MICROFABRICATED CARTRIDGES FOR ISOELECTRIC FOCUSING WITH WHOLE COLUMN IMAGING DETECTION AND NANO-ELECTROSPRAY MASS SPECTROMETRY

By

FUNMILAYO OYEDIRAN

A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Science

in

Chemistry

Waterloo, Ontario, Canada, 2008

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

ABSTRACT

Microfluidic chips have gained wide applications in various fields, including medicine, environmental sciences and forensic investigations. They are used for the separation of proteins, blood, bacterial cell suspensions, antibody solutions, and drugs.

Microfluidic chips display significant advantages, which include faster analysis time, reduced amounts of samples and reagents volumes, flexibility in design and increased separation efficiency.

Whole column imaging detection (WCID) exhibits significant advantages compared to other detection methods that are widely used for detecting analytes after the separation of these analytes using isoelectric focusing. With these other methods, there is a need to mobilize the focused sample bands past the detector after separation but with WCID, there is no need for mobilization step.

The aim of this research is further development of WCID by characterizing microfluidic chips fabricated for the detection system, to enhance its detection so that high efficiency can be obtained. The chips were fabricated using soft lithography technology at the Microfluidic laboratory, University of Waterloo and they were used to perform isoelectric focusing of various proteins in our laboratory.

The fabricated chips with straight channel design were used to carry out isoelectric focusing of some proteins and the results obtained were compared with the results obtained using commercial cartridges. The chips with tapered channel design were used to carry out isoelectric focusing of proteins in which thermally generated pH gradient principle was employed. The samples after separation were sprayed into a mass spectrometer using nano-electrospray interface to obtain their molecular masses.

Compatible cartridges for nano-electrospray mass spectrometer were developed and these cartridges were used to carry out capillary isoelectric focusing of low molecular pI markers and proteins. These cartridges were also connected to the nano-electrospray mass spectrometer to obtain the mass to charge ratios of some proteins.

The fabricated microfluidic chips with straight channel design were also used to investigate the interaction between drugs and protein.

ACKNOWLEDGEMENT

DEDICATION

I dedicate this thesis to GOD, the King and Maker of my life. You will forever be my King.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

CA   Carrier Ampholytes

CAD   Computer aided design

CCD   Charge-coupled device camera

CE   Capillary electrophoresis

CEC   Capillary electrochromatography

CGE   Capillary gel electrophoresis

CIEF   Capillary isoelectric focusing

CIEF-WCID   Capillary isoelectric focusing-whole column imaging detection

CITP   Capillary isotachorphoresis

CZE   Capillary zone electrophoresis

DNA   Deoxyribonucleic acid

EOF   Electroosmotic flow

IEF   Isoelectric focusing

LIF   Laser induced fluorescence

MEKC   Micellar electrokinetic capillary chromatography

MS   Mass spectrometry

PDMS   Polydimethylsiloxane

pI   Isoelectric point

pIs   Isoelectric points

PVP   Poly vinyl pyrrolidone

Tris-HCl   Tris (hydroxymethyl aminomethane)

WCID   Whole column imaging detection

UV	Ultra-violet
Vis	Visible

CHAPTER ONE

1.0 INTRODUCTION

Microfabricated cartridges (chips) have gained wide applications in various fields of science, especially in separation techniques. They have been widely used in capillary electrophoresis (CE), which is a promising separation method for proteins and other zwitterionic compounds. In recent years, the use of chips for the separation of proteins, peptides and other biomolecules has increased because these chips have significant advantages, which include reduction in analysis time, reduced use of samples and reagents, high efficiency and ease of fabrication.

1.1. CAPILLARY ELECTROPHORESIS

Electrophoresis is one of the widely used separation techniques. It involves the movement of electrically charged particles in a conductive medium under the influence of an electric field. CE is a type of electrophoresis performed in a capillary format.

This separation method was introduced in the 1960s. CE is a family name for some techniques used for the separation of various compounds. These various techniques include capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE), micellar electrokinetic capillary chromatography (MEKC), capillary electrochromatography (CEC) and capillary isotachophoresis (CITP).

CE is a powerful technique used for the separation of ionic species such as proteins, peptides and other zwitterionic biomolecules. The analytes, which are electrically charged under the influence of an electric field move in a conductive medium and the

