6}-10^{-7} M) concentrations of angiotensins in mixtures. ITP-TOFMS of a single mixed zone of five angiotensins (3 x 10^{-7} M) showed that ion suppression due to the co-elution of angiotensin III in the electrospray significantly reduced the ionization of other analytes. A practical solution to the detection difficulties for ITP mixed zones involved the insertion of a CE separation between the ITP and TOFMS for online preconcentration, separation, and identification in one system.
ACKNOWLEDGMENTS

My research and the completion of this dissertation would not have been possible without the generous support of many people to whom I would like to express my gratitude.

I dedicate this volume of work to my wife, Trisha Jensen Bowerbank, who has been by my side as we have traveled roads of success and difficulty together during graduate school. I also wish to thank my children, Rachel (5), Katie (3), and Josh (1), for their understanding and patience as Daddy went to "work" at all hours of the day and night, even on some "Daddy stay home days". I also thank my parents, M. Ryan and Norma J. Bowerbank, and my parents-in-law, Ned L. and Kathleen E. Jensen, without whose support this work would have never been possible. I likewise thank other members of my extended family who offered their generous support.

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<th>Definition</th>
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<tbody>
<tr>
<td>A/D</td>
<td>analog to digital</td>
</tr>
<tr>
<td>C</td>
<td>centigrade</td>
</tr>
<tr>
<td>C_L</td>
<td>leading electrolyte concentration</td>
</tr>
<tr>
<td>C_S</td>
<td>sample concentration</td>
</tr>
<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CEC</td>
<td>capillary electrochromatography</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>E</td>
<td>electric field</td>
</tr>
<tr>
<td>EDM</td>
<td>electrical discharge machining</td>
</tr>
<tr>
<td>EOF</td>
<td>electroosmotic flow</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HAc</td>
<td>acetic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>I.D.</td>
<td>internal diameter</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>ITP</td>
<td>isotachophoresis</td>
</tr>
<tr>
<td>k</td>
<td>proportionality constant</td>
</tr>
<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LE</td>
<td>leading electrolyte</td>
</tr>
<tr>
<td>LIF</td>
<td>laser induced fluorescence</td>
</tr>
<tr>
<td>LOD</td>
<td>limit of detection</td>
</tr>
<tr>
<td>M</td>
<td>molarity (mol L⁻¹)</td>
</tr>
<tr>
<td>m</td>
<td>mass</td>
</tr>
<tr>
<td>m</td>
<td>meter</td>
</tr>
<tr>
<td>m</td>
<td>milli (10⁻³)</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>O.D.</td>
<td>outside diameter</td>
</tr>
<tr>
<td>PEEK</td>
<td>polyetheretherketone</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethyleneglycol</td>
</tr>
<tr>
<td>PMMA</td>
<td>polymethylmethacrylate</td>
</tr>
<tr>
<td>PTFE</td>
<td>polytetrafluoroethylene</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinylalcohol</td>
</tr>
<tr>
<td>RSD</td>
<td>relative standard deviation</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SFC</td>
<td>supercritical fluid chromatography</td>
</tr>
<tr>
<td>TE</td>
<td>terminating or trailing electrolyte</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
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<tr>
<td>TOFMS</td>
<td>time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>µ</td>
<td>micro ($10^{-6}$)</td>
</tr>
<tr>
<td>µ&lt;sub&gt;ep&lt;/sub&gt;</td>
<td>electrophoretic mobility</td>
</tr>
<tr>
<td>µL</td>
<td>microliter ($10^{-6}$ liter)</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer ($10^{-6}$ meter)</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Vis</td>
<td>visible</td>
</tr>
<tr>
<td>z</td>
<td>charge</td>
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CHAPTER I
INTRODUCTION

I.1. CAPILLARY ELECTROPHORESIS

I.1.1. Background

The popularity of capillary electrophoresis (CE) for the separation of charged species is due to its numerous useful characteristics including high separation selectivity, small sample volume requirements, applicability to a wide variety of analytes, high speed, variety of separation modes, high separation efficiency, excellent mass sensitivity, low reagent/buffer consumption, high recovery when surface adsorption is minimized, and more recently, its applicability to microchip-scale separation systems. Since the early CE experiments in 1967 by Hjerten [1] and later in 1974 by Virtanen [2], the past few decades have experienced remarkable growth in the number of CE applications developed. Beginning with the success of Everaerts [3] and Jorgenson [4] in the latter part of the 1970’s and early 1980’s, CE has been applied to a wide variety of analytical problems including complex biological and environmental samples, high-throughput and combinatorial chemistry screening, genomics and proteomics.

Despite the rapid growth of CE, its major drawback is that of a poor concentration limit of detection (LOD) [5]. This is often cited as the major roadblock to the implementation of new CE-based applications in other scientific disciplines [6-10]. The primary reason for the poor CE concentration LOD is limited sample capacity which results from the use of small internal diameter (I.D.) capillaries that intrinsically have a very short optical path length. A similar constraint is placed on microchip-based systems
which often utilize channels with widths as small as tens of microns. A field of study which has available only small quantities of sample and which foresees using CE as a research tool is the discipline known as ultramicroanalysis. CE is especially attractive because of its ability to rapidly separate charged species in very small sample volumes. If CE capabilities expand to the point where it is capable of analyzing low nanoliter and picoliter sample volumes, numerous chemical and biochemical applications will certainly appear. One such area where CE would find immediate application is single-cell analysis [11-13].

However, before such small samples will be possible, further improvements in decreasing the concentration LOD of CE must be realized. In an effort to improve the CE concentration LOD and hence broaden its applicability, a number of novel techniques have been used. In particular, three techniques have shown the greatest promise: derivatization, improvements in detection instrumentation, and online preconcentration prior to CE separation.

I.2. EFFORTS TO IMPROVE CE SENSITIVITY

I.2.1. Derivatization

A major drawback to using detection techniques which require native absorbance or fluorescence is that some analytes in a sample mixture may show weak signals. A common method to enhance absorbance or fluorescence characteristics is to chemically derivatize the compound using tailored derivatization agents. In CE, derivatization can take place pre- or post-column, or in an online configuration. Labeling using a fluorescent tag is the most common method in combination with laser-induced
fluorescence (LIF) detection. Numerous structural functionalities including carbonyl, amine and hydroxyl groups have been derivatized.

A side benefit to derivatization is that, because additional functional groups are added to the compound of interest during the derivatization process, often the CE separation improves due to (1) more favorable electrophoretic characteristics, (2) addition of ionic character to an otherwise neutral or weakly charged compound, (3) improvement in the separation from co-eluting or interfering compounds, or (4) prevention of premature compound degradation in the CE environment. Comprehensive reviews of derivatization for CE can be found in the literature [14-16].

1.2.2. Improved detection techniques

Improvement in CE concentration detection limits have been realized by improvements in many detectors including ultraviolet/visible (UV/vis) absorbance, LIF, electrical conductivity detection, and mass spectrometry (MS) [17,18], and their mass and concentration detection limits are listed in Table 1.1. Nearly all commercial CE instruments come standard with on-column UV/vis detection, which makes improvements even more difficult because of the small dimensions of the capillary. Absorbance detection methods using shorter wavelengths of light (less than 200 nm)[19], modified detection cells such as the bubble cell [17], Z-cell [20], and multi-reflection cells [21], and indirect UV [22] have all reported improved detection limits, with indirect UV showing the greatest enhancement of up to 50-fold.

As a result of pioneering work by Jorgenson et al. [4], Zare et al. [23], and advances in derivatization technology, LIF has become the next most popular CE
Table I.1. Detection limits for capillary electrophoresis detectors [18].

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<th>Detection Method</th>
<th>Mass Detection Limit (mol)</th>
<th>Conc. Detection Limit (mol L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultraviolet-visible absorbance</td>
<td>$10^{-13} - 10^{-16}$</td>
<td>$10^{-3} - 10^{-8}$</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>$10^{-15} - 10^{-17}$</td>
<td>$10^{-5} - 10^{-9}$</td>
</tr>
<tr>
<td>Laser-induced fluorescence</td>
<td>$10^{-18} - 10^{-21}$</td>
<td>$10^{-10} - 10^{-16}$</td>
</tr>
<tr>
<td>Amperometry</td>
<td>$10^{-18} - 10^{-20}$</td>
<td>$10^{-5} - 10^{-11}$</td>
</tr>
<tr>
<td>Conductometry</td>
<td>$10^{-15} - 10^{-16}$</td>
<td>$10^{-4} - 10^{-8}$</td>
</tr>
<tr>
<td>Indirect amperometry</td>
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<td>$10^{-5} - 10^{-8}$</td>
</tr>
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<td>Indirect fluorescence</td>
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<td>$10^{-5} - 10^{-7}$</td>
</tr>
<tr>
<td>Mass spectrometry</td>
<td>$10^{-16} - 10^{-17}$</td>
<td>$10^{-8} - 10^{-9}$</td>
</tr>
</tbody>
</table>
detection technique after UV/vis. LIF has shown the highest reported sensitivity in CE, detecting concentrations in the sub-femtomolar range [24]. Also, electrochemical detection has also shown improvements in such areas as decoupling of the electrochemical detection potential from the potential and current in the CE column, optimization of the detection cell position, and improving the ruggedness and reproducibility of electrochemical cell performance [6,25].

Of most interest in recent years has been advances in CE coupled with MS. Several CE-MS reviews have been published [10,26,27]. The most common interface for CE-MS is electrospray ionization (ESI) which uses an applied voltage at the CE column outlet to induce a spray which disperses the ions into tiny droplets. The droplets are then desolvated prior to introduction to the MS. Various interfaces can be used, with the most common being the sheath-flow interface [28], liquid junction interface [29], and the sheathless interface [30]. Applications of microchip CE-ESI-MS have also appeared more frequently in the literature in recent years [31-39].

1.2.3. Preconcentration techniques

In CE, the use of small I.D. (10-150 µm) capillaries presents both advantages and disadvantages. The major advantages are rapid heat dissipation to reduce localized heating and minimization of thermal gradients within the capillary, small sample requirements for injection which reduces sample waste, minimal sample loss from adsorption on the capillary surface because of the reduced surface area, and dead volumes that are much smaller compared to other separation systems [40]. The major disadvantage, as discussed earlier, is the short detection path length. Another
disadvantage of capillary separation systems is their limited sample capacity. Efforts to
develop sample introduction techniques which provide on-line sample preconcentration
have given rise to numerous on-column solute focusing methods that can accommodate
larger sample volumes. Introduction of a preconcentrated sample increases the amount of
analyte on-column compared to conventional electrokinetic or hydrodynamic injection
alone, giving rise to decreased concentration LOD's. Among the preconcentration
techniques commonly used with CE are sample stacking [41-45], field amplification [46-
49], and isotachophoresis (ITP) [50-55]. Other techniques such as isoelectric focusing
(IEF) [56-59], a physical barrier [60], transient isotachophoresis [61,62], hollow fibers
[63,64], sweeping concentration [65], and a variety of ligand-binding techniques [66-76]
can also be used but will not be discussed here.

1.2.3.1. Sample stacking

Hydrodynamic injection, which is a common sample introduction method for CE,
does not provide any sample concentration benefits. If a sample is dissolved in the run
buffer and placed on the CE column hydrodynamically, the width of the sample plug
determines the width of the solute zone that is subjected to the CE separation conditions,
and no concentrating effect occurs. In sample stacking, however, the sample is dissolved
in a buffer that is of lower conductivity than the run buffer [41]. The sample is then
hydrodynamically injected, resulting in a plug of lower conductivity introduced
immediately behind the run buffer. When a voltage is applied across the capillary, a
higher electric field develops in the injected sample plug due to its higher resistivity.
Since electrophoretic velocity is directly proportional to the strength of the electric field,
the analytes in the sample quickly migrate to the boundary between the sample plug and
the run buffer, where they encounter a lower electric field at the run buffer interface. As
a result of this electrophoretic velocity difference between the two buffers, analytes stack
and are concentrated at the boundary. The analytes then move through the capillary as
narrower zones than would exist under conventional hydrodynamic injection conditions.
The sample stacking process is illustrated in Figure 1.1. Concentration factors using this
method are typically from the low tens up to several hundred [77]. A drawback to
sample stacking is that all charged species in the sample are concentrated, including
potentially interfering compounds, which may hinder the separation of the analyte(s) of
interest.

1.2.3.2. Field amplification

Although similar in concept to sample stacking, field amplification is used when a
sample is introduced electrokinetically instead of hydrodynamically. In electrokinetic
injection, the CE capillary is first completely filled with the run buffer, and the capillary
inlet is inserted into a sample vial. When the injection voltage is applied, analytes
migrate from the sample vial to the CE capillary. With field amplified injection, the
sample is prepared in similar fashion to sample stacking using a dilute buffer having a
lower conductivity than the run buffer. Under these conditions, there is an amplified field
at the capillary inlet, and the analytes migrate rapidly from the high electric field in the
sample solution to the lower electric field in the CE capillary. Since the electrophoretic
Figure I.1. Schematic of sample stacking with a sample dissolved in a lower conductivity solution. Top: sample plug is hydrodynamically injected. Middle: voltage is applied and analytes migrate to the run buffer interface and concentrate. Bottom: concentrated analytes migrate through the capillary in a narrower zone than the original sample plug and begin to separate.
velocity decreases when the sample ions encounter the run buffer, they slow down and become concentrated. The advantage of field amplification over sample stacking is that problems associated with large volume samples, often required for hydrodynamic injection and sample stacking of dilute samples, are minimized [78]. Also, field amplified injection results in higher concentrating factors than sample stacking, with factors up to one thousand reported [47-49]. Again, a drawback to field amplification, like sample stacking, is the simultaneous concentration of potentially interfering compounds and contaminants.

1.2.3.3. Isotachophoresis

Isotachophoresis (ITP) differs from both sample stacking or field amplification in that it can also be used as a stand-alone separation technique. ITP is an electrophoretic separation technique that, similar to CE, separates compounds based on differences in electrophoretic mobility. However, whereas CE uses a single background buffer as a separation medium, ITP uses a discontinuous buffer system. As shown in Figure 1.2, the sample is placed between a leading electrolyte (LE) and trailing or terminating electrolyte (TE). The LE is selected such that it has a higher electrophoretic mobility ($\mu_{ep}$) than any analyte in the sample. Similarly, the TE is selected such that it has an $\mu_{ep}$ lower than any analyte of interest in the sample.

When an electric field is applied across the capillary, the analytes arrange or “stack” themselves in order of $\mu_{ep}$ from highest to lowest, with the analyte zone having the highest mobility positioning itself next to the LE, and the analyte zone with the lowest $\mu_{ep}$ lying next to the TE. After the analytes distribute themselves accordingly, a steady
Figure 1.2. Pictorial representation of the ITP separation mechanism. L = leading electrolyte, T = trailing electrolyte, A,B,C = individual analytes, with A having the highest electrophoretic mobility, followed by B and C.
state equilibrium is reached, and the LE, analyte zones, and TE all travel through the capillary towards the detector at the same velocity, \( v_L \), which is the velocity of the LE zone. This is where the name “isotachophoresis” is derived, meaning “iso” for same and “tacho” for velocity. The velocity of the LE zones is given by the equation,

\[
v_L = \mu_{ep} E
\]  

(I.1)

where \( E \) is the electric field strength. Thus, for the entire ITP zone stack, the equation

\[
v = \mu_L E_L = \mu_1 E_1 = \mu_2 E_2 = \mu_T E_T
\]  

(I.2)

applies where the subscripts L and T refer to the leading and trailing electrolytes, respectively, and 1 and 2 refer to individual analyte zones. Since each analyte has a different electrophoretic mobility, a different electric field strength is required in each analyte zone to maintain constant velocity, thereby establishing a potential gradient. The highest electric field is found in the TE, which has the lowest mobility.

Sharp boundaries develop between ITP zones because of the self-sharpening effect of ITP. If an analyte migrates into a neighboring zone, it experiences a different electric field strength, which changes its velocity. For example, if a solute migrates into a zone of analytes with higher \( \mu_{ep} \), that solute will experience a lower electric field, and, based on Equation I.2, will slow down and return to the zone having the appropriate \( E \) for its mobility.
Once the analytes have migrated into their proper zones and the ITP steady state is reached, the analyte concentration in each band becomes approximately the same. This concept is derived from the Kohlrausch regulating function [79] where the concentration of the solute (analyte) ions are related to the concentration of the LE [80],

\[ \frac{C_L}{C_S} = \left[ \mu_L(\mu_L + \mu_C) \right] \left( \frac{\mu_S + \mu_C}{\mu_S} \right) \] (I.3)

where \( \mu \) is the electrophoretic mobility, and the subscripts C, L, and S represent the counter-ion, leading electrolyte ion, and solute ion, respectively. The final concentration of the solutes in the ITP zones is given by,

\[ C_S = C_L k \] (I.4)

where \( k \) is a proportionality factor. When electrophoretic mobilities are similar, \( k = 1 \) and the concentrations of the analytes within the zones become the same as the concentration of the LE. For most analytes, the value of \( k \) is between 0.2 and 1 [81]. This concentration parity across the ITP zone stack at steady state means that analytes which are at higher concentrations in the original sample than the LE will be spread out until their concentrations within the zone match the LE concentration. Likewise, dilute analytes will form narrow zones in an attempt to reach the LE concentration.