separation of these analytes is based on their size to charge ratios. This technique is very simple and rapid. Figure 1 below shows a simple set up for the separation of analytes.
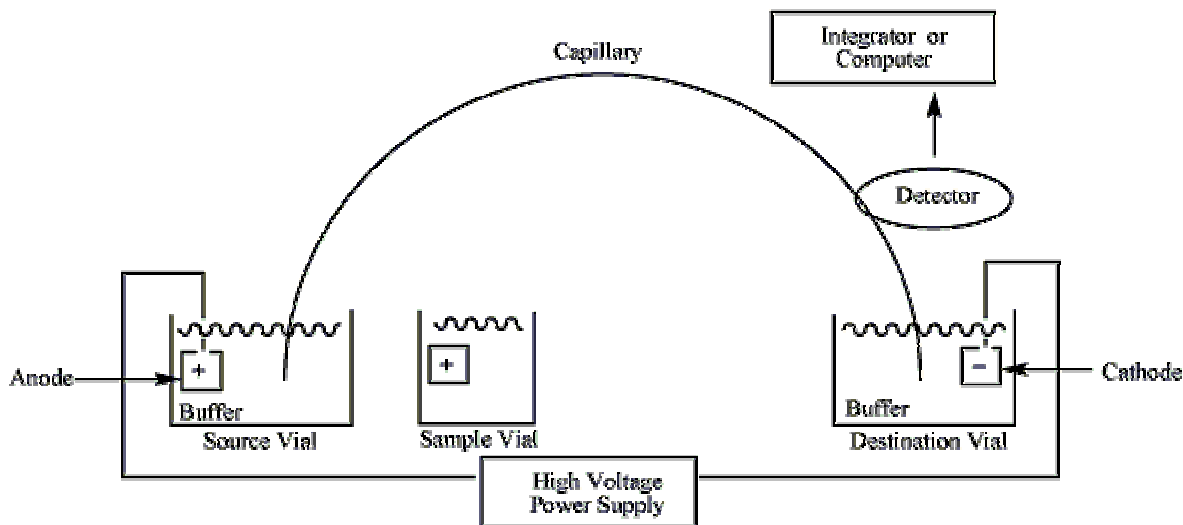
Figure 1: Capillary electrophoresis set up.[1]

The instrumentation set up as shown in Figure 1 above includes the sample, source and destination vials, the electrodes (anode and cathode), a capillary, a high voltage power supply, a detector and an integrator or a computer to process the data obtained. Various detection methods have been employed in CE namely ultra violet- absorbance detector, fluorescence detector, mass spectrometry detector, amperometric detector, conductivity detector, radiometric detector, raman-based detector and refractive-index detector.[2]

The procedure for the separation is simple; an aqueous buffer solution is filled into the source and destination vials and the capillary. The sample is injected into the capillary through pressure, capillary action or by siphoning. An electric field is applied between the source vial and the destination vial, which makes the analytes to migrate by electroosmotic flow and the analytes separate due to their electrophoretic mobilities. The

analytes are then detected by the detector and processed by the integrator/computer and displayed as electrophoregrams.[1]

The advantages of CE include speed, high efficiency (because the solutes move through the capillary column in a plug flow - constant velocity of flow in every part of a system), small amounts of sample and reagents, easy automation, cost effective and quantitative analyses can be done.[3, 4] CE is a separation technique that is widely used in different fields. It is commonly used in the clinics and hospitals for routine analyses and in pharmaceutical companies for chiral separations. Recently, this separation method has gained a wide use in forensic investigations and also in food and drug regulations.[5] It has also been reported that electrophoresis is the workhorse method for in vitro studies of protein-DNA interactions.[6]

1.2. CAPILLARY ISOELECTRIC FOCUSING

Capillary isoelectric focusing, CIEF is the focusing type of capillary electrophoresis in which the solutes are separated based on the differences in their isoelectric points (pIs).[7, 8] CIEF was introduced by Hjerten in the early 1980.

The focusing of analytes is usually accompanied by an exponential drop in the focusing current with constant applied voltage.[7, 9] This drop in the focusing current is unique with CIEF and there is a low residual current at the end of the focusing.[9]

CIEF is a high resolution technique used for the separation of ampholytes (proteins, peptides and other zwitterionic biomolecules) based on the differences in their isoelectric points.[7, 10] Isoelectric point is an identity of a protein and it stands as one of the most important physicochemical parameters of proteins and other zwitterionic biomolecules.[10]

The pI value is a measure of the total surface charge of an ampholytic species. The composition (and sequence) of amino acid residues determines the isoelectric point of peptides.[11] The charge of an ampholyte depends on the difference of its pI with local pH and the higher the difference, the greater the charge. The sample composition and the pI value can be determined using this separation method.

This method is an equilibrium method whereby the separation proceeds until equilibrium is reached with each component of the analyte focusing and settling at its pI, unlike other CE modes in which the component moves out of the capillary.[12] In this CE mode, discontinuous electrolytes and buffers are involved unlike other CE modes.[8, 10] The advantages of this CE mode includes simplicity, speed, low sample and reagents consumption, high resolution, standard instrumentation quantitation analysis and also, it requires little training to perform.[13, 14] It is used routinely to confirm the identity of proteins, to assess the purity of proteins, to determine the isoelectric point (pI) and monitor protein stability.[15] Other applications of isoelectric focusing include separation and identification of bacterial and yeast cells, detection of oligoclonal IgM, preparative-scale enantiomer purification, monitoring of protein refolding, measuring the activity of α-amylase.[16-21]

1.2.1 CONCEPTS OF CIEF

Mobility is the fundamental parameter of electrophoresis. Mobility is the charge-to-mass ratio of a solute. CIEF is usually carried out in a free solution and the focusing process produces electrically neutral solutes.[22] The concentration of the ampholyte used for most applications is between 0.5-2.0 %. The ampholyte exhibits two behaviours:

1. The zwitterion is positively charged and migrates toward the cathode at a pH below the solute's pI, and

2. At a pH above the solute's pI, the zwitterion is negatively charged and migrates toward the anode.

The pH values of the electrode buffer solutions are important parameter in CIEF. The anolyte (acidic electrolyte) must have a pH that is lower than the pI of the most acidic ampholyte and the catholyte (basic electrolyte) must have a pH higher than the pI of the most basic ampholyte. A net flow is usually directed toward the cathode.

An increase in solute concentration by a factor of more than 200 is produced by the focusing power of CIEF.[22] It is very important to always start at the lower concentrations to minimize the potential of precipitation upon focusing.

1.2.2 CARRIER AMPHOLYTES

Carrier ampholytes (CA) is a mixture of a high number of synthetic chemical species that possess slightly different pI values over a selected pH range. CA is a series of polyamide, polycarboxylic acids or polyamine-polysulfonic acids that are used in CE to establish pH gradient.

1.2.2.1 PROPERTIES OF CARRIER AMPHOLYTES

1. They possess a certain buffer capacity in their isoelectric states to maintain the local pH.

2. They have sufficient solubility in water.

3. They have low absorbance at 270 nm or longer to facilitate UV-detection of analytes.

4. They have a certain conductivity contribution to conduct current.

5. They can be without hydrophobic group to prevent possible interaction with analytes.[4, 22]

A narrow range of CA is used to obtain high resolution separation of closely related amphoteric species while a wide range is used to separate protein samples with widely different isoelectric points. It is also used to estimate the pI of unknown protein.[4, 22]

In CIEF, the capillary is filled with a mixture of carrier ampholytes and the sample. An electric field is applied, which creates a pH gradient in the capillary as shown in Figure 2 below. The pH gradient is heterogeneous inside the capillary upon the application of voltage. The series of ampholytes selected determines the breadth of the pH gradient that would be formed.
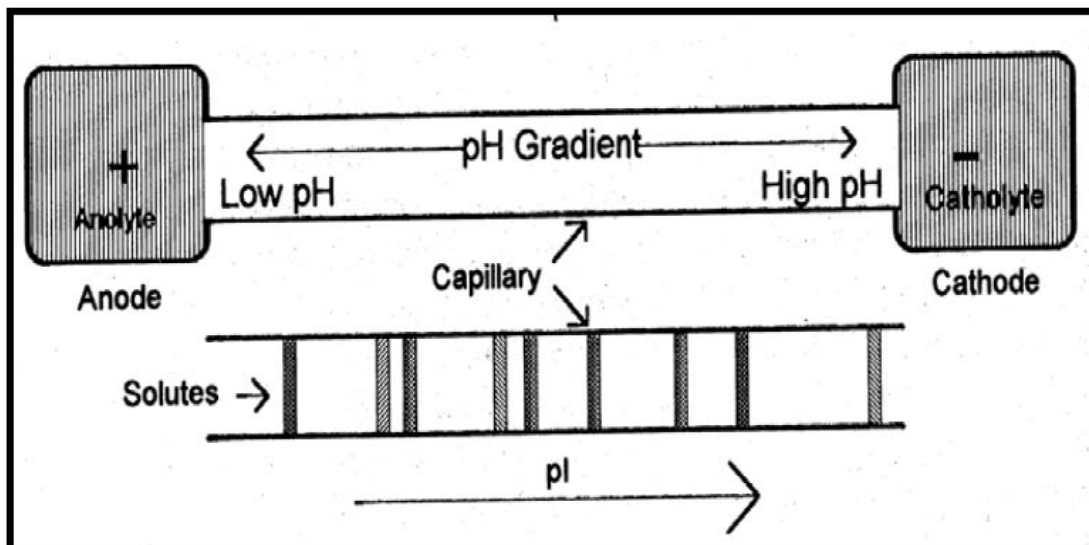
Figure 2: Formation of pH gradient.[23]

The electrolytes are used to confine ampholytes within the separation channel while the sample and the carrier ampholytes migrate through the capillary and focus into narrow zones within the capillary due to the differences in their pIs. As the analytes and the carrier ampholytes migrate, they reach a point where they are uncharged, i.e. where pH= pI. A steady state is attained at this point and this is indicated by a decrease in the current to a stable value. Upon the completion of the focusing, the solution is forced past the detector to generate the electrophoregrams. Both the buffer and the neutral molecules also move under the influence of an electric field due to electroosmotic flow (EOF). For CIEF, EOF has to be eliminated or reduced; therefore, the capillary is usually chemically modified or dynamically coated. EOF is the bulk flow of movement through the system and results from the surface charge on the inner side of the capillary wall.[4]

1.2.3 THE MECAHNISM OF SEPARATION

There are two main factors that drive the separation of analytes namely electrophoretic velocity (electrophoretic mobility) and electroosmotic flow. The mobility of the analytes and the EOF determines the velocity of the analytes. Electrophoretic mobility is the movement of the analyte in the capillary due to applied electric field. Electroosmotic flow is the bulk flow of solution.[24]

$$v = (\mu_e + \mu_{eof})\, E \qquad\qquad (1)$$

where v = velocity of the analyte

μ_e = electrophoretic mobility of the analyte

μ_{eof} = EOF

E = field strength

$$\mu = \frac{qE}{6\pi\eta\, r} \tag{2}$$

where q = charge of the analyte

E = applied field strength (V/cm)

η = viscosity of the mobile phase

r = radius of the analyte (related to the mass)

Electrophoretic mobility results from the equilibrium of frictional and electrical forces. The charge of the analyte and the magnitude of the applied electric field determine the movement of the analyte through a conductive medium either toward or away from an electrode. Hence, the movement and separation depends on the size-charge ratio of the analyte.