The typical analyte profile in ITP differs from the readily recognizable gaussian peak shape that results from other separation methods such as gas chromatography (GC).
Figure I.3. Zone shapes in isotachophoresis resulting from (A) sufficient concentration, (B) insufficient concentration (L = leading electrolyte, T = terminating electrolyte, S = sample).
Figure I.4. ITP separation with both UV (254 nm) and conductivity detection. The conductivity trace shows the typical ITP stair-step pattern. Note that analytes 1, 3, 5 and 6 have no UV absorption. Reproduced by permission, Elsevier Scientific Publishers [82].
and liquid chromatography (LC). In ITP, analytes which have "sufficient" concentrations appear as adjacent flat-topped zones once the ITP steady state has been reached. Since the ITP zones stack immediately next to each other to maintain electrical continuity in the circuit, there is very little distance between the zones and they appear as steps, with each step representing one of the analyte zones. The width of each zone is directly proportional to the amount of analyte present and can be easily used for quantitation. During the process of reaching this equilibrium state, the analyte concentration within each zone increases in an attempt to match the concentration of the LE, which is typically introduced at a concentration at least three orders of magnitude higher than the individual analyte concentrations in the sample. Once the LE concentration has been reached within the zone, the zone width appears with a flat-top profile, illustrated in Figure I.3A. Each adjacent analyte zone also has a defined width, and the resulting stack of zones appears as a stair-step pattern in an electrical conductivity detector. An example of an ITP analysis is illustrated in Figure I.4.

In looking at the boundary between adjacent zones, careful examination shows that ITP zones are not fully separated from each other and, in reality, have overlapping boundaries due to a fixed boundary width. Samples which contain "insufficient" analyte concentrations are not able to reach the LE concentration and, as a result, form a peak-shaped mixed zone. As shown in Figure I.3B, the leading and trailing edges of the zones never fully separate. This also poses a challenge for electrical conductivity detectors commonly found on commercial ITP instruments. For a dilute (insufficient) sample, the minimum zone length in ITP is determined by the zone boundary width. Since this minimum width for ITP is small (µm), a very narrow detection window is required to
obtain good spatial resolution of the analytes. However, the typical gap between two electrodes using conductivity detection for ITP is about 200 µm (0.2 mm). For a high frequency contact-less conductivity detector, the width of the detection window is about 400 µm. Because of this, the detection of individual analytes in ITP mixed zones have proven difficult. The basic requirement for narrow overlapping zone detection is high spatial resolution and selectivity. Therefore, ITP is more suitable as a preconcentration and injection method for CE than as a stand-alone separation method for dilute samples.

The ability of ITP to concentrate dilute samples and remove interferents is one of the most cited ITP applications, since the final concentration does not depend on the original sample concentration. Also, ITP is capable of analyzing much larger sample volumes (µL’s) than can be injected in CE. Thus, for samples containing trace analytes, the concentrating effect of ITP can bring analytes, which previously were below the LOD in CE, to levels which are detectable.

I.3. ISOTACHOPHORESIS COUPLED TO CAPILLARY ELECTROPHORESIS

1.3.1. Introduction

A common use of ITP, besides being a stand-alone separation technique, is a sample preparation/preconcentration technique for CE. While other methods such as field amplification and sample stacking concentrate all of the sample constituents, including contaminants and interferants, ITP has the advantage that it can remove potentially interfering compounds prior to CE separation. A number of different configurations which combine the preconcentrating advantages of ITP with the high
Figure I.5. Schematic of ITP-CE configurations with (A) a single column, (B) coupled columns, and (C) coupled columns using a t-junction.
resolution and high speed analysis capabilities of CE are published in the literature. These configurations can be classified into three general categories and are illustrated in Figure 1.5: single-column ITP-CE, coupled-column single-pass ITP-CE, and coupled-column comprehensive ITP-CE.

I.3.2. Single column ITP-CE

I.3.2.1. Transient single column ITP-CE

As the name suggests, single-column ITP-CE uses only one capillary to perform both ITP and CE steps sequentially. Two approaches for the single column technique have been reported. One method, known as transient ITP-CE, occurs when migration of solutes gradually changes from the ITP mode to the CE mode. In this method, the column is first filled with LE and the terminating end of the capillary is inserted into the LE buffer. The sample is then loaded onto the column hydrodynamically, and the leading end of the capillary is immersed in TE. Once the voltage is applied across the capillary, ITP takes place and the sample analytes are separated into zones and concentrated. After stacking, the TE vial is replaced with an LE vial, and the sample is separated by CE since the LE serves as the background electrolyte by virtue of its presence both in front of, and behind, the original ITP stack. The advantage to this transient technique is that larger volumes of dilute sample can be loaded onto the column without overloading than by using either electrokinetic or hydrodynamic injection alone. Increased sample loading between 30 and 50-fold have been reported using this transient ITP-CE technique [83-88].
1.3.2.2. Counterflow single column ITP-CE

Another technique which has been used primarily to counter EOF effects is single column counterflow ITP-CE [89,90]. This counterflow is usually introduced by applying pressure to the outlet buffer reservoir at the terminal end of the CE column. Since the magnitude of EOF is relatively small, the required pressure is not difficult to achieve to counterbalance the EOF. However, the effect of the counterflow on ITP zone shape has kept this method from gaining widespread use. In the single capillary format, the LE, sample, and TE are introduced in similar fashion to the transient ITP-CE technique described earlier. An advantage to the counterflow technique is that because the counterflow is applied prior to the ITP stack reaching the end of the capillary, more of the capillary can be filled with sample compared with the transient method, which would not have adequate capillary length to provide CE separation following ITP pretreatment of such a large sample volume.

The counterflow can be applied both during and following ITP preconcentration. It is applied during the run if either a large sample volume fills a majority of the capillary, or if the EOF in the capillary does not allow the sample to reach ITP steady state before reaching the end of the capillary. By applying the counterflow while the voltage across the capillary is on, the zone dispersing effect of the parabolic flow profile of the counterflow is offset by the zone self-sharpening effect in ITP [91]. If adjusted properly, the counterflow in the opposite direction can be used to exactly match the EOF in the forward direction, thus suspending the ITP stack in a static position within the column. By balancing these forces, more time is allowed for the ITP stack to reach its steady state before CE separation is performed.
A more common use of the counterflow is to push the ITP zones back towards the column inlet following completion of the ITP concentrating process. During counterflow infusion, the TE is removed from the column and is replaced with LE by inserting the column inlet into an LE reservoir. The CE separation of the concentrated zones then occurs, with LE acting as the background electrolyte. Drawbacks of this counterflow technique are that the parabolic flow profile of the pressure-induced counterflow causes disruption and dispersion of the ITP zones, which necessitates a higher electric field strength in order to maintain ITP equilibrium in the presence of the counterflow. In addition, the small volumes of the 50-150 µm I.D. CE capillaries, combined with the difficulty in precisely controlling the counterflow using a pneumatic pump, make counterflow ITP-CE using capillary-sized columns even more problematic.

1.3.3 Coupled-column ITP-CE

In order to subject an even larger volume of sample to ITP-CE than is possible using the single capillary format, the coupling of a larger inner diameter column with a smaller column has been used [92,93]. The ITP-CE methodology is the same as for the single capillary technique, only a larger sample volume can be injected. The large volume of dilute sample is concentrated and stacked under ITP conditions, and either transient or counterflow techniques are then used to effect the CE separation.

Coupled column systems using either two or three column arrangements have been used and are noted in the literature [5,94-96]. In the dual column system, a large capillary is attached directly (e.g., using epoxy) to a smaller I.D. capillary [97]. A drawback to this type of configuration is that CE column overloading which results in
poor CE separation can occur. With this type of system, it is difficult to estimate how much sample can be injected to avoid this overloading. A more common configuration used in commercial ITP instruments is the three column system [98] which uses a t-junction to join the capillaries, and is illustrated in Figure 1.5C. In these systems, overloading of the CE capillary is avoided by splitting the sample eluting from a large (e.g., 800 µm I.D.) ITP capillary between a CE capillary and another large I.D. split capillary that lies orthogonal to the ITP and CE capillaries. In this method, ground potential is applied to both the splitting and CE capillaries simultaneously, resulting in two electrical circuits that effectively split the sample. Control of the ground location can also be used to selectively remove individual contaminant ITP zones prior to CE separation. Numerous papers exist in the literature describing this t-junction system [99-107]. The disadvantage of the three-column configuration is that by splitting the sample, trace analytes, although enriched via ITP, may not be in high enough concentration following splitting to be detected. In an effort to analyze all of the components of a sample including trace analytes without sample splitting, comprehensive ITP-CE was developed.

1.3.4. Comprehensive ITP-CE

In comprehensive two-dimensional separations [114-120], all components separated in the first dimension are subjected to separation in the second dimension. The comprehensive ITP-CE separation technique combines the advantages of ITP and CE into a two-dimensional arrangement whereby both techniques are performed sequentially using coupled capillary columns having different internal diameters. Initially, the method
is similar to coupled column ITP-CE using a counterflow whereby analytes are first separated and focused into narrow bands in the ITP capillary, after which a portion of the focused ITP zones is introduced into the CE capillary. However, instead of splitting off the remainder of the sample that was not injected into the CE capillary, the remaining ITP zones are pushed back into the ITP capillary using a hydrodynamic flow of LE. Once the previous CE injection has had adequate time to move down the CE column, the ITP zones are once again allowed to migrate down the column where another CE injection occurs. This process, illustrated in Figure 1.6, is repeated multiple times in a comprehensive manner until all ITP zones have undergone CE separation. Since only a portion of the ITP zones is injected into the CE capillary each time, column overloading, a common problem in single column ITP-CE techniques, is largely avoided. The comprehensive ITP-CE preconcentration and separation technique decreases the concentration LOD by performing multiple CE injections and separations, which can be added together. By so doing, the signal-to-noise ratio is increased, thus allowing trace analyte detection at lower levels than currently attainable using single pass techniques.

The first example of comprehensive electrophoretic separations using coupled column ITP-CE was shown by Chen et al. [121]. The comprehensive ITP-CE research in this dissertation (Chapters II-IV) is derived from this earlier work, and the instrumentation and methodology are reviewed here to provide sufficient background for later discussions. The instrumentation for the early ITP-CE instrument is represented schematically in Figure 1.7. High voltage was applied to two of the three buffer reservoirs (BR1 and BR2) using a single high voltage power supply (30 kV maximum)
Figure 1.6. Comprehensive ITP-CE method. Voltage switching was used to apply the voltage across the ITP or CE columns independently, or across both for CE injection. No voltage is applied during counterflow infusion, or across the ITP column during CE separation, resulting in diffusion of the ITP zones. LE = leading electrolyte, TE = terminating electrolyte.
Figure 1.7. Schematic representation of a comprehensive ITP-CE instrument. BR1 = buffer reservoir 1, BR2 = buffer reservoir 2, BR3 = buffer reservoir 3, V1 = valve 1, V2 = valve 2.
and a high voltage relay. The ITP column was fixed between BR1 and BR2, and the CE column was fixed between BR2 and BR3, with the bifurcation point located in the center of BR2. The position of the ground was also controlled by a high voltage relay and was applied to either BR2 or BR3. The three buffer reservoirs were machined from Plexiglass, and the screw plugs in the buffer reservoirs and the valves were made from Delrin and Nylon, respectively. Fused silica columns of 320 µm I.D. × 430 µm O.D. and 50 µm I.D. × 190 µm O.D. were used as ITP and CE columns, respectively, and a UV absorbance detector was used for on-column CE detection. A programmable syringe pump was used to infuse the counterflow.

In preparation for an ITP-CE run, the following procedure was followed. First, the entire system was filled with LE. Flat sections of membrane tubing were used to suppress hydrodynamic flow within the system and were kept in position at the top of BR2 and BR3 by Nylon screw plugs and rubber O-rings. Once the capillaries were flushed and bubbles removed, the LE was removed from BR1 and replaced with TE. The sample (5-10 µL) was then injected using a syringe into a small, machined area in front of the ITP column inlet. For initial ITP focusing, a voltage was applied across the ITP capillary only, with BR1 serving as the voltage application point and the ground at BR2. The ITP zones then formed and migrated down the ITP column towards the bifurcation point (CE column inlet). Once the current in the ITP column reached a predetermined value, the voltage was turned off and the ground was switched to BR 3 for CE injection. The system current was monitored, and when it dropped by an assigned value, ΔI, the voltage was again turned off. The syringe pump then infused LE at the bifurcation point.
and pushed back the remaining ITP bands away from the CE column. Using this method, only a portion of the focused ITP zones was allowed to enter the CE column.

Following CE injection and LE infusion, the voltage relays were switched and the voltage was applied across BR2 and BR3 for CE separation. During this time, the zones that were pushed back into the ITP column remained stagnant and began to diffuse and mix, thus losing their sharp zone profiles since no potential existed across the ITP capillary. Following the CE separation, the voltage was again switched and applied across the ITP capillary to begin ITP re-focusing, and the entire ITP-CE process was repeated as many times as was necessary. This comprehensive ITP-CE system provided easier, more reproducible injections of large volume samples by using an ITP preconcentration step prior to CE without the need for an ITP detector prior to the bifurcation point. It also was capable of improved sensitivity due to the ability to perform multiple analyses and mathematically sum the results.

For the complete analysis of complex samples, multidimensional separation techniques such as ITP-CE show great promise. Since ITP is an effective preconcentration method for CE, its use is beginning to extend into trace analysis of complex matrices, especially those of biological origin such as blood and urine. In these types of fluids, the concentration of ionic species is high and can interfere with efficient separation of the analytes of interest. These types of samples are the focus of ITP-CE to remove interfering compounds and concentrate the analytes of interest for improved concentration LOD.
I.4. OBJECTIVES

The main objective of this research was to continue development of novel ITP-CE instrumentation to carry out comprehensive electrophoretic separations. Efforts to improve the instrument design, construction, installation and performance are discussed. This dissertation provides detailed descriptions of the changes made to comprehensive ITP-CE instrumentation to improve reliability, reproducibility, and ease of use. The coupling of ITP and ITP-CE to a time-of-flight mass spectrometer (TOFMS) is also reported.

Chapter II discusses the design and characteristics of an improved comprehensive ITP-CE system. A description of changes in comprehensive ITP-CE methodology is also discussed. Chapter III reports on the application of comprehensive ITP-CE to samples containing analytes of both sufficient and insufficient concentrations using dual ultraviolet (UV) detectors. The coupling of ITP and comprehensive ITP-CE to TOFMS is summarized in Chapter IV. Difficulty with ion suppression in the electrospray interface for samples containing analytes of insufficient concentration during ITP-TOFMS is discussed. Drawings, circuit diagrams, and control software screen diagrams for the ITP-CE system are included in Appendices A through C.
1.5. REFERENCES


42. Gebauer, P.; Thormann, W.; Bocek, P. J. Chromatogr. 1992, 608, 47.
44. Quirino, J.P.; Terabe, S. Electrophoresis 2000, 21, 355.


61. Toussaint, B.; Hubert, Ph.; Tjaden, U.R.; van der Greef, J.; Crommen, J. J. 


CHAPTER II

PRACTICAL ASPECTS OF COMPREHENSIVE ISOTACHOPHORESIS-
CAPILLARY ELECTROPHORESIS INSTRUMENTATION: DESIGN,
INSTALLATION, OPTIMIZATION AND TROUBLESHOOTING

II.1. INTRODUCTION

During the development of analytical instrumentation such as that for comprehensive isotachophoresis-capillary electrophoresis (ITP-CE), an understanding of the factors affecting performance plays a vital role in optimizing separations. As is the case with most separation methods, numerous factors affect the quality of an ITP-CE separation, and the purpose of this chapter is to explain in detail the design, instrument setup, and the effects that various changes in the operational parameters have on the separation quality when using the comprehensive ITP-CE instrument.

Whenever a novel instrument is being developed, problems arise which may, or may not, be specific to that instrument. The source of the problem must be quickly identified and remedied. These problem-solving opportunities contribute to the increased understanding of the methodology and instrumentation and, subsequently, provide a better understanding as to the capabilities and drawbacks of the instrument. On occasion, problems with similar ITP-CE instrumentation are noted in journal articles, and books written by experts in the field [1-4] are also valuable resources. However, not all problems encountered have been previously observed or reported in the literature, especially when developing novel instrumentation. Thorough reading of the literature
may provide a starting point, but often the solution will be specific to the newly developed instrument. In this chapter, the various components and operating parameters used for comprehensive ITP-CE will be discussed in detail, paying particular attention to their effect on the overall operation of the instrument.

II.2. PREVIOUS COMPREHENSIVE ISOTACHOPHORESIS-CAPILLARY ELECTROPHORESIS INSTRUMENTATION AND METHODOLOGY

First, a brief overview of the instrumentation used in a previous comprehensive ITP-CE instrument [5] is provided so that differences with the present comprehensive ITP-CE system can be highlighted. As discussed in Chapter I, the primary purpose for the comprehensive ITP-CE arrangement was to be able to analyze all of the concentrated zones from a single large volume sample. In other ITP-CE arrangements, only a portion of the original sample is analyzed to prevent CE column overloading.

In the previous comprehensive ITP-CE instrument design, several homemade components were used, together with a single high voltage power supply and two voltage switching relays. Referring to the schematic in Figure 1.7, Plexiglas blocks (BR1, 2, and 3) were machined to serve as buffer reservoirs. Nylon screws and plugs were also machined as supports for electrodes, columns and membrane tubing, and to act as a sample introduction valve (V1). The high voltage output, relay switching, system current read, data acquisition start and syringe pump infusion were all controlled using a Visual Basic (Microsoft, Redmond, WA, USA) program together with a 12-bit analog-to-digital converter card installed in a PC-compatible computer.
Although it is more common to use two detectors for ITP-CE, this system utilized a single detector positioned at the terminal end of the CE column and a system current monitoring process to initiate CE injection. As a result, an accurate timing routine was not required for CE injection. The following is a brief description of the operation of the instrument [5]. The system was first flushed and filled with LE. A syringe was then positioned near the ITP capillary inlet in a small mL-size region inside BR1, and a μL-size sample volume was injected. A voltage was applied across the ITP capillary where sample preconcentration occurred. It was assumed that the entire sample volume eventually moved onto the ITP capillary via electromigration. During ITP, the system current was monitored by the computer. When it reached a preset value determined prior to the run using crystal violet dye as a surrogate, the voltage was turned off and the ground was switched via relay from the terminal end of the ITP column to the terminal end of the CE column. By switching the location of the ground, the electrical circuit now extended through both the ITP and CE capillaries. The voltage was re-initialized and the ITP bands were allowed to migrate past the CE capillary inlet, resulting in a small portion of sample moving onto the CE column. The voltage was turned off and the syringe pump was started so that a counterflow was introduced at the bifurcation point located in BR2. The voltage was once again turned off and the high voltage application was switched from the ITP capillary inlet to the bifurcation point/CE capillary inlet in buffer reservoir 2 via the second relay. With the voltage now applied across only the CE capillary, CE separation occurred. After completion of the CE separation, both the voltage and ground were once again switched to their original positions. With the voltage now applied across only the ITP capillary, ITP concentration and migration towards the CE capillary inlet
once again occurred. This was repeated multiple times until all of the sample was analyzed.

One of the most significant drawbacks to this comprehensive ITP-CE system was the time required for complete analysis requiring multiple injections. For a sample requiring four CE injections, nearly two hours was needed for completion, despite each CE separation occurring in only 2.5 min. The long analysis time can be attributed to two steps in the ITP-CE method, which were addressed in the redesigned instrument. The first was that when the voltage was switched from the ITP column to the CE column, the remaining ITP bands, without any current in the capillary, dispersed and lost their previous steady state condition. Thus, prior to another CE injection, the bands had to be refocused, which required an additional 45 min to bring the zones back to equilibrium. This had to be repeated after each CE injection.

The other significant time-consuming step was the actual CE injection itself. Using the previous methodology, the value of the system current at the point where a crystal violet zone was 1.5 cm from the bifurcation point was used as a set point for CE injection. When the ground was switched so that the circuit included both the ITP and CE columns, migration onto the CE capillary was extremely slow due to the addition of the small diameter CE capillary to the circuit. Although using only a single detector is attractive in terms of lowering the overall cost of an ITP-CE system, the benefits of this minor cost savings may not outweigh the ramifications of the additional time required for a complete analysis.

In this work, each component of the previous instrument was scrutinized and redesigned. Although much was changed, some important aspects of the previous
instrumentation were retained. For example, in conventional ITP-CE, the ratio of the ITP column I.D. to the CE column is usually less than 3-to-1, since higher ratios make it more difficult to inject a small sample onto the CE column without overloading. In the comprehensive ITP-CE system, ratios larger than 4:1 can be used without overloading. In the current system, the CE column I.D. was kept at 50 µm to reduce the effect of hydrodynamic flow within the column following LE counterflow infusion. The hydrodynamic counterflow, when introduced at the bifurcation point, takes the path of least resistance. In this case, with a column I.D. ratio 4 or larger, the majority of the flow passed into the ITP column. Also, small column diameters were preferred since Joule heating effects were reduced due to rapid heat dissipation.

II.3. INSTRUMENTATION DESIGN

Since comprehensive ITP-CE involves the analysis of all ITP-concentrated zones, column dimensions, as well as the design of other system components, must ensure complete transfer of the analytes from the initial ITP sample injection to CE detection. The presence of a bifurcation junction between the ITP and CE capillaries and the use of an LE counterflow further increases the importance of careful design to minimize loss. Other aspects which could disturb the ITP steady state, such as having one buffer reservoir level higher than the other, must be also be carefully considered since siphoning or introduction of hydrodynamic flow in either direction can cause the ITP zones to be disrupted. A diagram of the redesigned comprehensive ITP-CE instrument is shown in Figure II.1 and details of its design follow.
Figure 11.1. Schematic of the comprehensive ITP-CE system. (1) High voltage power supply, (2) high voltage power supply, (3) high voltage relay for splitting voltage application, (4) LE buffer reservoir, (5) TE buffer reservoir, (6) injection valve, (7) ITP column, (8) CE column, (9) syringe pump, (10) rinsing shut-off valve, (11) rinsing shut-off valve, (12) UV detector for ITP column, (13) UV detector for CE column, (14) computer for control of power supplies, high voltage relay, and ITP column data acquisition, (15) computer for CZE detector data acquisition and control.
II.3.1. Construction materials

Since the materials used in the instrument design come in direct contact with the electrolyte solutions used for ITP and CE, the choice of materials for the overall design including the electrodes, injection valve, and column connection points (bifurcation point, CE terminus, etc.) was important. It was especially important to ensure that contamination did not occur as a result of contact with the materials. Materials such as silicone grease may cause contamination, thus, mechanical methods for forming a closed system were necessary.

Other requirements of the system included the ability to withstand high voltage, as well as tolerate organic solvents and extreme acidic or basic conditions which may be used during method development. Materials such as Plexiglass, Nylon, and Delrin have all shown to be compatible materials for electrophoretic applications, and Plexiglass is the material of choice due to its ease of machining, low cost, and availability.

II.3.2. Sample introduction

In looking at the following equation [3],

\[ c_i = 2(c_i^1/V_i)V_i^1 \]  

where \( V_i^1 \) is the initial volume, \( c_i^1 \) is the initial concentration of an analyte in a dilute ITP zone, \( V_i \) is the volume after ITP focusing and \( c_i \) is the maximum concentration of the analyte after ITP focusing, the maximum concentration of an analyte in a concentrated ITP zone increases linearly with the injection volume. Therefore, ITP-CE concentration
sensitivity increases linearly with increased injection volume. The key advantage of ITP over other sensitivity enhancing techniques is its ability to use large volume samples. Typical CE injection volumes are in the nL range, whereas ITP can analyze sample volumes as large as 1000 µL (1 mL). Although ITP can be performed using conventional CE electrokinetic and hydrodynamic injection techniques, fixed volume sampling is more common. The use of electrokinetic injection limits the sample volume that can be placed in the capillary, and has the negative aspect of sample discrimination caused by differences in the electrophoretic mobilities of the analytes. Hydrodynamic injection can also be used, but the actual volume introduced into the capillary can only be estimated.

ITP injection is typically carried out in similar fashion to LC injection. A sample loop having a known volume is filled with sample with the valve in its “load” position. Following sample loading, the valve is switched to its “sample” position, and the sample is placed in-line with the rest of the ITP system. The only commercially available ITP-CE instrument (ItaChrom EA 101, J&M Analytische Mess und Regeltechnik GmbH, Robert-Bosch-Straße, Aalen, Germany) utilizes a 30 µL sample loop, which is compatible with the large I.D. fluoropolymer capillary (800 µM) used for ITP separation.

II.3.3. ITP sample valve design

An aspect of a sample valve that can have a negative effect on a separation is unswept void volume. The greatest concern in ITP and CE is the presence of voids along the sample path where air may get trapped during rinsing and filling, resulting in the formation of an air bubble in the system which cuts the system current and terminates an analysis. This was particularly troublesome in the previous ITP-CE instrument sample
valve. These voids are typically found in areas not directly in the path of the main flow stream, such as around sharp corners or inside grooves. The most common location of void volume in capillary systems is at the mating point between the capillary and external fittings.

To help minimize void volume, a number of precautions can be taken. First, it is necessary to ensure a good square cut is achieved at each end of the capillary prior to mating with a fitting. This can easily be done by using one of the many commercially-available capillary cutting tools. Although capillary scorers are effective, a better cut can be achieved using one of the commercial tools. Although not as critical for this work, the integrity of the capillary ends becomes even more of an issue when using extremely narrow bore columns (e.g., <50 μm I.D.) A second precaution is to make sure that the internal diameter of the capillary matches the I.D. of the union to which it is to be joined. For example, one drawback of the previous injection valve was that the region for sample introduction was much larger than the I.D. of the ITP column. Thus, when the sample was introduced, it was not confined to a small space, but rather was diluted, and it was impossible to know whether or not the entire sample migrated onto the ITP column.

In an attempt to reduce the overall void volume and improve the previous injection method, an injection valve was designed with dimensions that minimized extra-column volume. The key to the design was matching the ITP capillary I.D. with the I.D. of any drill-through holes, ports, orifices, or mating points within the valve. In addition, the sample port I.D. was designed to be as small as possible to accommodate the O.D. (365 μm) of the ITP capillary while still allowing for large volume (10 μL) sample introduction. To meet this requirement, a tapered sample area was used with a large
opening at one end and a very small outlet at the point where the ITP capillary met the sample valve. The valve was comprised of three main components that came together to form the complete sample valve unit. This format was necessary to allow adequate access for high tolerance machining of critical angles and grooves. The previous sample valve was constructed using a single piece of plexiglas, which proved to be too difficult to modify due to inaccessibility for the machinist to perform precision work.

The schematics of the injection valve are given in Appendix A. Despite the considerable design work, both production cost and manufacturing physical limitations prevented the valve from being produced. However, this discussion and the valve schematics were included for future reference. The main manufacturing complication was that the minimum size for a drill bit for holes meeting the size requirement of a small capillary column is 0.008" (203 µm). In the design, the size of the required holes (178-203 µm) was smaller than the smallest drill bit. Other manufacturing techniques such as wire electrical discharge machining (EDM) are not suitable for most polymeric materials, as it would simply melt the polymer and not guarantee the correctly sized hole. Therefore, capillaries having an O.D. of greater than 178 µm must be used if the port in the sampling valve were to be made. Since this would require the use of larger columns, the injection valve design was abandoned and commercial alternatives were sought.

Although valve injection is common for ITP, the only vendor of commercial ITP instrumentation (J&M) does not sell sample valves separately. Also, the dimensions for the valve use 800 µm I.D. ITP capillaries, whereas 200 µm I.D. capillaries were used in the current instrument. Valve injection options in CE are not common and few attempts are noted in the literature, presumably due to the difficulties associated with fabricating a
sample loop which has both nanoliter volume capacity and a liquid path that is electrically isolated when voltage is applied. Although ITP sample volumes are much larger, the electrical isolation requirement limited the valve selection choices. Several valves are manufactured for use in capillary LC and supercritical fluid chromatography (SFC) applications. Valco Instruments (Houston, TX, USA) manufactures rotary valves commonly used in SFC that use sample rotors as small as 60 nL, but the rotor is contained in an all metal housing which is not compatible with CE due to electrical discharge and/or grounding problems.

Upchurch Scientific (Oak Harbon, WA, USA) issued a micro injection valve (Part number M435) which was marketed as being specifically designed for systems using all-capillary flow paths, although it had not yet been used for electoseparation applications. The valve used ceramic internal components and claimed to fully insulate the flow stream. The sample loop was comprised of a short section of fused silica capillary whose length and I.D. could be easily changed by the user. The advantage of an interchangeable, custom sample loop is that capillary column of any I.D. (and therefore any volume) could be installed. For ITP-CE, a 31.8 cm section of 200 μm I.D. capillary was installed to give a total sample volume of 10 μL.

Claims of full insulation were quickly discovered to be invalid. Using components from the previous ITP-CE instrument, the Upchurch valve was installed and tested using a solution of $3 \times 10^{-4}$ M angiotensin III (Aldrich, Milwaukee, WI, USA) as a test solute. Whereas the ITP migration time of angiotensin III was approximately 45 min in earlier runs, use of the Upchurch valve extended the run time to over 2 h despite identical electrolyte and run conditions (+20 kV, 10 mM triethylamine LE), 10 mM
acetic acid TE, 30 cm uncoated fused silica column, UV detection at 215 nm). The capillaries were held in place against the ceramic valve ports by a series of polyetheretherketone (PEEK) ferrules adjacent to the ceramic housing, and were tightened down by a stainless steel cover plate. The cause for the discharge was likely due to the cover plate and housing. It is probable that there was not adequate sealing between the capillaries and ceramic insert inside the valve, despite the fact that the valve was tightened as much as possible. Despite assurances from Upchurch that the valve would work for this application, the micro injection valve failed to adequately isolate the sample stream, and a new valve was sought.

Valco recently modified its Cheminert® series of polymeric valves and rotors such that the pathway was completely polymeric, including the housing and all fittings. A Valco Model C22Z six port valve was purchased which used any internal diameter PEEK tubing as the sample loop and whose advantages are similar to the sample loop characteristics of the Upchurch valve. A 8.8 cm x 380 μm I.D. section of PEEK tubing was installed and angiotensin III was again used to characterize the integrity of the sample valve. ITP was again performed in triplicate under similar conditions as described above, with migration times of approximately 45 min. Run-to-run variability was less than 1.5%. This valve was used for all subsequent comprehensive ITP-CE analyses.

II.3.4. Bifurcation and CE terminus tees

The previous instrument (Figure 1.7) used a large buffer reservoir block to house both the buffer reservoir and the bifurcation region. The area for bifurcation was a drilled
region in the center of BR2. Both the ITP and CE columns were held in place by septa supported by Delrin screw plugs secured into the side of the reservoir block. The 0.25 in I.D. Tygon tubing that connected the LE syringe on the syringe pump to the bifurcation block was much larger than was necessary to deliver such a small volume (4-10 µL). The short duration pulse of LE from the pump to the LE instigated flow throughout a much larger volume of electrolyte in the tubing leading to the bifurcation block, and precise control of a few µL delivered to the bifurcation point was questionable. One observation made during early trials with this setup was a lag effect of the counterflow on actual ITP column flow. After the syringe pump had delivered its preset LE volume, flow could still be seen emptying from the ITP column inlet. This residual flow may also have further contributed to the long ITP refocusing times mentioned earlier.

To minimize the total volume of the counterflow system, changes were made to both the bifurcation point and the infusion line. Only a small volume of buffer is required at the bifurcation point to maintain system integrity, and the reduction in total volume at the junction was made possible by the use of small quartz tees custom made by InnovaQuartz (Phoenix, AZ, USA). The dimensions of these fittings were such that the I.D.’s were only a few µm larger than the O.D. of the capillary columns to be used. Since the total volumes of the quartz unions were small, this helped minimize the flow lag discussed earlier. Schematics of the tees and unions are shown in Figures 11.2 and 11.3.

The change to the infusion line was simply a conversion from the 0.25 in I.D. Tygon tubing to 200 µm I.D. capillary column. The reduction in size of both the infusion line and the bifurcation region reduced the total volume from approximately 6.3 mL to an estimated 35 µL. Under these conditions, LE counterflow infusion resulted in almost
Figure II.2. Schematic of the bifurcation point four-arm tee.
Figure 11.3. Schematic of the CE column outlet three-arm tee.
negligible lag flow from the ITP capillary once the syringe pump was stopped. This greater control of the counterflow allowed for more precise evaluation of the effect of the counterflow on ITP-CE separations.

A similar change to the CE column outlet was made by installing a small 3-arm quartz tee (InnovaQuartz) which had one arm leading to an LE buffer vial and the other arm leading to a back flush valve used to flush the entire ITP-CE apparatus following an analysis. In the previous ITP-CE system, dialysis tubing was used with a series of O-rings to create a closed system to suppress hydrodynamic flow in the forward direction upon introduction of the counterflow LE. By using a shutoff valve at the flushing arm, and a buffer vial with a sealed lid on the LE buffer vial, a simple closed system was created which was much simpler to construct and maintain than the previous buffer reservoir block (BR3).

II.3.5. Single and dual power supplies

Another capability added to the ITP-CE system was a splitting voltage at the bifurcation point using a second computer-controlled power supply. A splitting voltage refers to a small \( \sim 2 \) kV bias placed near the union of the ITP and CE columns to help propel charged analytes into the CE column rather than along the outside since the ITP column had an I.D. much larger than the I.D. of the CE column. To add to the functionality of the splitting voltage, a high voltage relay was placed inline between the power supply and bifurcation point to allow for rapid switching on and off of the splitting voltage. The relay position was controlled by computer. The effects of this splitting voltage are discussed in Chapter III.4.7.
II.3.6. Multiple CE injection method: single vs. dual detectors

As was discussed briefly in Chapter I, comprehensive ITP-CE involves multiple, sequential CE injections of a preconcentrated ITP sample. The approach used to accomplish the injections had two forms, the first of which was based on a drop in the system current as analytes were transferred from the ITP column to the CE column; this approach was used by Chen et al. [5]. In this work, an approach whereby the voltage is left on was used to decrease the required analysis time and prevent the ITP zones from dispersing during the CE step. This approach necessitated the use of a second UV detector placed just prior to the bifurcation point to detect when the ITP zone had reached the inlet of the CE column. The CE injection method using this second detector is thoroughly described in Chapter III.3.7.

II.3.7. LabView programming

Control of the ITP-CE instrument, including both power supplies and the high voltage relay, was performed using LabView 5.0 (National Instruments, Austin, TX, USA) programming software. The software was also used to set and monitor the voltage and current on each power supply, rather than rely on front panels for their values. The schematics for the LabView virtual instrument are included in Appendix B.

II.3.8. Column technology

The evaluation of ITP and CE columns should be based on a number of criteria, including coating type, coating uniformity, surface characteristics including EOF and adsorption, and separation reproducibility. Improved preconcentration and separation in
ITP and CE occurred when the EOF was minimized. This can be accomplished by a number of methods, the most common of which is coating the capillary column to suppress active silanol groups on the inner surface of the capillary wall. In past work, coated columns (e.g., Ucon and polyvinylalcohol (PVA)) that were well rinsed after each run performed the most consistently. Other work using polymethylmethacrylate (PMMA) hollow fibers reported poor thermal conductivity (excessive Joule heating) and strong UV absorbance below 260 nm, with reduced column lifetimes in the range of 20 to 30 runs [6]. However, both PVA and Ucon-coated fused silica columns performed well (<3% RSD) after more than 50 runs. In addition, coated capillaries provided narrower ITP zones and better CE resolution than untreated capillaries.