Electroosmotic flow results from the surface charge on the inner side of the capillary wall and this leads to the formation of a double-layer when an electric filed is applied. This flow usually occurs as a plug flow rather than laminar flow.
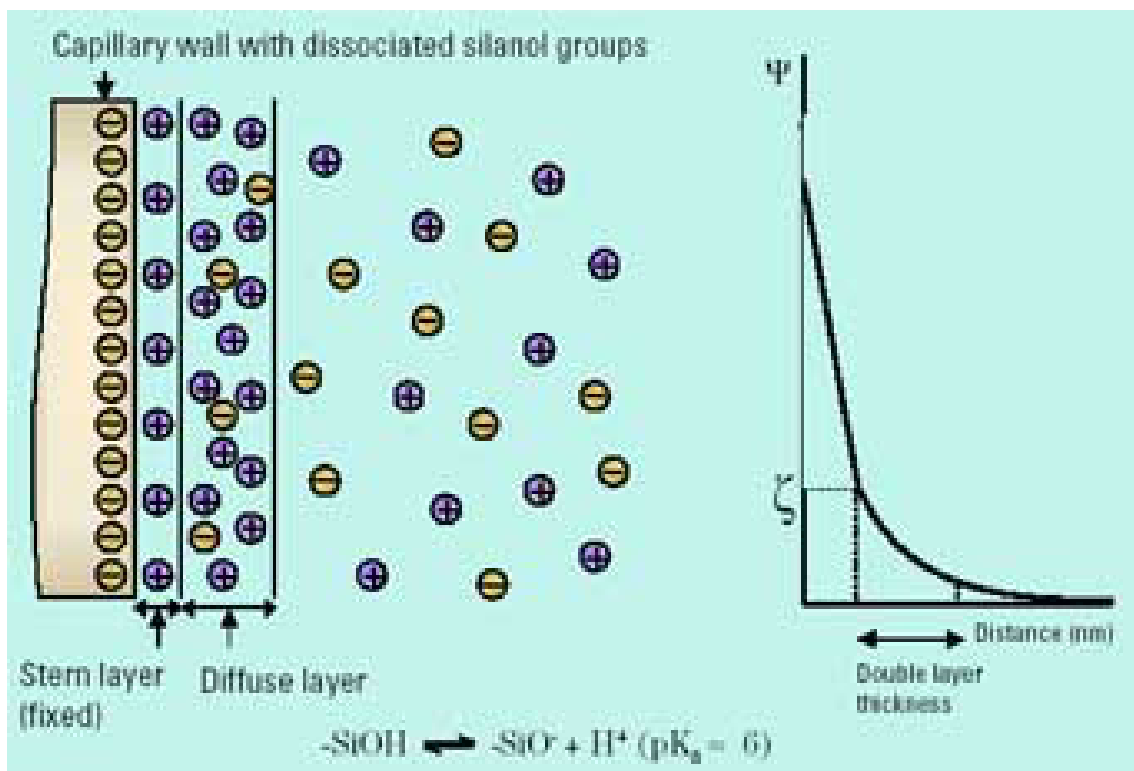
Figure 3: The build up of ions at the capillary wall. [5]

The movement of the analyte is usually less than the magnitude of the EOF and they are moved through the capillary because of EOF. This flow may be too great leading to short separation time at high pHs. The capillary is usually coated in CIEF to reduce EOF and also to minimize protein adsorption. There are various reasons to reduce/eliminate EOF as explained below:

- Cathodic drift of the pH gradient- the EOF may flush out the analytes past the detector before the completion of the focusing.

- Band broadening.

- Non-linearity of the pH gradient- the low pH anolyte can enter the capillary in the presence of EOF. [22]

1.3. CAPILLARY ISOELECTRIC FOCUSING-WHOLE COLUMN IMAGING DETECTION (CIEF-WCID)

In CE experiments, the optimization of the separation requires labour and time.[25] The method requires an additional time for the analysis of the sample after the optimization of the separation conditions. The diffusion of molecules broadens sample zones, which leads to a decrease in the concentration sensitivity and separation efficiency of the method. With this method, the analyte cannot be traced after injection into the column and the progress of the separation cannot be monitored as well.[25]

The analysis of proteins and other peptides using conventional CIEF has displayed some limitations. After the focusing process, the focused sample zones in the separation capillary have to be moved through the detection point located at the end of the capillary column for measurement and record purposes.[14, 26-30] There is non-linearity with this mobilization and the movement is usually done by EOF, hydrodynamic flow or electrophoretic force. This mobilization process results in distortion of the established pH gradient, increase in analysis time (the focusing time is 2-6 mins while the mobilization process takes 10-30 mins), uneven resolution, poor reproducibility, protein precipitation, incomplete pattern detection of the sample at points near the end of capillary and difficulty in the determination of pI values for the analytes from elution time.[14, 26-36]

Due to the difficulties with the mobilization, a single- point detection method becomes a non-ideal detection method for CIEF.[25] Whole column imaging detection (WCID) has

been developed to solve the problems of mobilization process. There have been some whole column detectors that have been developed for CIEF, which include optical absorption imaging detector, universal refractive index gradient imaging detector and fluorescence imaging detector.[14, 27]