II.4. INSTRUMENT PREPARATION AND SETUP

II.4.1. ITP and CE column dimensions

When determining the column lengths for either the ITP or CE columns, the desired electric field strength was determined, and then the overall column length for the system, which includes the section of capillary column going from the TE buffer vial to the sample valve, the length of the valve sampling loop, the length of the ITP column, the distance from the ITP column detection window to the bifurcation tee, the length of the CE column, the distance from the CE column detection window to the end of the CE column, and the distance from the CE terminus tee to the buffer vial which contains the ground electrode, were calculated.

For example, in order to have a value of 350 V cm\(^{-1}\) using an applied voltage of 20 kV, the overall length of the ITP-CE setup must be 57.1 cm. Since the TE capillary and sample loop are both 5 cm, and the distance from the CE detection window to the
grounded buffer vial is 7 cm, the overall length of the ITP and CE columns combined cannot exceed approximately 40 cm. Once this distance is known, a determination of appropriate column lengths is determined based on the complexity of the samples to be analyzed. If the sample is highly complex, more residence time in the ITP column may be needed to reach the steady state for the ITP step, and thus a longer ITP column may be required. In any case, when one column is lengthened, a compromise in the other is made. Unless the desired electric field value is changed, it is difficult to initially determine whether or not shortening the ITP or CE column is the appropriate step. In most cases, shortening the ITP column is the best choice. Since ITP in the ITP-CE method is performed at such high voltage, the samples tend to reach steady state quite rapidly, and having a longer CE column is more of an advantage for better CE separation.

Choosing the I.D. values of the ITP and CE columns was dependent on a few factors. One was that a small I.D. for the CE column was desired to obtain good separation efficiency, and to reduce Joule heating with its associated band dispersion that arises when a temperature gradient is present in the column. Joule heating can negatively affect a separation in a number of ways, including the occurrence of convection causing mixing of the zones, the creation of a spatial dependence on electrophoretic mobility, and sample degradation from the increase in temperature inside the column [7].

For ITP, larger diameter columns allow for a larger sample volume to be introduced. However, constraints are placed on the maximum I.D. and O.D. of each column in this system by the dimensions of the bifurcation tee (365 µm I.D.) In other ITP instruments, large bore columns (800 µm I.D.) are commonly used. However, systems which use large bore capillaries are not configured in a comprehensive
arrangement. The advantages of large I.D. columns for ITP are that they have a large sample capacity and are rapidly flushed between analyses since they require only a low back pressure to achieve a strong hydrodynamic flow. For capillary-scale separations, 320 µm I.D. columns are considered somewhat large; however, they performed effectively for this work. A drawback to larger I.D. columns is the effect of hydrodynamic flow during LE infusion. A strong flow occurred when such a large volume of buffer was mobilized, and more time was required for the ITP column to once again reach a minimized flow state. This may be due in part to the parabolic flow profile which can significantly disturb the ITP stack, which can be more adverse when using the larger bore (>320 µm I.D.) capillaries.

When looking at the minimum I.D. to use for either the ITP or CE steps, several considerations were addressed. First, the chosen I.D. for the ITP capillary had to be capable of handling the proposed sample size. Column overloading can lead to inadequate focusing of the ITP bands as well as bands which are extremely wide. This can increase the overall sample analysis time since multiple injections of only a single band must be made to prevent overloading of the CE column. Introducing a single, long sample zone onto the CE column before the counterflow is initiated will likely result in only one analyte being introduced onto the column. Or, if more than one analyte is injected for CE, the column length now available for CE separation is reduced. For CE, in addition to the advantages of low joule heating and higher separation efficiencies, the smaller I.D. (i.e. 50 µm) prevents hydrodynamic flow from seriously affecting the CE separation since the majority of the counterflow follows the path of least resistance, which in this case is the larger I.D. of the ITP column. A disadvantage of the smaller I.D.
is the difficulty in adequately rinsing the column due to a higher required back pressure. Also, a small I.D. ITP column may require a longer column in order to accommodate an increased sample volume. However, an advantage of the comprehensive ITP-CE arrangement is that sample size can be controlled by altering the CE injection timing, rather than having to continually change the I.D. of the CE capillary.

II.4.2. Evaluation of column coating

The integrity of a coated capillary column was evaluated using EOF as an indicator of coating efficiency and uniformity. For all of the work performed in these studies, capillary columns (50, 75, 150 or 200 µm I.D. X 365 µm O.D., Polymicro Technologies, Phoenix, AZ, USA) were used. Once the coated capillaries were cut to the appropriate length (~70 cm), dimethylsulfoxide (DMSO) was used as an electroosmotic flow (EOF) marker. As a general rule of thumb, an elution time of approximately 2 h was considered an indication of sufficient coating. In some isolated cases, the DMSO did not elute after 4 h. Replicate runs gave similar results. However, there was no significant separation improvement in ITP-CE between the two columns despite their different EOF values.

After determining the uniformity of the capillary coating procedure, the next step was choosing an appropriate electrolyte to act as the background CE buffer. However, it must be remembered that the buffer must likewise act as the LE in the ITP step, so the buffer must have a mobility that is higher than any of the analytes to be separated. Once an optimized separation is obtained, reproducibility is then evaluated to ensure the
stability of the column coating. In most cases with a well-coated capillary, retention time RSD values in the range of 2-5% were obtained.

II.4.3. Column installation

During installation of the capillary columns in the ITP-CE instrument, extreme care was necessary to not break the capillary detection windows. The windows were formed by placing the column at the desired position inside a tightly coiled wire, whereupon by application of a voltage, the wire heated and subsequently burned off the outer polyimide coating. This left a short, translucent detection window (~2 mm) suitable for on-column detection. Since the polyimide coating is used as a protection for the fused silica, removing it obviously increases the likelihood for scratches and breakage. Despite the inherent strength of fused silica, the heat from the removal process in the window burner weakens the capillary, making it more fragile. Extreme bending of the capillaries during installation caused them to break at the detection window, resulting in the need to prepare another column.

After forming the detection windows, the outside of the column, as well as any septa used in the system, were cleaned with water and methanol before installation. This removed any dirt and organic material which could contaminate the buffer solutions. Since both buffer vials in the ITP-CE instrument used septa to either seal the vial or hold the capillary and platinum electrode in place, columns could be blocked after passing them through the septum and into the vial. A short section of column was removed after insertion through a septum. This also allowed the user to verify whether or not the end of the column had a straight or jagged cut.
II.4.4. ITP-CE instrument preparation

Prior to initial use and following each analysis, the ITP-CE system was thoroughly flushed with LE to remove any residual sample or TE. This included rinsing all syringes, filters, tubing, buffer vials, and capillaries. Since cleaning and rinsing involve disassembling portions of the system, care was given to ensure that any air bubbles were flushed completely out of the system. Air bubbles cause an interruption of the current inside the capillaries, forcing an ITP-CE run to terminate. The most common places for bubbles to form were in the injection valve and in the capillaries leading to the buffer vials. Best results were found when the system was rinsed with LE for at least 10 min. Since the syringe pump was capable of holding two syringes, it was convenient to flush both the ITP and CE columns simultaneously using the flush lines connected to both tees. In addition, the sample loop could also be flushed by simply moving the valve to its “sample” position.

After flushing the system, the valve attached to the column leading to the CE terminus was closed and a syringe containing fresh LE was attached to the column leading to the bifurcation point and mounted in the syringe pump. After reconnecting the buffer vials, the system was allowed to re-equilibrate. The syringe pump was then turned on momentarily and any residual back pressure originating from the LE in the syringe was allowed to bleed from the system through the ITP column. This also ensured that there was no counterflow lag between when the syringe pump was turned on and when LE flow began.
II.4.5. ITP injection

After ensuring that both the sample and load positions of the sample valve were adequately flushed and that the system had come to a pressure equilibrium, the valve was turned to its load position. To ensure adequate mixing, the sample was sonicated for 2-3 min. The sample was then introduced using a 50 μL syringe. The sample was pushed into the valve until the sample loop (10 μL) was filled and extra solution was seen in the section of tubing leading to waste. Again, the key was to introduce the sample so that bubbles in the sample loop were avoided. Too rapid infusion lead to voids forming in the liquid stream. Also, there was no advantage to infusing rapidly since the sample was introduced into the system by moving the sample valve from its load to sample position. Once the valve was in the sample position, the power supply was turned on and the data acquisition system was started to begin the ITP-CE process.

II.5. INITIAL TESTING OF SYSTEM

II.5.1. Leak testing

Two types of leakage can occur with the ITP-CE system. The first is the loss of solution from openings in valves or other connection points. A difficulty in designing this system was that minimal contact with materials other than fused silica was desired. Thus, the use of the polymeric rotary valve and the quartz tees with minimal volume were used.

The second type of leakage, and the one which proved most difficult to overcome, was current leakage or electrical discharge. There are numerous points along the instrument where the electrical current can jump from the system to a nearby ground.
Both detector housings, the syringe pump, and the sampling valve all served as potential destination points for the current when arcing occurred. The most common cause of current leakage was when the syringe for the counterflow was not adequately isolated, even after being wrapped with Teflon tape. Arcing occurred between the tip of the syringe and the syringe pump housing. Audible arcing sounds could be heard when this occurred, as did rapid fluctuations in the system current as monitored by the power supply.

Another common source of current leakage was at the quartz tees. Careful attention had to be paid when constructing the bifurcation point and the CE terminus tee. Because there was a small gap (a few µm) between the inner wall of the quartz fittings and the outer wall of the fused silica capillaries, Superglue was able to penetrate between the two and resulted in both immobilization and sealing of the capillary in the fitting. However, at the tip of the quartz fittings was a short tapered region which allowed for simplified insertion of the capillaries. In this region, a small amount of glue would periodically accumulate which required a long time to fully cure. If the glue was not allowed to cure completely, the application of high voltage caused the electrolyte solution to work its way through the glue until eventually an arc was established between the end of the quartz fitting and a ground. Best results were obtained when the fitting and capillaries were joined and allowed to cure overnight. Minimum time for curing was at least 2 h, with improved results directly proportional to the amount of curing time allowed.
II.5.2. Testing of LabView program

The LabView module written specifically for the ITP-CE system was thoroughly tested to ensure that proper feedback was established between the power supplies, high voltage relay, and the computer. One common occurrence was a voltage spike in the data acquisition system electropherogram when the high voltage relay was switched to the "on" position. However, this did not appear to adversely affect the separation. An initial concern was that this spike would adversely affect the control circuit, but its effect was suppressed by the spike-limiting circuit (see Appendix C) inserted between the computer and power supply.

II.5.3. Initial testing of system with crystal violet dye

To initially test the ITP-CE system, crystal violet dye was used as a marker. Crystal violet has the advantage that it can be monitored visually on the system, giving an easy-to-follow indication of whether or not charged compounds follow the desired path in the system. Often, the crystal violet migrated towards the source of current discharge and identified the location of the problem.

In ITP, the time required for ITP focusing is directly related to the magnitude of the current in the system. Thus, ITP performed at high current reaches its steady state in less time. However, this must be carefully offset with the length of the ITP capillary, as the ITP bands may travel the length of the capillary before they have time to fully focus when high current is used. Reduction in EOF can help this process. In this system, the system current is limited by the Joule heating effect inside the capillaries as well as the ability of other external components such as the electrodes to handle high voltage/current.
Using this system, voltages of up to 25 kV were used. However, at that high voltage, the quartz tees would often arc. At 20 kV, the system showed good reliability.

II.5.4. ITP injection

In the previous comprehensive ITP-CE system, the ITP focusing voltage used was extremely small (1-2 kV). However, in the current instrument, much higher voltages were used without a problem, seemingly due to the improvements associated with the sample injection valve. However, some ITP injection problems occurred often enough to warrant their mention here.

After turning on the voltage, it was helpful to look at the current in the system. When the value of the current was too high relative to when the system was filled only with LE, a leak in the current due either to a broken ITP or CE column, or arcing from one of the tees to a nearby ground was indicated. When the current was lower than expected, it was likely that a bubble formed in the system, or that mistakenly the TE was used to initially fill the system rather than the LE. Also, hydrodynamic flow can occur in the forward direction anytime the system becomes open due to a leak either in one of the tees or in the connection between the syringe in the syringe pump and the capillary connecting it to the bifurcation tee.

II.5.5. Reproducibility

Numerous factors affected the run-to-run reproducibility of the ITP-CE system. When testing the same sample repetitively, rinsing of the system between adjacent runs was important to adequately evaluate reproducibility. A conscious effort was made to
remove factors which caused fluctuations in the system current, which ultimately led to a change in both the ITP focusing time and CE separation time. One of the causes of retention time change was the failure to remove all of the TE or sample from a previous run, which led to lower system current and longer total analysis times.

Another factor that can affect reproducibility was the use of the same buffer for an extended period of time. It was found that keeping the TEA and HAc buffers in the refrigerator when not in use resulted in the best run-to-run consistency. When the buffers were left out on the lab bench and used over an extended period of time, the need to degas the buffer multiple times led to a possible TEA loss due to its moderate volatility, as evidenced by its fish-like odor. ITP focusing times increased over the course of multiple days when using the same buffer for the entire run sequence. Similarly, the angiotensin samples were also kept in the refrigerator to prevent degradation. When the samples were left out at room temperature for an extended period of time, peak areas began to show slight declines.

11.5.6. Adsorption

Adsorption of analytes to the wall of fused silica capillaries, which can be either transient or irreversible adsorption, results in poor reproducibility for both ITP and CE, and indicates the need to install new columns. This was evident by the appearance of peak tailing which became more pronounced with each CE separation. Proteins and peptides are known for their adsorption due to multiple charged sites, and numerous applications of column technologies have focused on improving the CE of these classes of compounds [8,9]. For example, one method is to directly immobilize either cationic (polybrene [10], polyamine [11]) or neutral hydrophilic polymers (PVA [12], PEG [13]).
onto the capillary walls, thus masking silanol groups and making them unavailable for interaction with the proteins. In this work, PVA-coated capillaries showed minimal adsorption and improved reproducibility over bare fused silica.

II.5.7. Sample types

One drawback to the ITP-CE system is the difficulty of using non-pretreated samples (e.g., high salt content) having high conductivity. On-column ITP-CE is most suitable for low conductivity samples since high conductivity can degrade preconcentration by not allowing adequate time to reach the ITP steady state. In essence, high conductivity samples require much longer times to come to equilibrium due to the high concentrations of ionic species, whereas samples of low conductivity progress along the column slowly and reach an isotachophoretic steady state much more rapidly.

Due to the difficulties and complications arising from high conductivity samples, separations are possible but not always easily achievable. Comprehensive ITP-CE is particularly difficult with these samples because of the necessity to refocus the remaining ITP bands following a CE injection. High conductivity samples require more counterflow to be infused in order to compensate for the higher current, hence, faster progress along the column. This can adversely affect the refocusing dynamics, resulting in only partially focused bands reaching the bifurcation point. This obviously leads to a degradation in CE separation.

The application of comprehensive ITP-CE to analytes embedded in complex matrices complicates the focusing and separability due to the high concentrations of charged species such as salts and proteins. Typically, a pretreatment step such as solid
phase extraction (SPE) is required to desalt the sample and remove these substituents to make it more suitable for analysis. When attempting to analyze highly complex samples such as blood plasma, it is often necessary to filter the sample and remove the large number of proteins present. However, a sample concentrated by SPE can also have high conductivity, which leads to poor ITP focusing. Thus, ITP-CE may prove difficult for highly complex mixtures whose sample preparation has not been well characterized.

II.5.8. Limit of detection

Low concentration angiotensin standards were prepared in 10 mM TEA LE by diluting stock solutions kept in the refrigerator. These samples were then allowed to come to room temperature (23°C). Each standard was then injected and the signal-to-noise ratio measured. For angiotensins II and III, a detection limit of 3 nM was achieved. For angiotensins I, I(1-7) and IV, a detection limit of 5 nM was observed.

II.6. TROUBLESHOOTING

II.6.1. Decrease or loss of system current

After sample injection, a low current can indicate the presence of an air bubble forming somewhere in the system. Typically the source of the air bubble was at the injection valve where the ITP column meets the valve rotor. Although Valco advertises that the union between the valve and column has zero dead volume, bubbles still may occur. The only remedy is to flush the system with LE to remove the air bubble. Another source of bubble formation was generated from the buffer system or sample itself. If either the sample or electrolytes were not degassed properly, bubbles formed
either in the sample plug or elsewhere in the ITP-CE system once the voltage was applied. For the sample, since degassing is not an option for such a small volume, keeping the sample cold until just before injection proved adequate and reduced the occurrence of bubbles. Poorly prepared buffers can also be the cause. If the buffers were not fresh and degassed thoroughly, bubble formation quickly occurred. It was often necessary to prepare a buffer daily for best results. Another cause, although less frequent, was periodic clogging of the small diameter CE column. If the ITP-CE system was filled with electrolyte and allowed to sit overnight, evaporation of the electrolyte left deposits in the capillaries. Therefore, the system was flushed with purified water at the end of each day.

If a higher current than the initial value at the start of the run suddenly developed during an analysis, a break in the column or electrolyte leakage at either one of the unions or at the syringe pump was investigated. Simply wrapping the syringe barrel in a thicker layer of Teflon tape often solved the problem. If the source of electrical discharge is at one of the quartz tees, it was necessary to dismantle the entire apparatus and remount the capillaries.

II.6.2. Baseline noise

From time to time the ITP-CE system was subject to an increase in baseline noise at one or both UV detectors. Usually this was due to a current leak, with the detectors reacting to the influence of the nearby discharge. Another source of baseline drift or noise occurred when the photodiode near the bifurcation point was near a bifurcation tee which was not properly sealed. Since the photodiode can serve as a ground, small
coronas would form at the ends of the tee arms. When current leakage such as this occurred, it could be identified both visually with the naked eye and audibly.

II.6.3. No appearance of peaks

The most common cause of no peaks appearing in the CE electropherogram was that the timing delay for the CE injection was too short, thus no peaks were allowed to migrate onto the CE capillary and, instead, were pushed back into the ITP column. A simple increase in the delay time almost always remedied the situation.

Two other possible sources of no peaks were degradation of the sample and high EOF. If the sample integrity was verified using a separate CE instrument, it was likely that the ITP step did not adequately focus the analytes by either forming discrete zones or a single mixed zone. Poor ITP focusing was also a result of inadequate column coating, which resulted in high EOF in the column. Because such a short length of capillary is used for ITP (15 cm), high EOF caused the analytes to elute before ITP focusing could be accomplished.

II.6.4. Sensitivity and migration time variability using coated columns

Coated columns have an associated lifetime. The column lifetime is affected by pH, buffer compatibility, and analyte adsorption. As columns reached the end of this usable lifetime, their performance began to deteriorate and was usually accompanied by a loss in sensitivity and higher retention time RSD. This was caused by sample adsorption onto the walls of the column, which typically first appeared as tailing peaks in the CE separation. Poor rinsing between runs can partially account for this adsorption. Usually
if the column was rinsed well with LE after each run, the coated capillary lifetime was extended.

At the end of each day, the entire apparatus was rinsed with purified water to flush any remaining electrolyte solutions and sample out of the columns. Leaving the solutions overnight resulted in salt formation after the evaporation of water. They also had the potential for degrading the coating on the inside of the capillary. A 30 min rinsing gave reproducible results the following day. Finally, the use of old buffers (>2 days old), or an old sample, periodically resulted in both sensitivity decrease and migration time variability. It was best to keep the stock electrolyte solution in a refrigerator and only remove a small volume for use during the day. Repeated degassing was necessary, depending on the performance of the instrument and the length of time the buffer had been left out at room temperature.
II.7. REFERENCES


III.1. INTRODUCTION

As discussed in Chapter I, the coupling of isotachophoresis (ITP) with capillary zone electrophoresis (CE) in an online, comprehensive arrangement is a promising technique for combined trace enrichment and analysis of samples requiring low limits of detection (LOD) [1]. The ability to introduce large sample volumes in the µL range, compared with nL-size injections in conventional CE, along with the 10- to 1000-fold preconcentration factor of ITP [2] is attractive for the analysis of low concentration analytes in complex matrices.

The advent of lab-on-a-chip and other small volume manipulation technologies has brought detection of trace biological compounds to the forefront of contemporary separation science challenges [2]. Detection of low concentration analytes remains a difficult task due to the small volume (pL) of samples. Detectors with higher sensitivity such as laser induced fluorescence (LIF) [3] are often employed to compensate for this concentration-dependent limitation. Although typical sample volumes are considerably larger in CE than for lab-on-a-chip, CE also suffers from concentration sensitivity limitations [4]. A number of efforts have been made to increase CE sensitivity, many of which have centered around improving the detection scheme and were discussed in Chapter I.2 [3,5-9]. Despite impressive advances in these new detection capabilities,
alternatives to single-pass UV/Vis detection other than the use of a Z-cell have not gained widespread commercial interest.

UV/Vis detection continues to be the most common type of detector used for CE due to its ease of use, robustness, and relatively low cost. Thus, attempts to enhance CE sensitivity while preserving the use of the UV/Vis detector are likely to find the most rapid acceptance. To increase CE sensitivity with a conventional UV/Vis detector, a sample preconcentration technique can be employed. As was discussed in Chapter I.2.3, a number of methods have been used as sample preparation techniques for CE, including liquid chromatography [10,11], field amplification [12,13], isoelectric focusing (IEF) [14], membrane preconcentration [15,16] and ITP [17].

ITP is one of the most promising sample concentration techniques for CE since the CE background buffer also serves as the leading electrolyte (LE) in the ITP separation. Various configurations can be used for the combination of ITP with CE (see Chapter I.3), including transient ITP-CE [18,19], directly coupled columns having different diameters [20,21], and columns combined via a t-junction [22].

Recently, comprehensive ITP-CE using a configuration with the CE column directly inserted inside the ITP column was introduced [17]. In this method, multiple, sequential injections of an ITP stack can be made for subsequent CE separation. Using this configuration, it was assumed that all of the sample (~10 µL) eventually was subjected to CE separation, with the results of these multiple injections being summed to give a single electropherogram with reduced single-to-noise. However, drawbacks of the system were that the timing for ITP injection was determined using a current-monitoring method developed by Reinhoud et al. [23,24], which can be difficult to standardize from
one run to another due to variance in the overall conductivity and buffer conditions. In addition, it is also possible that analyte was lost along the outside of the CE capillary with no method of determining whether loss actually occurred.

In this chapter, a modified comprehensive ITP-CE method is introduced whereby a second UV/Vis detector is placed just prior to the bifurcation point to improve injection timing and to simplify the application of the technique without having to rely on the measurement of the system current. Valve injection using a polymer rotary valve was also used to increase ITP-CE reproducibility compared with the previous instrument. Analyte loss in the modified ITP-CE system was also evaluated.

III.2. EXPERIMENTAL

III.2.1. Materials and chemicals

Angiotensins were purchased from Aldrich (Deerfield, IL, USA). Reagent grade triethylamine was obtained from Fisher Scientific (Fair Lawn, NJ, USA) and glacial acetic acid was purchased from EM Science (Gibbstown, NJ, USA). Cyanoacrylate ester (Superglue) for use in constructing the ITP-CE column interface was obtained from Loctite Corporation (Rocky Hill, CT, USA). Deionized water for buffer solutions and rinsing of the ITP-CE apparatus was from a Milli-Q water purification system (Millipore, Waterford, MA, USA). Small bore (365 µm I.D.) quartz junctions (see Figures II.2 and II.3) for the bifurcation point and CE column terminus were custom made by Innova Quartz (Phoenix, AZ, USA) and were designed to minimize the buffer volume inside the bifurcation tee, leading to better volumetric control of the counterflow buffer. The columns were held in place in the tee using superglue, which proved to be an
effective means of ensuring that the tees were sealed and reusable. As capillaries are prone to break, simple submersion in an acetone bath frees the capillary, allowing the tee to be reused. Untreated fused silica capillary columns (250 µm I.D. x 365 µm O.D., and 50 µm I.D. x 190 µm O.D. for ITP and CE, respectively) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Syringes for sample injection (50 µL, glass) and LE infusion (1 mL, plastic) were from Hamilton (Reno, NV, USA).

III.3. INSTRUMENTATION AND METHODS

III.3.1. Preparation of coated capillaries

Both ITP and CE capillaries (200 µm I.D. and 50 µm I.D., respectively) were coated with polyvinyl alcohol (PVA) prior to use to suppress electroosmotic flow. The procedure is similar to that used by Clarke et al. [25]. Briefly, a solution of 6% PVA in water was prepared and thoroughly degassed using both sonication and vacuum. Then 2.5 m of fused silica capillary was attached to a small pressure vessel which contained a vial of the 6% PVA solution. The PVA solution then filled the capillary and flowed continuously through the column for 1.75 h at 100 psi. The column was then emptied following removal of the front of the column from the PVA solution vial by applying 30 psi of nitrogen gas for 1 h. Finally, the column was thoroughly dried using nitrogen gas at 20 psi for 1.25 h. The column was then placed in a GC oven (HP 5890, Hewlett-Packard, Palo Alto, CA, USA) and subjected to a temperature program of 40°C to 145°C at 5°C min⁻¹, followed by a hold at 145°C for 5 hours. The entire process was then repeated again to ensure proper coating, only this time the capillary was reversed and filled from the opposite end.
III.3.2. High voltage power supplies and computer control

The two power supplies used for this work consisted of a 60 kV/500 μA max SL60PN30 from Spellman (Hauppauge, NY, USA) and a 30 kV/300 μA max CZE1000R (Spellman), both used in the positive mode. A 12-volt high voltage relay with two outlets was obtained from Kilovac (Santa Barbara, CA, USA). Each power supply was controlled via computer using an in-house LabView v. 5.1 (National Instruments, Austin, TX, USA) programming module. Two National Instruments data acquisition cards (models Lab-PC-1200/AI and PC-AO-2DC) were used having analog input and output capability for both setting of the output voltage/current and feedback monitoring of the actual voltage/current present in the system. The high voltage relay status was also controlled by the same LabView programming module. Remote control of the programmable PHD 2000 syringe pump (Harvard Apparatus, Holliston, MA, USA) for infusion of the LE counterflow was performed using Symphony version 1.1 software (Harvard Apparatus). Voltage isolation boards and analog-to-digital control boards were constructed in-house.

III.3.3. ITP injection

A model C22 Cheminert two-position six port valve (Valco Instruments, Houston, TX, USA) was fitted with a 10 μL sample loop constructed of polyetheretherketone (PEEK) tubing (Valco) and used for ITP sample introduction. The inlet buffer vial was brought inline using a short length of fused silica tubing (5 cm x 250 μm I.D.) connected to a small 2 mL screw cap vial fitted with an open top screw cap and a Thermolite septum.
The screw cap was left loose to prevent hydrodynamic flow from being introduced to the system due to pressure build-up when the vial is tightly closed. The sample valve was placed on an elevated Plexiglas block so that the column sat parallel to both the ITP and CE columns in order to prevent hydrodynamic flow in either direction due to siphoning.

To further prevent hydrodynamic flow in the forward direction, the terminal end of the CE column was placed in a closed buffer system using a quartz tee (InnovaQuartz) whose three arms were attached to (1) the CE column, (2) a small length of capillary (8 cm x 200 µm I.D., 365 µm O.D.) inserted into another 2 mL screw cap vial and septum which served as the buffer vial source for ground, and (3) an additional length of capillary (30 cm x 200 µm I.D., 365 µm O.D.) fitted with a polymeric stopcock valve (Upchurch Scientific, Murieta, CA, USA) used to flush the entire system with LE following an ITP-CE run. After passing the capillary for the buffer vial through the septum, the screw cap was tightened onto the filled buffer vial and the system was allowed to come to pressure equilibrium prior to sample introduction. This was evident by the absence of buffer flowing at the front-end buffer vial. Thus, a closed system was formed which prevented hydrodynamic flow in the forward direction. A schematic of the ITP-CE system is shown in Figure II.1.

III.3.4. Detection and data acquisition

For the ITP column, a FOCUS scanning/triple-wavelength UV absorbance detector with a fiber optic assembly was purchased from Thermo Quest (San Jose, CA, USA) for on-column detection. Photodiodes with extended cable lengths suitable for the
fiber optic assembly were purchased from Phoenix Scientific (Santa Fe, NM, USA). Small UV-transparent windows were formed by removing a small portion of the polyimide coating using a frit burner from Innovatech (Stevenage, Hertfordshire, United Kingdom). Data were processed using PC1000 version 3.0.1 detector control and data acquisition software (ThermoQuest) on a Pentium-class computer with an OS/2 version 3 operating system (International Business Machines, Armonk, New York, USA). For the CE capillary, a UV3000 UV/Vis detector (ThermoQuest) was used, with detector control and data acquisition performed using ChromQuest version 2.54 (ThermoQuest) running on a Pentium-class computer running the Windows 2000 operating system (Microsoft, Redmond, WA, USA). Both detectors were set to 215 nm for detection of angiotensins.

### III.3.5. ITP-CE procedure

Prior to ITP injection using the rotary injection valve, the entire system (including the buffer vials) was flushed from the terminal end (CE terminus) with freshly degassed LE to remove any residual sample or terminating electrolyte (TE) remaining from previous runs. Buffers were thoroughly degassed using both house vacuum (250 torr) and sonication. After filling the LE and TE buffer vials, the system was closed and allowed to pressure equilibrate. The opposite end of a short 5 cm section of capillary tubing (200 µm I.D., 365 µm O.D.) placed in the “mobile phase in” port of the sample injection valve was inserted into the TE buffer vial, making sure not to close the cap snug which would introduce hydrodynamic flow. The sample valve was then placed in its “load” position and the 10 µL injection valve sample loop was filled using a 50 µL glass-barreled, stainless steel blunt-tipped syringe (Hamilton). After switching the valve to
“sample”, the voltage was turned on and the ITP-CE procedure as described in the above section was initiated.

III.3.6. Determination of when sample reaches the bifurcation point

It is a commonly accepted method in ITP-CE to use the current as a monitor for the migration of analytes in the ITP or CE column [2,3]. In many coupled ITP-CE studies, a drop in current is used to indicate when analytes have passed from the ITP column into the CE column. However, using this approach it can be difficult to develop a standardized method for multiple sample types, since the current in a particular analysis system may vary depending on the type and concentration of the buffer and amount of analyte present in the system. Method development often requires performing “sacrificial” runs to determine the expected current values. Also, in other ITP-CE instruments, the current is measured across only the ITP capillary, after which the ground is switched from the ITP terminal side to the CE terminus. However, unlike the comprehensive ITP-CE instrument used for this study, these other systems are not used in an “always-on” mode where voltage is applied to both capillaries continuously, but rather make use of voltage switching routines. In the present comprehensive ITP-CE arrangement, the voltage was applied at the front end of the instrument and a voltage switching method was not employed. Rather, the voltage was maintained throughout the analysis and the system current was used to simply monitor the integrity of the system and confirm the presence of an electrical circuit.

Instead of using voltage switching and current monitoring as in other ITP-CE instruments, a more universal approach was used whereby a second UV/Vis detector was
placed just prior to the bifurcation point. In this system, the head of the CE column was inserted to a position immediately next to the ITP column detection window. After analytes appeared as ITP zones on the data acquisition system for the ITP detector, migration onto the CE column began, and optimization of the counterflow was performed so as to inject sufficient analyte onto the CE column without overloading.

III.3.7. CE injection method

In an earlier prototype of this instrument [17], a single power supply was used with two external high voltage relays to send voltage independently to either the ITP or CE column. A voltage switching technique was used after the initial ITP step whereby the voltage was turned off at the ITP column and the ground was switched from the bifurcation point to the CE column terminus. When the voltage was again applied at the head of the ITP column, CE injection would occur since the circuit extended from the ITP column through the CE column. Once CE injection was completed, the voltage was turned off at the ITP column and the counterflow was infused. At that point, a relay was actuated so that voltage was only applied across the CE column, leaving the ITP column, with the remainder of the analytes inside, electrically neutral. The absence of the voltage thus allowed the previously focused ITP bands to diffuse, necessitating additional refocusing ITP steps (~45 min each) after each CE injection. This was the primary source of the long run times in previous studies, especially when multiple CE injections were necessary.

The current instrument configuration uses either single or dual power supplies in an “always on” mode which removes the necessity for voltage switching between the ITP
and CE columns, and thereby effectively removes the need for numerous lengthy ITP refocusing steps. The key advantage to performing comprehensive ITP-CE in this mode is the significant reduction in sample analysis time. This is particularly evident when the analysis requires multiple CE injections in order to analyze a sample having several sufficiently-concentrated zones.

In the “always on” mode of operation, the voltage is applied at the TE buffer vial located prior to the injection valve. The ground is placed at the end of the CE column, with the bifurcation point electrically floating. CE injection occurs by allowing the ITP sample to migrate past the front of the CE column. A portion of the sample enters the CE column, and the LE counterflow is infused. However, since the voltage is still being applied to the entire system, only a minimal disturbance of the ITP bands occurs due to the self-sharpening mechanism of ITP which remains in effect. Because this zone sharpening continues, only a small volume of LE needs to be infused, and CE injection can then re-occur in only a fraction of the time required compared to the previous comprehensive ITP-CE system.

A key to the comprehensive ITP-CE injection technique is allowing sufficient time for the ITP zones to migrate onto the CE column. If the counterflow is infused too early, little or no analyte is transferred from the ITP column to the CE column. Enough sample must be transferred to allow for detection. If the amount transferred is too little, the resulting CE peaks go undetected since their concentrations are likely below the LOD of the detector.
III.3.8. Use of counter flow for multiple injections

When a counterflow is used in this comprehensive arrangement, great care must be taken to ensure the absence of air bubbles in the transfer line or formation of gas bubbles during infusion. It was found that infusion at too high a rate (>100 µL min⁻¹) caused random loss of current in the system. However, when the infusion rate was lowered to 60 µL min⁻¹ or less, no loss in current was observed. To monitor the effect of the counterflow infusion on the system integrity, the system current can be checked. During the transfer of analyte from the ITP column to the CE column, the system current drops slightly. However, during LE infusion, the remaining ITP zones are pushed back into the ITP column and the additional LE restores the system current to near its original value.

Ideally, injection could be controlled using a microprocessor-based timing circuit. However, this requires programming integration of the software of both detectors with the ITP-CE control software, which was beyond the scope of this study. As used, the ITP-CE system produced ITP separation time RSD values from 2-5%. More precise control of the counterflow infusion timing would likely decrease the system variability and show improved control of the amount introduced onto the CE column. However, under manual timing control with an experienced user, reproducible separations were possible and CE column overloading was not observed.

The key to successful CE injection in the comprehensive ITP-CE configuration is the timing and duration of the LE counterflow. The syringe pump was started after a short delay (usually 5-15 s) following the indication via the UV/Vis detector near the
bifurcation point that analytes were present. This allowed a portion of the ITP bands to migrate onto the CE column. The delay was timed manually using a stopwatch, which may explain the variation in retention times (RSD 2-5%) between injections.

III.4. RESULTS AND DISCUSSION

III.4.1. Overview of the comprehensive ITP-CE methodology

The ability of ITP to both concentrate desired analytes and purify complex samples is well known [26] and has been used to for a wide variety of separations including cations/anions, proteins, peptides and amino acids [27]. Assuming each analyte is of sufficient concentration, the sample constituents stack in discreet bands and are detected using either UV or electrical conductivity. However, if analyte concentrations are not sufficient to form discreet zones, single mixed zones result. A description of what constitutes sufficient and insufficient zones was discussed in Chapter 1.2.3.3 [17].