WCID is a real imaging detection because it allows the simultaneous detection along the entire length of a column.[25-26] A charge-couple device camera (CCD) images the entire separation column as the analytes are being focused in the capillary.[27] The advantages of WCID over single-point detection include real time detection, dynamic information of the analysis, speed, stability of pH gradient, optimization of separation conditions, high sample throughput, better resolution, better reproducibility compared with other modes of CE, accurate analysis and external factors can be ignored.[9, 14, 25-36] With the above mentioned advantages, CIEF-WCID is a reliable pI-based technique for protein characterization and analysis as it is an equilibrium method, which separates proteins based on the difference in their pIs.[9, 37] The peak positions are used to calculate the pI values of the focused analytes by comparing with a calibration curve obtained by using standard pI markers.[9, 32]

1.3.1 THE SEPARATION CARTRIDGE AND WCID INSTRUMENT

Figure 4: A picture of the separation cartridge

11

Figure 4 above illustrates a picture of the separation cartridge commercialized by Convergent Bioscience Ltd., Toronto, Canada. The side and the top views are shown in Figure 5 below.
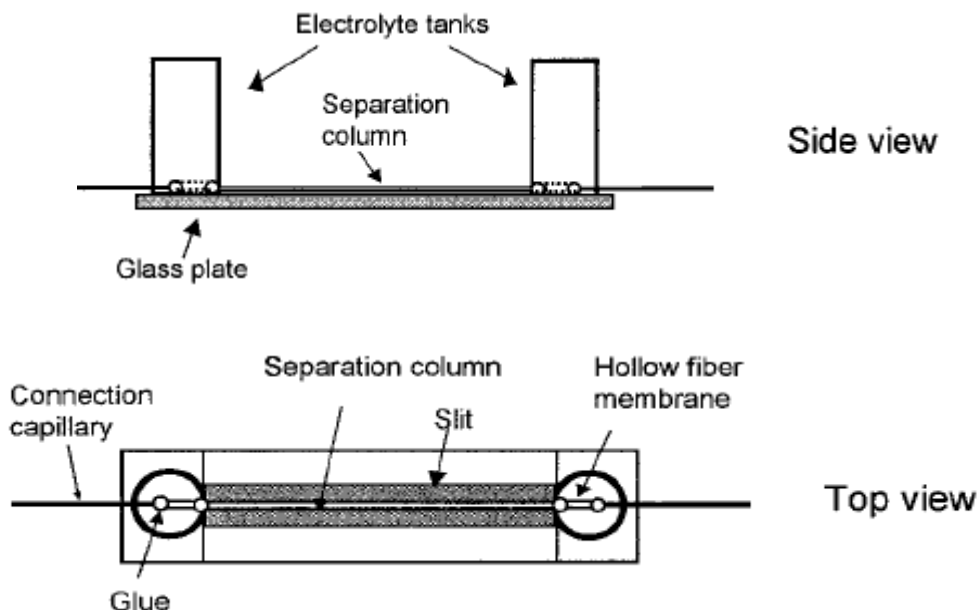
Figure 5: Schematic diagram of side and top views of the capillary cartridge. [32]

An advantage of the separation cartridge is that the sample solutions can be introduced into the separation column without the electrolytes being disturbed.[14] The focusing and the separation usually take about 5 minutes.[14, 25]

The separation channel is usually a short capillary (few cm long). The dimension is a 50 mm long, 100 µm ID X 200 µm OD silica capillary and the outside polyimide coating of the capillary is removed for whole-column detection while the inner wall is coated with either fluorocarbon or polyacrylamide to reduce or eliminate electroosmotic flow. An inlet and outlet capillaries are connected to the separation channel by sections of porous hollow fiber membrane. These junctions provide electric conduction and allow the passage of small ions (such as protons and hydroxyl ions), but confine large molecules

12

inside the capillary. The electrolyte tanks are filled with anolyte (usually 100 mM phosphoric acid) and catholyte (usually 100 mM sodium hydroxide).