The analysis of individual components contained in these mixed zones has proven difficult using ITP alone since the zones have only a single detector response (e.g., a single peak) in both UV and conductivity detectors, despite the fact that the zones contain multiple analytes. Because of this limitation in ITP, CE was coupled to ITP to provide an additional separation step which allowed for the detection of analytes in mixed zones [17]. In an effort to reduce the analysis time for multi-injection comprehensive ITP-CE, a revised methodology was used together with the redesigned comprehensive ITP-CE instrument described in Chapter II.

The representation in Figure III.1 illustrates the modified comprehensive ITP-CE method used for this study which differs from that used with a previous instrument.
Like the earlier method, the sample was preconcentrated under ITP conditions between the LE and TE. However, instead of using a voltage switching technique to move between the ITP, CE injection, and CE steps, the voltage was applied across the both columns during the entire comprehensive ITP-CE process (see Figure III.1). A key difference was the addition of the first UV detector positioned just in front of the bifurcation point, which indicated when the ITP-focused bands arrived. Once the ITP sample appeared, a short injection time delay was allowed during which the ITP stack continued to migrate past the front of the CE column, and a portion of the ITP stack was injected onto the CE capillary. Once the delay time finished, the remainder of the analytes which were not transferred into the CE column were pushed back into the ITP capillary using the LE counterflow from a syringe pump. Since the voltage was continually applied throughout this process, the portion of the sample that was pushed back into the ITP capillary continued to stay focused and returned to its ITP steady state after recovering from the slight disruption due to the parabolic flow profile of the LE counterflow.

Simultaneously while CE separation was occurring on the portion of the sample that was previously injected onto the CE column, the ITP sample (now with less total analyte than the previous ITP preconcentration step) began to once again migrate along the ITP capillary towards the front of the CE capillary in preparation for another CE injection. By the time the ITP zones reached the CE column, the previous CE separation had been completed. This process of multiple-injection ITP-CE was repeated until the entire sample had been analyzed. The end of an analysis was indicated when no analyte peaks appeared in the CE electropherogram following a CE injection.
Figure III.1. Modified comprehensive ITP-CE method. No voltage switching was used (compare the positions of the applied voltage with Figure I.6) and voltage was applied across the entire system during each step of the comprehensive ITP-CE process.

LE = leading electrolyte, TE = terminating electrolyte.
III.4.2. Counterflow volume and flow rate effect on peak shape

Since the comprehensive ITP-CE method calls for the voltage always being applied without any voltage switching, considerable attention must be given to the conditions under which the counterflow of LE is infused into the system. Variables such as infusion rate ($\mu$L min$^{-1}$) and infusion volume ($\mu$L) must be optimized, especially when looking to reduce the overall analysis time. Their effects on the analysis time, front edge ITP zone shape and CE peak shape were evident.

The effects of the rate and volume of LE infused were readily seen by observing the resulting ITP zones as they returned to the first UV/Vis detector located just before the bifurcation point following LE infusion. Due to the fact that the infusing LE is a hydrodynamic flow from the syringe pump whose flow profile is parabolic in nature, this inevitably causes some disruption in the flat leading edge of the ITP stack as it is pushed back away from the bifurcation point. Adequate time must be allowed for the ITP zones to return to their steady state with their associated sharp zone boundaries. Increasing the total infusion volume pushes the ITP stack farther back towards the injection valve, thereby allowing more re-equilibration time as the zones must travel a farther distance to reach the bifurcation point. However, this comes at the expense of overall analysis time. A better method is to find a compromise between infusion volume and infusion rate. One way to minimize the dispersion caused by the flow is to shorten the LE delivery time by increasing the infusion rate. It was found that with the current instrument configuration, a 4 $\mu$L volume provided the optimum conditions for rapid re-equilibration and short analysis times. When 4 $\mu$L of LE was infused, a much improved leading edge shape was
observed by increasing the rate from 10 µL min\(^{-1}\) to 60 µL min\(^{-1}\). Figure III.2 illustrates the effect of the LE infusion rate on the ITP leading edge.

Additionally, slower infusion rates had an adverse affect on the CE separation peak shape, causing some peak tailing. This was likely due to the prolonged increase in fluid pressure at the bifurcation point which caused additional diffusion of the peaks. However, the CE separation did not seem to be affected when the infusion rate was increased to 60 µL min\(^{-1}\). Infusion rates as high as 150 µL min\(^{-1}\) were also explored, but rates above 60 µL min\(^{-1}\) caused the current to drop to zero in each instance. Although the TE was thoroughly degassed before each run, the loss of current was presumably from the creation of bubbles in the system due to the rapid flow disruption from the buffer infusion.

III.4.3. Single mixed zones of analytes having insufficient concentrations

Samples having trace components of analytical interest pose the most difficult challenges in separation science due to the presence of background or matrix interferences present in most complex mixtures. ITP is especially promising in handling these types of samples since it can effectively remove interfering compounds as well as concentrate them to levels above the detector LOD. However, in many cases these samples do not contain sufficient analyte concentrations to form discreet flat-top zones consistent with sufficient concentration ITP zones. Rather, multiple trace analytes converge to form a single mixed zone, the boundaries of which are defined by the trailing edge of the LE and the leading edge of the TE. The analysis of these mixed zones likewise poses a challenge to traditional detectors (i.e. UV/Vis, conductivity) since they
Figure III.2. Effect of counterflow infusion rate (4 µL volume) on ITP leading zone shape.
appear as a single peak on the electropherogram. Therefore, the use of a separation step such as CE in combination with ITP greatly increases the amount of sample information that can be obtained.

To illustrate the usefulness of comprehensive ITP-CE, a mixture of four angiotensins at concentrations below which sufficient ITP zones form in the system (~1 x 10⁻⁸ M) was analyzed. The resulting electropherograms are shown in Figure III.3. For single mixed ITP zones, only 1-3 CE injections were usually required in order to subject the entire contents of the mixed zone to CE analysis. In the electropherograms, all four angiotensins are present, since a single mixed zone was sampled each time. Completion of an ITP-CE run is indicated by the absence of analyte peaks at the CE detector following a CE injection. During the analysis of single mixed zones, CE peak heights are similar from injection to injection until the final CE injection is made, which shows peak heights considerably lower than previous injections (see Figure III.3).

The advantages of summing multiple ITP-CE electropherograms from the same sample for increased sensitivity have been discussed previously [17]. Figure III.4 shows the summed electropherogram for the sample whose individual electropherograms are shown in Figure III.3, with a detection limit of approximately 2 nM (S/N = 3).

III.4.4. ITP zones of analytes having sufficient concentrations

When a sufficient quantity of analyte is present in the sample to form the recognizable flat-top ITP zones, great care must be given to the CE injection so as to not overload the CE column. By carefully controlling the timing of the LE infusion, only a portion of the front ITP zones are introduced into the CE column during injection. In
Figure III.3. Comprehensive ITP-CE of a mixture of insufficient concentration angiotensins forming a single mixed ITP zone. Conditions: 20 cm x 200 µm I.D. (365 µm O.D.) PVA-coated ITP column (15 cm effective separation length) coupled with a 20 cm x 50 µm I.D. (187 µm O.D.) PVA-coated CE column (15 cm effective separation length), 10 µL injection volume, approximately 1 x 10⁻⁸ M angiotensins, +20 kV for initial ITP concentration, reduced to +8 kV for subsequent ITP and CE steps. Peak identifications: (1) angiotensin III, (2) angiotensin I, (3) angiotensin IV, (4) angiotensin II.
many cases, if the entire zone or more than one zone were allowed to be injected, column overloading and deterioration of the CE separation would occur. A typical ITP-CE run with a sufficient amount of sample typically results in the first injection consisting of only the first analyte with the highest electrophoretic mobility. Subsequent injections then contain either just the single zone or portions of two to three zones, depending on the relative concentration of each zone. In Figure III.5, the analysis of a sample consisting of four angiotensins at sufficient concentrations is shown. In the first injection, only angiotensin III was transferred onto the CE column. The next injection shows that the injected amount consisted mainly of the remaining angiotensin III along with a small amount of angiotensin I. Later injections show a similar trend of containing either a single or just a few analytes. Using comprehensive ITP-CE, samples containing multiple analytes can be analyzed in a piecewise manner, thereby reducing the possibility of interferences. The individual electropherograms can then be summed to give a complete analysis (Figure III.6).

III.4.5. Reduction in analysis time

A key characteristic of ITP is its zone self-sharpening property as it moves towards the steady state. Once discreet analyte zones are formed and stacked next to each other, a different electric field strength is present in each zone. The zone containing the highest mobility analyte experiences the lowest electric field, and similarly the zone with the lower mobility has the highest electric field strength in order to maintain the same velocity as the rest of the bands. This is understood by looking at equation I.2.
Figure III.4. Summed comprehensive ITP-CE electropherogram for the sample containing components of insufficient concentration shown in Figure III.3.
Figure III.5. Multi-injection comprehensive ITP-CE of a sample containing components of sufficient concentration. $1 \times 10^{-5}$ M angiotensins. Conditions and elution order same as in Figure III.3 except +9 kV used for CE separation.
Should a portion of a zone migrate into its neighboring zone, it experiences either a higher or lower electric field strength, thereby increasing or decreasing its velocity. As a result, it then returns to its original zone. This is particularly useful when the ITP system is disturbed such as by a counterflow, as it allows for rapid re-equilibration back to steady state.

The comprehensive ITP-CE system with dual UV detectors and no voltage switching has a number of advantages over the previous system. First, the need for full re-focusing of the ITP bands following counterflow infusion is eliminated. In the previous instrument, the voltage was turned off at the ITP column and was switched to the CE column, thereby removing any current through the ITP column. This additional time in an indeterminate state for the ITP capillary resulted in diffusion and mixing of the ITP bands, compounding the diffusion effects from the parabolic LE counterflow infusion flow profile. The previous comprehensive ITP-CE study [17] required 40-60 min of ITP re-equilibration time between subsequent CE injections. However, using the current setup, re-equilibration times were reduced to 4-7 minutes depending on the LE counterflow infusion volume.

In the current comprehensive ITP-CE configuration, the application of a continuous voltage to the entire system accomplishes an important time-saving advantage; that is, CE separation and ITP refocusing are allowed to occur simultaneously. The remaining ITP bands are allowed to re-focus and migrate towards the CE capillary while returning to the ITP steady state. The earlier system only allowed ITP refocusing to take place after the CE separation had finished. Also, the removal of the high voltage relay switching steps simplifies the required instrumentation.
Figure III.6. Summed comprehensive ITP-CE electropherogram of the sample containing components of sufficient concentration shown in Figure III.5. Conditions same as in Figure III.5.
An effective method for decreasing the total analysis time while maintaining acceptable CE resolution was accomplished by changing the value of the applied voltage following the initial ITP step. The length of time required for ITP to reach its steady-state condition is inversely proportional to the value of the applied current [26]. Therefore, to decrease the overall analysis time, higher voltage was applied (20 kV) during the initial ITP step, and was reduced to 10 kV immediately following the first CE injection. The application of an initial high voltage for the first ITP step substantially decreased the total analysis time. Whereas in the earlier system the ITP focusing time was usually 15 times longer than the CE analysis time, it was now reduced to a factor of 8-10. Even higher voltages (>20 kV) for the first step were tried with the system, but arcing and excessive joule heating within the capillaries compromised the performance.

Another option that was explored for decreasing the analysis time was performing rapid CE injections by turning off or reducing the continuous LE counterflow. In this method, the counterflow was initially set to a low value (~0.5 µL min⁻¹) during the ITP step, and was rapidly increased following CE injection to move the remaining ITP zones back into the ITP column. However, it was observed that the continuous infusion of LE caused a significant increase in the analysis time required for re-equilibration of the ITP bands due to ITP zone diffusion and disruption from the parabolic flow profile in the ITP capillary.

III.4.6. Evaluation of analyte loss at the bifurcation point

Considerable attention was given to whether or not analyte loss occurred at the bifurcation point. During instrument setup, great care was given to ensure that the
bifurcation tee and the syringe used for LE infusion at the syringe pump were electrically
"floating" and not the source of a secondary ground. If it were so, the analytes traveling
in the ITP column would have two circuits from which to choose once they reached the
bifurcation point. The plastic syringe and tip were wrapped in Teflon tape to further
isolate the syringe from grounding at the syringe pump. Analyte loss was evaluated by
comparing the integrated areas of a single ITP stack and the summation of multiple
injections of the same sample in the comprehensive ITP-CE mode. Comparisons showed
that sample loss was minimal, ranging from 1-4 (±2)% . This was expected since the
front end of the CE column was placed a considerable distance (3-4 cm) into the ITP
column until it reached a point just prior to the detection window. In order for sample to
be lost from the system, the ITP stack would have to travel a considerable distance before
LE infusion took place. Since the syringe pump was turned on only a few seconds after
the sample appeared at the first UV/Vis detector, loss was anticipated to be minimal. The
sample loss observed could be due to sources such as the presence of residual ground at
the bifurcation line leading to the syringe pump or the sample not being pushed far
enough back away from the CE column during the LE infusion process.

III.4.7. Splitting voltage

The effect of a splitting voltage applied at the bifurcation point on sample transfer
to the CE column and on CE separation peak shape was investigated. In systems which
utilize a ground switching routine, the possibility exists for residual ground effects
causing a portion of the analytes to travel towards the residual ground rather than into the
CE column. To prevent this, a splitting voltage can be applied at the location of the
previous ground to ensure that the analytes travel along their intended route. However, no increase in CE sample transfer amount was observed with splitting voltages between 0.5-2 kV compared with CE injections with no applied splitting voltage.

III.5. CONCLUSIONS

Comprehensive ITP-CE was used to analyze both insufficient and sufficient quantities of angiotensins present in standard samples. The original sample introduced by ITP was subjected to analysis in the second CE dimension without significant sample loss. Multiple injections of the ITP band(s) were accomplished using a periodic counterflow, with only minor effects on the CE peak shape. By carefully controlling the injection time, CE column overloading was not observed. Large volume (10 µL) injections were used with reproducibilities between 2-5% RSD.
REFERENCES


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CHAPTER IV

ISOTACHOPHORESIS AND COMPREHENSIVE ISOTACHOPHORESIS-CAPILLARY ELECTROPHORESIS COUPLED TO TIME-OF-FLIGHT MASS SPECTROMETRY

IV.I. INTRODUCTION

Capillary electrophoresis (CE) has shown wide applicability to separations demanding high resolution while requiring only very small sample volumes [1,2]. Electroseparation methods have been especially useful for proteomics [3] and the human genome project [4], and are the methods of choice for lab-on-a-chip systems [5,6]. However, an often-cited drawback of CE, as discussed in Chapter I, is its concentration limits of detection (LOD) due to the short path length associated with small internal diameter (I.D.) capillaries (50-100 µm) and the low sample capacities (10-100 nL) of these capillaries [7-9]. This LOD limitation is especially apparent when conventional detectors such as ultraviolet/visible absorption (UV/Vis) or electrical conductivity are used for trace analysis. In many biological specimens, analytes are often present at concentrations below the detector LOD, necessitating a method in which analytes are preconcentrated before final high resolution separation.

In Chapter III, the online combination of capillary isotachophoresis (ITP) preconcentration with CE separation in a comprehensive arrangement was discussed [10,11]. Although ITP can serve as a stand-alone separation technique, in the ITP-CE configuration, it serves both as a sample preconcentration step and as a CE injection method.
An important concept in ITP and ITP-CE of trace analytes is that of "sufficient" or "insufficient" concentration zones (See Chapter I.3). These terms refer to the amount of each analyte present in the injected sample and, relative to the LE concentration and I.D. of the capillary, whether or not this amount is adequate to satisfy the equation [12],

\[ c_i^1 V_i^1 \geq (c_i/2) V_i \]  \hspace{1cm} (IV.1)

where \( c_i^1 \) and \( V_i^1 \) are the initial concentration and volume of the analyte in a sample zone, and \( c_i \) and \( V_i \) are the sample concentration and volume following ITP focusing, respectively. If this condition is satisfied, unique ITP flat-top zone profiles result. Otherwise, failure to reach the sufficient concentration results in peak-like zones forming that may contain multiple trace analytes [12-14].

One of the most challenging aspects of ITP involves the analysis of single mixed zones which form as a result of analytes not reaching the "sufficient" criteria. In these mixed zones, multiple trace-level analytes combine together into a single peak-shaped band whose boundaries are defined by the leading and trailing edges of the adjacent LE and TE zones (see Figure I.3A). When "sufficient" concentration zones are produced (Figure I.3B), detection is simplified by the fact that the bands are easily distinguished by their change in detector response (i.e., absorption or conductivity) at the zone boundaries. However, when faced with a single mixed zone containing multiple co-eluting analytes, conventional UV and conductivity detectors are unable to perform the required signal deconvolution necessary for analyte differentiation.
ITP is well suited for combination with mass spectrometry (MS) [15-18], which is the detector of choice for most chromatographic and electrophoretic separations. MS has many advantages over other detectors. In addition to electrophoretic separation, MS generates a second dimension of mass information which can be used to identify a separated compound. Moreover, it allows for peak deconvolution when analytes of different mass-to-charge (\(m/z\)) ratios co-elute. Unfortunately, inherent scanning characteristics of most mass spectrometers (ion trap, quadrupole, and sector instruments) prevent their use as detectors for either fast separations or ITP insufficient zones, both of which can require high data sampling rates to detect narrow peaks (i.e., \(w_{1/2} < 1.0 \text{ s}\)). High-speed separations performed using methods such as CE, ultrahigh pressure liquid chromatography (UHPLC), and GC can produce peak widths that are less than 1 s, which are too narrow to be adequately defined by full spectrum scanning mass spectrometers. They simply cannot acquire data fast enough to fulfill the 10 points peak\(^{-1}\) requirement.