The prepared sample solution is injected into the separation channel and the cartridge placed inside the instrument for separation. The volume of sample in the separation channel is 0.4 μL. The sample can be injected into the separation channel manually using a syringe or automatically via commercially available liquid chromatography (LC) autosampler. A 3 kV voltage is applied to the cartridge and the analyte inside the separation channel is focused to a certain pH, which is equal to the pI of the analyte. The focused bands of the analyte are imaged with a CCD camera as shown in Figure 6.
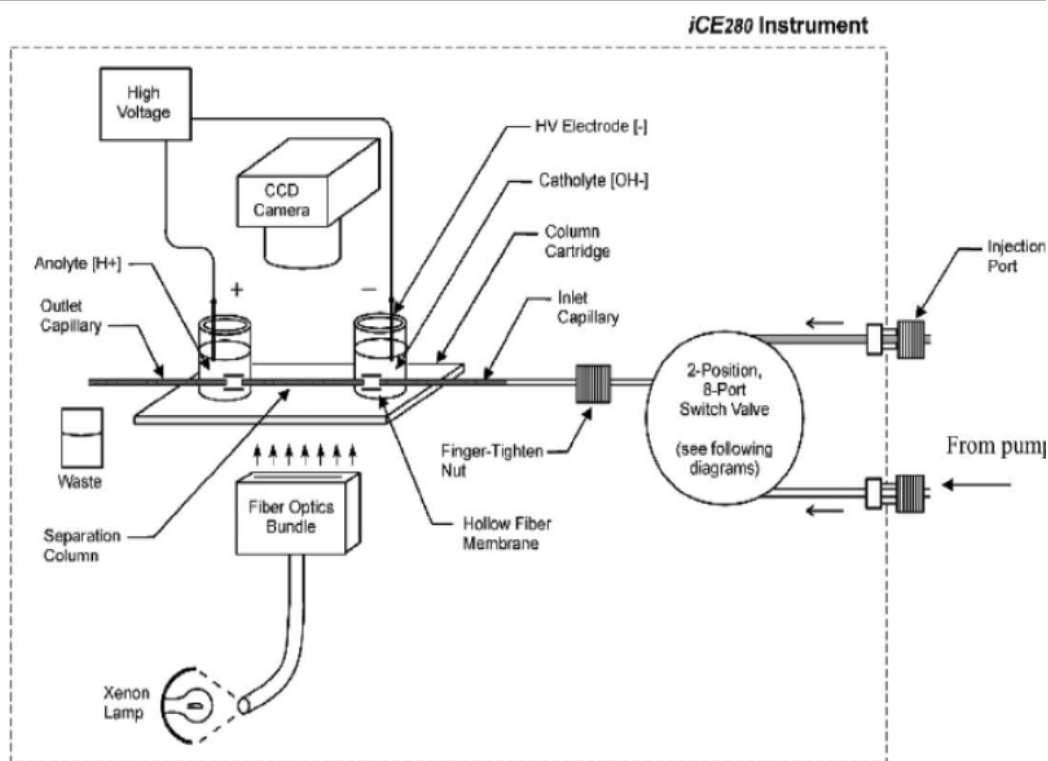
Figure 6: A block diagram of the iCE280 instrument.[14]

The light source in the instrument is a xenon lamp. A bundle of optical fibers and a cylindrical lens from the lamp focuses light beam onto the column cartridge. Since most proteins absorb at wavelength 280 nm, a 280 nm bandpass filter is used in the iCE280 instrument. The whole-column image is captured by a camera and this camera includes an imaging lenses and a CCD sensor. There is possibility of enhancement of signal-to-noise ratio by averaging method with this fast CCD sensor. [26]

A PC computer is used to control the focusing and image detection. There is quantitation software in the iCE280 instrument for rapid processing of the results obtained.

Figure 7 shows the separation of Hemoglobin control (AFSC Hemo control) and two pI markers using CIEF-WCID.
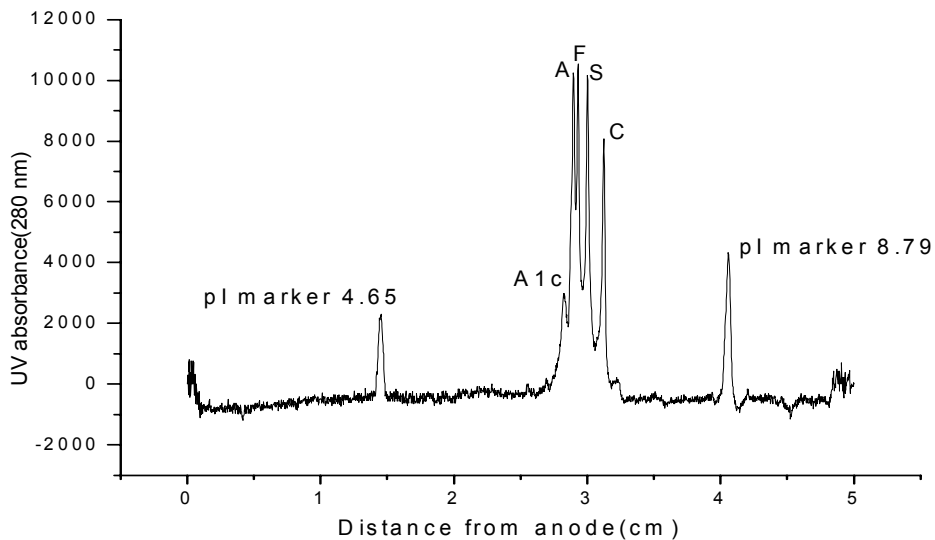
Figure 7: Separation of a mixture of AFSC Hemo control (1 μL) and two pI markers. (Carrier Ampholytes pH 3-10, 1 % PVP to coat the separation channel, Catholyte; 0.1 M H_3PO_4 1 % PVP, Anolyte: 0.1 M NaOH 1 % PVP).

The pI values of the variants were determined using the peak positions (pixel) of the two pI markers and the calculated pI values were 6.96, 7.02,7.13 and 7.32 for A, F, S, C, respectively. The reproducibility of peak positions for the Hemoglobin control mixed with the two pI markers (pIs: 4.65 and 8.79) in four consecutive separations is shown in table 1. The RSD values are within 0.45%.

	pI 4.65	Hemoglobin	pI 8.79
Run	Pixel	Pixel	Pixel
1	595	1200	1661
2	595	1209	1672
3	596	1208	1663
4	598	1212	1675
Average	596	1207.25	1667.75
SD	1.4	5.1	6.8
RSD (%)	0.2	0.4	0.4

Table 1: Six consecutive separations of Hemoglobin control

1.3.2 RESOLVING POWER

The pH range of carrier ampholytes determines the resolution of CIEF. A narrow range pH range of CA enhances the resolution of separation. The resolving power, ΔpI is described by the following equation:

$$\Delta pI = 3\sqrt{\frac{D(dpH/dx)}{E(d\mu/dpH)}} \tag{3}$$

where D = diffusion coefficient

E = field strength

$$\mu = \text{mobility of the analyte}$$

The change in the buffer pH per unit capillary length is represented by d pH/dx and this is an adjustable parameter and can be controlled by an appropriate capillary length and ampholytes pH range.[22]

The equation illustrates that a high-field strength, a low diffusion coefficient, a high mobility slope and a narrow pH range favour good resolution.[22, 32]

1.4 ISOELECTRIC FOCUSING IN A TAPERED CHANNEL

The focusing of analytes in CIEF is usually carried out with the use of carrier ampholytes for the creation of pH gradient. However, there are limitations involved with the use of CA, which include the followings:

- Interaction with the analytes (formation of complexes).

- Elimination in preparative IEF is difficult.

- They are relatively expensive.

- Reduction in the sensitivity of UV detection.

- Ion current increment when CIEF is coupled to MS.

- Purification of proteins can be complex.[38-43]

Due to these limitations, various methods have been developed for the formation of pH gradient, which include changing the temperature of the buffer, electrophoresis of water, steady-state rheoelectrolysis, thermal method, sample autofocusing, changing the dielectric constant and the use of controlled flows of solvolytical ions in a quadrupole electromigration column.[38, 42-44]

Due to the simplicity and capability of forming a relatively stable and well defined pH gradient and ability of pH gradient manipulation during analysis, the thermally generated pH gradient is a promising method among the methods mentioned.[41-43]

The temperature gradient has formed an effective way of creating pH gradient because the method is fast, cheap, short-wavelength UV detection, little interaction and unnecessary changes are not introduced into the system being studied.[44-45] The method is fast as compared to other standard methods of pH gradient formation because of high-temperature conductivity of the buffer solution. The pH gradient is formed immediately the temperature distribution is established.[36]

The initial method of utilizing pH gradient formed in an electrolyte in the presence of a temperature gradient was performed by using a system of two circulating baths at different temperature attached to each end of the separation container. However, there is inconvenience with this method and Joule heating results in instability of the temperatures formed.[39] The need of temperature baths can be eliminated with the use of Joule heating whereby the diameter of the separation channel is narrowed to form the temperature gradient and the resistance of the medium. The amounts of heat generated along the capillary are varied.[38-40]

This method of using thermally generated pH gradient was initially used for the separation of dilute protein samples.[38]

1.4.1 HYPOTHETICAL TEMPERATURE AND pH GRADIENT

An example of hypothetical temperature and pH gradients created in a tapered channel for focused zones is illustrated in Figure 8 below.
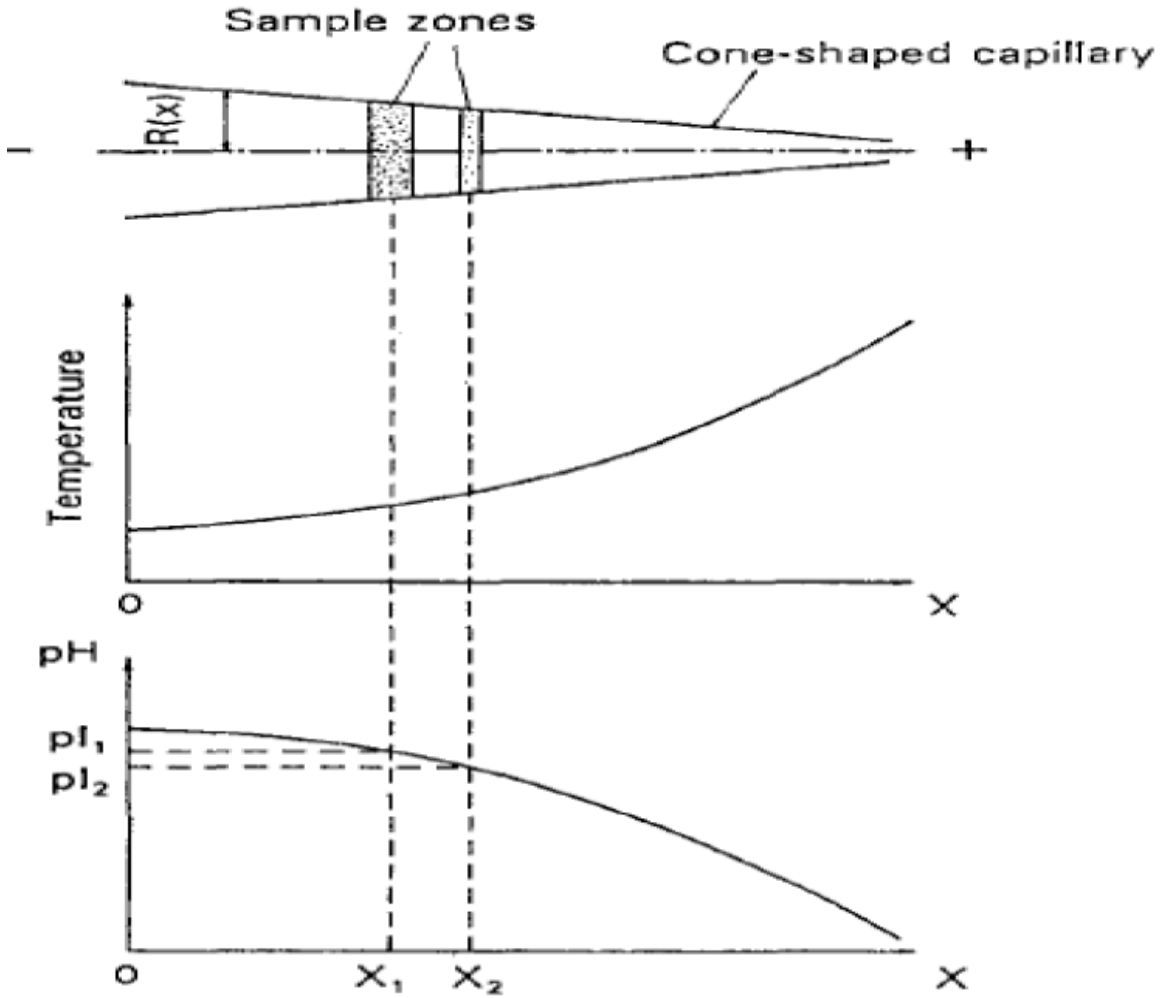
Figure 8: Hypothetical temperature and pH gradients created in a tapered channel.[39]