In recent years, time-of-flight mass spectrometry (TOFMS) has grown to be a powerful, relatively high-resolution, high-speed detector. For samples containing analytes in insufficient quantities, ITP zone widths can be narrow, and the full spectrum data acquisition capability of the TOFMS makes it the detector of choice for ITP-MS and comprehensive ITP-CE-MS. As zone widths in ITP and peak widths in other separation methods decrease with the advent of faster separation technologies, the need for mass spectrometers that are capable of collecting peak information at high speeds is becoming increasingly important. TOFMS has the unique ability to generate a complete mass spectrum with each pulsed ion extraction. This allows the TOFMS to generate mass spectra at a much faster rate than other forms of mass spectrometry, including
quadrupole, sector, and ion trap instruments. The TOFMS instrument used in this study can record, sum, and store complete mass spectra at rates as high as 100 spectra s\(^{-1}\). In addition to speed, other characteristics of TOFMS, such as high duty cycle, high ion transmission, theoretically unlimited mass range, and ease of peak deconvolution all make TOFMS an ideal detector for fast separations.

In this chapter, the feasibility of coupling both ITP and comprehensive ITP-CE with TOFMS for the analysis of both sufficient and insufficient concentrations of analytes was studied. The full-spectrum scans produced by the TOFMS allowed for monitoring of multiple ions of interest simultaneously.

**IV.2. EXPERIMENTAL SECTION**

**IV.2.1. Materials and chemicals**

Fused silica capillaries of 50 µm I.D. x 187 µm outer diameter (O.D.) and 200 µm I.D x 365 µm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). Deionized water for buffer solutions and rinsing of the ITP and ITP-CE capillaries was from a Milli-Q water purification system (Millipore, Waterford, MA, USA). HPLC grade acetonitrile, methanol, and triethylamine were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Glacial acetic acid was obtained from EM Science (Gibbstown, NJ, USA) and ammonium acetate was from Mallinckrodt (Hazelwood, MO, USA). Angiotensins I, I(1-7), II, III and IV, as well as polyvinylalcohol (PVA, 99+ %, average MW 89,000-98,000) were purchased from Aldrich (Milwaukee, WI, USA). All buffers were degassed under house vacuum with sonication. Compressed nitrogen and helium were obtained from Airgas (Salt Lake City, UT, USA). Nitrogen was used as a curtain
gas to help desolvate compounds emerging in the electrospray, and helium was used as a sparge gas for degassing the buffers.

**IV.2.2. Electrospray interface**

The electrospray ionization interface using sheath flow to provide electrical contact with the column effluent was built in-house [19]. The liquid sheath flow rate was controlled by syringe pump (Model 11, Harvard Apparatus, Holliston, MA, USA) with a 250 µL glass Gastight® syringe fitted with a 2-in. 22-gauge stainless steel needle (Hamilton, Reno, NV, USA). The syringe was connected to a 50 µm I.D. x 180 µm O.D. fused silica capillary transfer line (Polymicro) using a Chemfluor® Teflon straight union (Norton Performance Plastics, Akron, OH, USA). A Chemfluor® tee connected the transfer line to the analytical CE column. As the liquid sheath entered the tee, it flowed between the CE column and the inside of the 27-gauge stainless steel electrospray needle to which a voltage (2-4 kV) was applied. The needle was tapered at the tip using fine-grit sandpaper to help promote the creation of a stable Taylor cone at the electrospray tip. A schematic of the interface is shown in *Figure IV.1*. Protonated molecular ions were routinely generated using ESI for the angiotensins, with angiotensin III giving the strongest signal under identical ESI conditions. The ESI mass spectra for the individual angiotensins are shown in *Figure IV.2*. To produce these spectra and for subsequent separations, a liquid sheath solution of methanol/water (70:30) with 0.1% acetic acid was used at a flow rate of 1.0 mL min⁻¹.

The interface was mounted on an XYZ stage (Series 462, Newport, Engelwood, CO, USA) to aid in the optimization of the electrospray needle position relative to the
Figure IV.1. Schematic of the liquid sheath interface between the analytical column and electrospray needle.
Figure IV.2. Electrospray ionization (ESI)-TOFMS spectra of angiotensins.
Figure IV.2. Electrospray ionization (ESI)-TOFMS spectra of angiotensins (continued).
Figure IV.2. Electrospray ionization (ESI)-TOFMS spectra of angiotensins (continued).
TOFMS sample orifice. A microscope fitted with a 15-60x adjustable objective (Edmund Scientific, Barrington, NJ, USA) was used together with a Series 41722 fiber optic illuminator (Cole-Parmer, Arcade, NY, USA) to observe the integrity of the Taylor cone at the electrospray tip.

IV.2.3. Isotachophoresis (ITP)

A Crystal CE Model 300 (UNICAM, Madison, WI, USA) capillary electrophoresis instrument with both electrokinetic and hydrodynamic injection capabilities and a voltage limit of +30 kV was used for ITP. The capillary column was initially rinsed and filled with LE by immersing the inlet end of the capillary in the LE buffer vial and applying a pressure (1000 psi). The inlet of the ITP column was then placed into a sample vial and approximately 10 µL were inserted into the column hydrodynamically using pressure (1000 psi). The column and a platinum electrode were then inserted into the TE buffer vial and a voltage was applied to begin the ITP process. All of these steps were programmed to run automatically using the Crystal CE programmable interface.

IV.2.4. Comprehensive isotachophoresis-capillary electrophoresis (ITP-CE)

The instrumentation and methodology for ITP-CE used in this work were described in detail in Chapters II and III [10]. Briefly, the ITP-CE instrument consisted of a Valco (Houston, TX, USA) Model C22 four-port manually actuated sample valve (10 µL sample loop) connected to both the ITP column and the TE buffer reservoir with a platinum electrode attached to a Spellman (Hauppauge, NY, USA) 60 kV/500 µA
SL60PN30 high voltage power supply. A custom made quartz tee (365 µm I.D., InnovaQuartz, Phoenix, AZ, USA) was used at the bifurcation point between the ITP and CE columns to minimize dead volume and residual flow effects after LE counterflow infusion. A UV3000 UV-Vis detector (ThermoQuest, San Jose, CA, USA) was positioned just before the bifurcation point to signal when the ITP stack arrived at the junction between the ITP and CE columns prior to infusion of the LE counterflow from a Harvard Apparatus (Holliston, MA, USA) Model 22 syringe pump.

**IV.2.5. Time-of-flight mass spectrometer (TOFMS)**

A commercial Jaguar™ TOFMS (LECO, St. Joseph, MI, USA) with orthogonal extraction was used as a detector. The Jaguar™ TOFMS used a heated (80°C) nitrogen curtain gas running counter to the flow of ions into the mass spectrometer to desolvate ions emerging from the electrospray tip. After desolvation at atmospheric pressure, ions are carried through a nozzle, skimmer and an RF-quadrupole to the ion pulsing region (~2 x 10^-6 mbar). In this region, a portion of the ion beam receives a pulse of kinetic energy applied orthogonal to the direction of the ion beam. The ions then travel the length of the flight tube and are detected using a microchannel plate (MCP) detector. Ions separate in the flight tube according to the differences in their mass to charge ratios. Detection is rapid, and complete averaged mass spectra with mass range of up to 6000 m/z can be collected at a rate of up to 100 spectra s^-1.

The electrospray voltage applied to the 27 gauge stainless steel needle which housed the fused silica capillary and sheath flow was between 2.0 and 4.0 kV, with the counter electrode (the interface plate of the Jaguar TOFMS) voltage held constant at
+650 volts. Data were collected at a rate of 12.5 spectra s⁻¹. A schematic of the instrumentation, including both ITP-TOFMS and comprehensive ITP-CE-TOFMS is shown in Figure IV.3.

**IV.2.6. Preparation of PVA-coated capillaries**

The capillaries for both ITP and CE (Polymicro Technologies) were coated prior to use with polyvinyl alcohol (PVA) to suppress electroosmotic flow. A coating procedure similar to Clarke *et al.* [20] was used and is described in Chapter III.3.1.

**IV.3. RESULTS AND DISCUSSION**

**IV.3.1. Coupling of isotachophoresis with time-of-flight mass spectrometry**

The initial motivation for pairing isotachophoresis with TOFMS detection was to take advantage of the TOFMS peak deconvolution capabilities and rapid full spectrum data acquisition, thought to be especially well suited for the analysis of narrow, insufficient concentration zones. One obvious limitation of this approach is that the analytes contained in a given peak must produce ions which can be differentiated. Different compounds which produce the same protonated molecular ion will likely not be differentiated unless some separation exists within the mixed peak.

Previous studies describing the coupling of ITP with a quadrupole MS using an electrospray ion source addressed concerns regarding such issues as the overall effect of LE loss on the movement and integrity of the ITP stack as it progresses through the electrospray interface, the effect of EOF when using an uncoated fused silica capillary, and the overall stability of the electrospray as the eluting solution changes from LE to
Figure IV.3. Schematic of the ITP-TOFMS and ITP-CE-TOFMS instrumentation.
sample to TE as the ITP stack moves through the interface [15]. When EOF was present in the column, the overall elution time was decreased; in most cases, however, the ITP steady state was achieved before the ITP zones eluted from the column. For this work, we chose to use PVA-coated capillaries to suppress EOF, thereby allowing the use of short ITP and CE columns (15 cm each). The PVA coating procedure is also attractive because of simplicity.

IV.3.2. ITP-TOFMS of a sample containing analytes of sufficient concentrations

ITP samples having analytes of sufficient concentration form flat-top zones, which is advantageous compared to other separation techniques in terms of MS requirements. With a sufficient concentration, each zone has an analyte concentration equal to the concentration of the LE, and the width of each zone serves as the basis for quantitative analysis calculations. For insufficient concentrations, more stringent MS dynamic range requirements are needed since analytes may be present at trace levels. For sufficient concentration analyses, each analyte zone elutes immediately after the other without analyte-free gaps or regions, thus maintaining electrical continuity throughout the ITP stack. Quantitation in ITP is highly dependent on obtaining good flat-top zone shapes with the leading and terminating boundaries of each zone distinctly defined. However, if two or more compounds having similar absorption coefficients are analyzed simultaneously, conventional detectors may record the result as only a slight step up or down, making quantitation more difficult. For example, the analysis of five angiotensins in Figure IV.4 shows an ITP analysis using UV absorption (215 nm) as the detector. While distinct bands are clearly visible for the first three analytes, a
Figure IV.4. ITP-UV separation of a mixture of angiotensins of sufficient concentrations. Conditions: 30 µM angiotensins, 10 mM ammonium acetate LE, 10 mM acetic acid TE, 20 cm sample plug (50 mbar, 0.8 min), 82 (70) cm x 150 µm I.D. PVA-coated capillary, +30 kV for 13 min, +15 kV for remainder of the run. Peak identifications: (1) angiotensin III, (2) angiotensin I, (3) angiotensin IV, (4) angiotensin I (1-7), (5) angiotensin II.
recognizable boundary between the fourth and fifth analyte bands is not apparent. In such a situation, the ability of the TOFMS to monitor single ions unique to each compound becomes a clear advantage.

A reconstructed ITP-TOFMS electropherogram of the sample described in Figure IV.4 is shown in Figure IV.5. Using single-ion monitoring of the parent ion peak for each analyte, the boundary between angiotensin I (1-7) and angiotensin II is now easily definable, and quantitation can be much more easily performed. It is also obvious that the boundary between the two analytes is not as distinct as would be found had the ITP stack reached its full steady state prior to arriving at the electrospray interface. This implies that the ITP conditions can be further optimized to allow the ITP zones to fully develop. An additional advantage of the single ion monitoring capability of the TOFMS is that it provides ITP zone information useful in studying the effects of ITP parameters on the overall separation. As noted by Udseth et al. [15] the lack of complete separation between two analytes having small differences in their electrophoretic mobilities may be an indication of the adverse effects of Joule heating and diffusion within the column. Such information may lead to a better understanding of the ITP separation process through computational modeling.

IV.3.3. ITP-TOFMS of a sample containing analytes of insufficient concentrations

Dilute samples which contain analytes at concentrations below the sufficient criterion present a challenge to conventional ITP analysis. As was mentioned earlier, detectors such as UV and conductivity are not able to distinguish between multiple analytes contained in a single zone, but rather record them as a single peak. Figure IV.6
Figure IV.5.  ITP-TOFMS separation of a mixture of angiotensins of sufficient concentrations. Conditions: Sample and ITP settings same as in Figure IV.4, except for 82 cm effective column length (electrospray positioned at the end of the capillary) and +30 kV for 15 min, +15 kV for remainder of the run; 1.5 µL min⁻¹ liquid sheath (70:30 MeOH/H₂O/0.1% HAc), 4 kV applied electrospray voltage, 80°C heated TOFMS interface plate for curtain gas. Peak identifications: (1) angiotensin III (931 m/z), (2) angiotensin I (1296 m/z), (3) angiotensin IV (775 m/z), (4) angiotensin I (1-7) (899 m/z), (5) angiotensin II (1046 m/z).
Figure IV.6. ITP-TOFMS of a mixture of angiotensins of insufficient concentrations. Peak identifications same as in Figure IV.5.

Conditions: Same as in Figure IV.5 except 3 µM angiotensins.
demonstrates the advantage of TOFMS to track multiple ions simultaneously when a dilute sample of angiotensins is injected. As can be seen in the reconstructed electropherogram, a slight separation of angiotensins III and I occurred, but it is obvious that without selected-ion monitoring there would be no indication that angiotensins IV and I(1-7) were present within the angiotensin II peak.

One issue that can arise when using electrospray ionization is that of ion suppression. During the analysis of a mixed ITP zone, ion suppression and discrimination can occur, depending on the relative concentrations of compounds within the zone and analyte ionization efficiencies in the electrospray. For example, when a mixture of angiotensins, each at an initial concentration of $3 \times 10^{-7}$ M, was introduced into ITP, a mixed zone was formed, but angiotensin III dominated the mass spectrum, as shown in Figure IV.7. Under these conditions, the ionization of other ions in the sample were greatly suppressed by angiotensin III, being barely discernable above the baseline noise or completely lost. While this may not occur with every sample, attention must be given to the potential influence of electrospray ionization efficiency and suppression. Regardless of this, the information obtained using TOFMS with ITP for insufficient concentration mixed zones surpasses that of conventional detectors.

IV.3.4. Comprehensive ITP-CE-TOFMS

While ITP-CE alone has shown potential for analyzing both sufficient and insufficient multi-component samples with UV detection [10,11], the mass information provided by the TOFMS further increases its ability to identify trace analytes. In addition, ion suppression due to co-elution of analytes in mixed zones using ITP-TOFMS
Figure IV.7. ITP-TOFMS of a mixture of angiotensins of insufficient concentrations. Peak identifications same as in Figure IV.5.

Conditions: Same as in Figure IV.5 except 0.3 µM angiotensins.
is largely avoided by including a separation step following ITP preconcentration. One of the key advantages of comprehensive ITP-CE using coupled columns, compared with other ITP-CE techniques that use sample splitting or heart-cutting techniques to avoid column overloading, is that the entire sample is analyzed in a comprehensive manner. In this arrangement, the ITP column has an internal diameter which is larger (200 µm I.D.) than the outer diameter of the CE column (187 µm I.D.). The larger I.D. of the ITP column allows for more sample to be loaded onto the system.

The injected sample volume (up to 10 µL in this study) is first concentrated and separated in the ITP column. The ITP zones are then allowed to migrate just past the inlet end of the CE column, thereby allowing some sample to enter the column. Using a counterflow of LE, the remaining analytes which were not moved onto the CE column are pushed back into the ITP column, where they continue to focus and migrate once again towards the CE column. This occurs simultaneously with separation of the sample introduced into the CE column. The infusion of buffer at the bifurcation point at the junction of the ITP and CE columns via counterflow acts as both background electrolyte in CE and as LE in ITP. These multiple injections are repeated until all of the sample has been analyzed, and the results of the CE injections are summed to provide a complete electropherogram. Since none of the large volume sample introduced via ITP is discarded, the potential for detection of trace analytes increases.

One difference between ITP-CE-UV and ITP-CE-TOFMS is that with UV detection, a window was placed on-column by removing a small portion of the polyimide coating, and a buffer reservoir was placed at the end of the column to serve as ground to complete the circuit. This buffer vial also produced a “closed” system whereby
hydrodynamic flow was suppressed inside the CE capillary when the LE counterflow was initiated at the bifurcation point. However, with the use of TOFMS as the detector, an electrospray is established at the end of the CE column, leaving it open to the atmosphere. In all separations with ITP-CE-TOFMS, some peak tailing was observed and can be attributed to the presence of hydrodynamic flow induced by the LE counterflow. Although not evaluated in this study, either the coupling of a larger I.D. ITP column or the use of a smaller I.D. CE column would favor the ITP column as the path of least resistance for the LE counterflow and decrease the flow inside the CE capillary.

IV.3.5. Comprehensive ITP-CE-TOFMS of a sample containing analytes of sufficient concentrations

When analyzing major components in a concentrated sample in ITP, typical flat-top zones result, and the use of ITP-CE-TOFMS for these types of samples may not be required since similar results could be obtained using ITP-TOFMS alone. ITP-CE-TOFMS reconstructed electropherograms of injections at various times during an analysis of angiotensins (3 x 10^{-5} M) are shown in Figure IV.8. Multiple successive injections were required to analyze the entire sample. This is a significant drawback to using the ITP-CE method when ITP alone would likely suffice. Although not studied in this work, the possibility that trace component mixed zones in complex samples may reside in the electrophoretic mobility windows between adjacent flat-top zones remains a topic for further study and one where ITP-CE would be a valuable tool.
Figure IV.8. Multiple injection comprehensive ITP-CE-TOFMS separation of a mixture of angiotensins of sufficient concentrations. Conditions: 20 cm x 200 µm I.D. (365 µm O.D.) PVA-coated ITP column (15 cm effective separation length) coupled with a 20 cm x 50 µm I.D. (187 µm O.D.) PVA-coated CE column (15 cm effective separation length), 10 µL injection volume, 30 µM angiotensins, +24 kV for initial ITP concentration, reduced to +10 kV for subsequent ITP and CE steps, 10 mM triethylamine LE, 10 mM acetic acid TE, 4 µL LE counterflow, 60 µL min⁻¹ infusion, TOFMS and liquid sheath conditions and peak identifications same as Figure IV.5. A) Injection 1, B) injection 4, C) injection 7, D) injection 10.
Figure IV.8. Comprehensive ITP-CE-TOFMS separation of a mixture of angiotensins of sufficient concentrations (continued).
An obvious difference between ITP and ITP-CE is the appearance of the resulting electropherograms, with ITP showing flat-top zones and ITP-CE yielding the more recognizable CE analyte peaks. One consequence of manually controlling injection time is the potential for either overloading the CE column or allowing the ITP stack to migrate too far past the front of the CE capillary. The latter is illustrated in Figure IV.8B where it is possible that a small portion of TE entered the CE column along with the analytes. Since LE was infused behind the sample plug at the bifurcation point, the LE (with its higher electrophoretic mobility) raced through the sample to the front of the plug, resulting in the separation of angiotensins III, I and IV. However, it appears that the small volume of TE and the higher concentrations of both angiotensins I (1-7) and II resulted in ITP-type zones.

IV.3.6. Comprehensive ITP-CE-TOFMS of a sample containing analytes of insufficient concentrations

A potential application of comprehensive ITP-CE is trace analysis. Because of the ITP detection difficulties (co-elution and ion suppression) stated earlier with analytes of insufficient concentration, the addition of CE allows compounds which otherwise would have gone undetected due to suppression to be separated from other analytes and detected as separate CE peaks. By preconcentrating trace analytes in the ITP step, the combination of ITP-CE and TOFMS is potentially a more useful method for detecting trace compounds than CE or CE-MS alone.
Figure IV.9. Comprehensive ITP-CE-TOFMS separation of a mixture of angiotensins of insufficient concentrations. Two successive injections are shown, with $t_0 = 10.0$ min for the second injection. Conditions: Same as Figure IV.5 except 0.3 µM angiotensin IV, 3 µM angiotensin III, 5 µM angiotensin I and I (1-7), and 8 µM angiotensin II. Peak identifications: same as in Figure IV.5.
To show the viability of the ITP-CE-TOFMS technique, a dilute (0.3 μM angiotensin IV, 3 μM angiotensin III, 5 μM angiotensin I and I (1-7), and 8 μM angiotensin II) mixture of angiotensins was used. Angiotensins III and IV were purposely added at lower concentrations to show the capability of the ITP-CE technique in separating trace compounds from more concentrated analytes. As can be seen in Figure IV.9, single ions representative of each compound were monitored for each CE injection by the TOFMS and summed to produce a single electropherogram. The obvious advantage of adding a CE separation step can be visualized by comparing Figures IV.6 and IV.9. As the compounds elute independently from the CE column, they no longer must compete with co-eluting analytes and, therefore, ion suppression is avoided. The detection of trace analytes, which may have been completely suppressed using ITP-TOFMS alone, is made possible by the addition of CE.

IV.3.7. Electrospray interface

Loss in separation efficiency can occur when using a liquid sheath electrospray interface. It is assumed that most of the efficiency loss occurs at the electrospray tip and is due to excessive mixing of the liquid sheath with the CE column eluent, thus inducing band broadening. In theory, this loss can be minimized by positioning the tip of the analytical column just outside the tip of the stainless steel needle such that electrospray occurs immediately after contact of the liquid sheath with the mobile phase. In this study, the best results and the most stable Taylor cone formation were obtained when the end of the CE column extended approximately 0.5 mm beyond the edge of the stainless
steel electrospray needle. The needle was then positioned approximately 1 cm from the TOFMS interface plate.

It should be noted that as the electrospray voltage (2-4 kV), curtain gas flow rate (0-900 mL min⁻¹), liquid sheath flow rate (0.5-3 µL min⁻¹), and analytical column tip distance (0-1 mm) outside the electrospray needle were adjusted, changes in ionization efficiency and TOFMS sensitivity were evident, and it was necessary to optimize the electrospray conditions each day. For example, the sensitivities of the angiotensins were highly dependent on the position of the CE column inside the electrospray needle and its distance from the TOFMS nozzle. In addition, both the liquid sheath flow rate and electrospray voltage also greatly influenced ionization efficiency.

IV.4. CONCLUSIONS

ITP and comprehensive ITP-CE were successfully coupled to TOFMS using an electrospray ionization interface for the analysis of angiotensin mixtures of both sufficient and insufficient concentrations. The suppression of electroosmotic flow inside the capillaries using polyvinylalcohol (PVA) as a capillary interior coating permitted the use of shorter capillaries than were used in previous ITP-MS studies. Multiple-injection CE analyses using the comprehensive ITP-CE technique were performed. The capability of TOFMS to resolve partially separated compounds by deconvolution based on the monitoring of single ions was shown for samples containing insufficient concentrations of analytes. This represents a major improvement over ITP coupled with conventional absorption-type detectors. Both ITP and ITP-CE are suitable for dilute samples where
sufficient volumes are available for analysis. The large volume injection capabilities of ITP and ITP-CE are favorable for trace component analysis.
IV.5. REFERENCES


CHAPTER V
FUTURE RESEARCH RECOMMENDATIONS

Capillary isotachophoresis (ITP) as a preconcentration step for capillary electrophoresis (CE) in a comprehensive arrangement as described in this dissertation is a potentially useful tool for the analysis of charged compounds, especially those present in trace amounts. Although still in its development stage, continued improvements in comprehensive ITP-CE instrumentation and methodology could result in improved separation and detection of trace components in environmental and clinical samples. The following areas should be addressed to further develop the comprehensive ITP-CE concept and more thoroughly explore the potential of the technique.

V.1. AUTOMATED SYNCHRONIZATION OF CE INJECTION TIME WITH UV DETECTOR RESPONSE

The most critical step and the one that principally determines the success of a comprehensive ITP-CE analysis is sample introduction from the larger diameter ITP column to the smaller diameter CE capillary. The current ITP-CE method utilizes dual UV/Vis detectors, one of which indicates when the ITP bands have approached the CE column inlet while the other is used as the CE detector. Currently, the amount of sample that passes onto the CE column is controlled manually using a delay time between when the ITP zones first appear at the first UV/Vis detector and when the syringe pump for LE counterflow infusion is turned on. However, significant improvement in run-to-run reproducibility can be achieved by automating this delay procedure. This involves
including a timing program that uses the output signal from the UV/Vis detector which would indicate when the leading edge of the ITP zones begins to pass the detector window. By measuring the slope of the detector absorbance, a programming routine could be used to start a timing counter. After a user-defined delay time, \( t \), has elapsed, the syringe pump would then be turned on. The advantage is that the delay time could be set by the user, and method optimization to prevent CE column overloading would be possible.

In programming this type of operation, it must be remembered that the time between LE counterflow infusion and re-appearance of the ITP zones at the UV detector following a CE injection will increase over the course of an analysis. This is due to the loss of ions from the ITP system following a CE injection. Thus, \( t \) will need to change to reflect the decrease in system current that occurs with each CE injection.

V.2. ITP-CE-MS

Mass spectrometry (MS), when used as a detector for comprehensive ITP-CE, greatly increases the applicability of the ITP-CE technique by providing mass information that can be used in conjunction with retention time to positively identify compounds. Other non-specific detectors (i.e., UV/Vis, fluorescence, conductivity, etc.) provide only a general confirmation of the presence of a compound. MS has its greatest utility when applied to rapid separations or separations of low-concentration analytes whose peak widths are very narrow. Mass spectrometers such as quadrupole, sector, and ion-trap instruments have full spectrum scan speeds which are too slow to adequately handle narrow peak widths.
Time-of-flight mass spectrometry (TOFMS) has the capability to collect spectra at rates of up to 5000 spectra s⁻¹, making it an ideal detector for ITP-CE analysis of trace components. CE half-height peak widths, especially for samples of insufficient concentration, may be only a few hundred milliseconds wide. Other mass spectrometers (e.g., quadrupole, ion trap) that scan over a reasonable mass range do not have the capability to adequately detect such narrow peaks with the requisite 10 points peak⁻¹. Further studies into the combination of TOFMS with comprehensive ITP-CE would likely provide valuable information for the analysis of trace components in mixtures.

V.3. ITP-MS OF MIXED ZONES OF INSUFFICIENT CONCENTRATIONS

Direct analysis of mixed isotachophoretic zones without the use of a second separation step such as CE is an area where little research has been performed. During studies on ITP-TOFMS of single mixed zones in Chapter IV, it was readily apparent that ion suppression by angiotensin III during the electrospray process significantly hindered efforts to simultaneously identify other angiotensins contained within the same mixed zone. It is not currently known to what extent ion suppression occurs with other classes of compounds under similar isotachophoresis/electrospray conditions. Other ITP-MS papers make no mention of ion suppression problems; however, in all cases only sufficient samples were analyzed. When sufficient samples are used, only a single analyte elutes from the ITP column at a time; thus, ion suppression is not expected. It is not known whether or not the degree of suppression which occurred with the angiotensins is common with other compound classes. If other sample types do not undergo similar
ion suppression effects, ITP-MS will be a useful tool in the analysis of trace components in complex mixtures.

V.4. COMPREHENSIVE ITP-CE-UV AND ITP-CE-TOFMS FOR REAL-WORLD SAMPLES

The work performed in this dissertation primarily focused on comprehensive ITP-CE instrumentation development, using standard sample mixtures to show proof-of-concept. In order to apply the comprehensive ITP-CE technique to real-world clinical or environmental analysis, more work must be done to test the robustness and general applicability of the ITP-CE technique. Although ITP in general has not gained wide acceptance for routine analysis, successful comprehensive ITP-CE examples may catch the attention of analysts currently seeking alternative methods for their analytical needs.

An area of ITP research which has not been given much attention is the analysis of insufficient isotachophoretic zones lying between zones of sufficient concentration. Since MS has not been commonly applied to ITP, insufficient zones would go unnoticed, since their detector responses would be very low compared to those of the sufficient zones. Using MS deconvolution algorithms, the presence/absence of other analytes could be verified. With any real-world sample, a wide range of analyte concentrations would be expected. ITP-CE-MS could serve as a useful tool in analyzing trace components in the presence of other major components.
V.5. EFFECTS OF SPLITTING VOLTAGE ON CE INJECTION

The effect of applying a splitting voltage at or near the bifurcation point (the point at which the ITP and CE columns meet) during ITP-CE has not been studied in-depth, and information regarding ion behavior as ions reach the ITP and CE capillary interface should be further investigated. Few papers make mention of the use of a splitting voltage to promote ions into the CE capillary following ITP. With the present comprehensive ITP-CE system, the region around the bifurcation point electrically floats according to the strength of the electric field resulting from the applied voltage at the injection valve. While the calculated analyte loss was determined to be minimal with an applied splitting voltage of 2 kV (see Chapter III.4.7), the effect of a higher splitting voltage (i.e., 5 kV) at the bifurcation point has should be studied.

V.6. MULTICHANNEL HIGH THROUGHPUT ITP-CE

The advent of high profile scientific advances such as genomics and proteomics, combined with the large sample libraries produced by combinatorial chemistry, has brought high throughput analysis to the forefront of analytical instrumentation needs. The current trend towards miniaturization using small microchips means that the use of sample volumes even smaller than the current nL-size samples in capillary CE will become routine. This places a tremendous burden on the detection capabilities of even the most sensitive detectors. ITP, with its ability to concentrate analytes online, may serve as a plausible solution for some analysis needs. Also, since ITP and CE are both electrophoretic techniques, multichannel systems can be constructed using current etched microchip technology. Great advances have been made in recent years in the areas of
injection and detection using chip-based technology, and these same advances can be applied to multi-dimensional systems such as ITP-CE on a chip.

Miniaturization of ITP and ITP-CE will allow for larger samples to be used than are currently feasible for chip-based separation systems. After concentrating by ITP, analytes which were initially present at concentrations below the limit of detection may be detected. Also, since small sample volumes challenge the sensitivity limits of absorption-based detectors such as UV and IR, other detectors not limited by sample pathlength including fluorescence, refractive index, Raman, MS, and electrochemical detectors will now find greater applicability to microchip-based separation systems due to the addition of ITP concentration.

V.7. ITP MIGRATION MARKERS

When the amount of analyte introduced in ITP is small, a single mixed zone containing multiple analytes can only be identified as a single peak in an ITP-UV electropherogram. In this case, non-UV-absorbing spacers with electrophoretic mobilities between those of each desired analyte are required to separate and quantify these trace sample components. However, mobility values for many analytes are not readily available. This is a major drawback to ITP quantitation of single mixed zones, since the choice of mobility spacers directly depends on mobility information about the analytes of interest. Using a single set of mobility markers with known electrophoretic mobilities, a standard reference set can be developed. This would aid in method development for ITP. One key consideration in the pursuit of mobility spacers is that, initially, research should be performed on sample sets containing only a few analytes
until general trends can be established for general classes of compounds under standard conditions (pH, buffer concentration, etc.). The mobility spacers can then be used as migration markers to provide general mobility information about specific analytes.

Another application of mobility spacers would be for ITP-TOFMS. The fast scan speeds of the TOFMS can be expected to resolve narrow adjacent zones separated by mobility spacers. This may reduce the effect of ion suppression when analyzing single mixed zones without spacers. As examples, the general mobility ranges of a variety of analytes are given in Table V.I.
Table I. Mobility ranges of various analytes and synthesized markers.

<table>
<thead>
<tr>
<th>Compound Class</th>
<th>Electrophoretic Mobility Range (x 10^-6 cm^2 V^-1 s^-1)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotides</td>
<td>17-40</td>
<td>4.7</td>
</tr>
<tr>
<td>Amino acids</td>
<td>6.9-29</td>
<td>8.6</td>
</tr>
<tr>
<td>Dipeptides</td>
<td>3.6-17.1</td>
<td>8.0</td>
</tr>
<tr>
<td>Proteins</td>
<td>~3-40</td>
<td>varies</td>
</tr>
<tr>
<td>Disulfonic acids</td>
<td>28-40</td>
<td>8.4</td>
</tr>
</tbody>
</table>
APPENDIX A

ITP SAMPLE VALVE SCHEMATICS FOR SMALL INTERNAL DIAMETER CAPILLARIES
NOTES:


2. REMOVE ALL BURRS AND BREAK SHARP CORNERS .005-.010 MAX.

SEE SHEET 2C

MSX.8 $ SHOWN

M5 X.8 $ SHOWN

SECTION A-A

SCALE 0.50

SECTION B-B

SCALE 1.60
NOTES:
2. REMOVE ALL BURRS AND BREAK SHARP CORNERS .005-.010 MAX.

SEE DETAIL C

SEE DETAIL B

SECTION A-A

 DETAIL B SCALE 8

 DETAIL C SCALE 14

SCALE 0.500

4X Ø .265 THRU
NOTES:
2. REMOVE ALL BURRS AND BREAK SHARP CORNERS .005-.010 MAX.

SEE DETAIL E

SECTION C-C

SEE DETAIL GROOVE

SECTION A-A

DETAIL GROOVE

SCALE 13.00

DETAIL D

SCALE 14.00

DETAIL E

SCALE 4.00

PRECISION MACHINING LABORATORY
NOTES:


2. REMOVE ALL BURRS AND BREAK SHARP CORNERS .005-.010 MAX.

SCALE 1.000

- ø .62
- .28
- 1.50
- ø .44
- .02
- .04
- 45°
- 30.00°
- .63
- ø .05 THRU
NOTES:


2. REMOVE ALL BURRS AND BREAK SHARP CORNERS .005-.010 MAX.
NOTES:


2. REMOVE ALL BURRS AND BREAK SHARP CORNERS .005-.010 MAX.
NOTES:

1. ALL DIMENSIONS AND TOLERANCES SHALL BE INTERPRETED
   AND CONTROLLED BY ANSI Y14.5M-1982.

2. REMOVE ALL BURRS AND BREAK SHARP CORNERS .005-.010 MAX.

SCALE 1.500
NOTES:


2. REMOVE ALL BURRS AND BREAK SHARP CORNERS .005-.010 MAX.

SCALE 1:500
NOTES:


2. REMOVE ALL BURRS AND BREAK SHARP CORNERS .005-.010 MAX.

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SCALE 2.00

---

SPACER

NUT

SCALE 2.00

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PRECISION MACHINING LABORATORY

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Figure B.1. Schematic of the high voltage isolation board.
Figure B.2. Schematic of the control section of the high voltage isolation board.
Figure B.3. Schematic of the voltage isolation components on the voltage isolation board.
APPENDIX C

ITP-CE SOFTWARE SCREENSHOTS AND WIRING Diagrams FOR DATA ACQUISITION AND POWER SUPPLY CONTROL VIRTUAL INSTRUMENT
Figure A.1. Screen shot of power supply and high voltage relay control panel.
Figure A.2. Wire diagram of the power supply control panel.
Figure A.3. Screen snapshot of the data acquisition front panel (signal intensity vs. scan number)
Figure A.4. Screen snapshot of the data acquisition front panel (signal intensity vs. time).
Figure A.5. Wire schematic of the data acquisition front panel.