The amount of heat generated per unit length at the dimension x, dQ(x)/dx is proportional to the electrophoretic current, I, and the magnitude of the electric field along the separation channel at this dimension, E(x) as illustrated in the equation below. [39,42]

$$\frac{dQ}{dx}(x) = E(x)\,I \tag{4}$$

Or

$$\frac{\partial Q}{\partial t} = I^2\,R(x) \tag{5}$$

The electrophoretic current, I, is constant along the capillary axis. This can be calculated by using the equation below. A(x) is the cross sectional area of the separation channel at given dimension x and κ is the electrolyte conductivity.

$$I = \kappa\,A(x)\,E(x) \tag{6}$$

Combining equations 5 and 6, we have:

$$\frac{dQ}{dx}(x) = \frac{I^2}{\kappa A(x)} \tag{7}$$

Equation 8 illustrates the relationship for a tapered channel, where r(x) is the capillary radius at given dimension x

$$\frac{\partial Q}{\partial t} = \frac{I^2}{\kappa \pi r^2(x)} \tag{8}$$

The amount of heat generated in a capillary geometry is in inverse proportion to the square of radius at a given cross section as illustrated in equation 8.

Equation 9 shows the relationship for a tapered channel with uniform channel thickness.

$$\frac{\partial Q}{\partial t} = \frac{I^2}{\kappa d w_{(x)}} \tag{9}$$

where w is the width of that given cross section.

Figure 9 below shows the longitudinal section of an ideal tapered capillary. The outer diameter of the capillary is represented by 'R' and it is constant while the inner diameter

is represented by 'r' and this inner diameter is a linear function of the position along z axis.
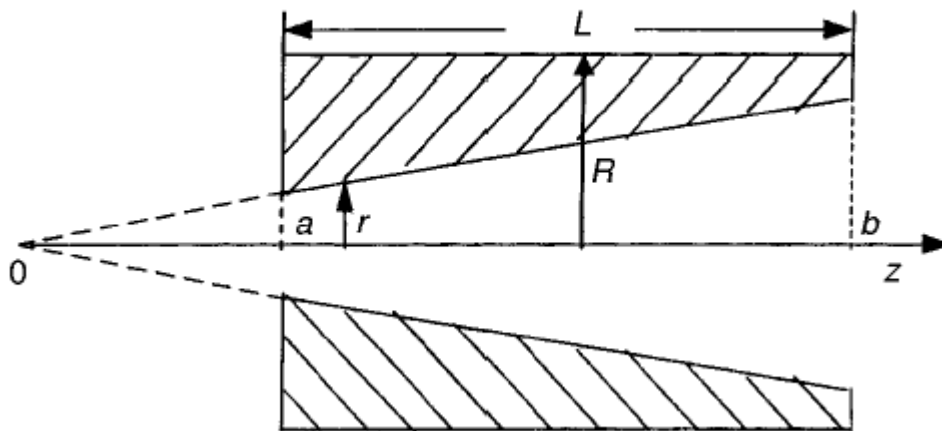
Figure 9: Longitudinal section of a tapered capillary.[44]

For isoelectric focusing in a tapered channel, there are two essential temperature gradients namely the longitudinal temperature gradient and transverse temperature gradient. The combination of the uniform Joule heating inside the channel and the non-uniform heat exchange in the channel walls results in transverse temperature gradient.[45] Complicated spatial distribution of the points where the analytes should be focused may occur because of the combination of both longitudinal and transverse temperature gradients. There may be longitudinal spreading of the focused zones if the isothermal surface is extended. A decrease in resolution may also occur. The optimal temperature region can thus be found by analyzing the temperature distribution in the capillary.[45]

The non-uniformity of EOF and temperature may cause the broadening of the focused zones as shown in Figure 10.

Radial distance

distribution of the isoelectric focusing points due to temperature or electroosmotic flow non-uniformity

capillary wall

capillary axis

analyte concentration

observed shape of focused zone

capillary axis

analyte zones focused at different radial distances
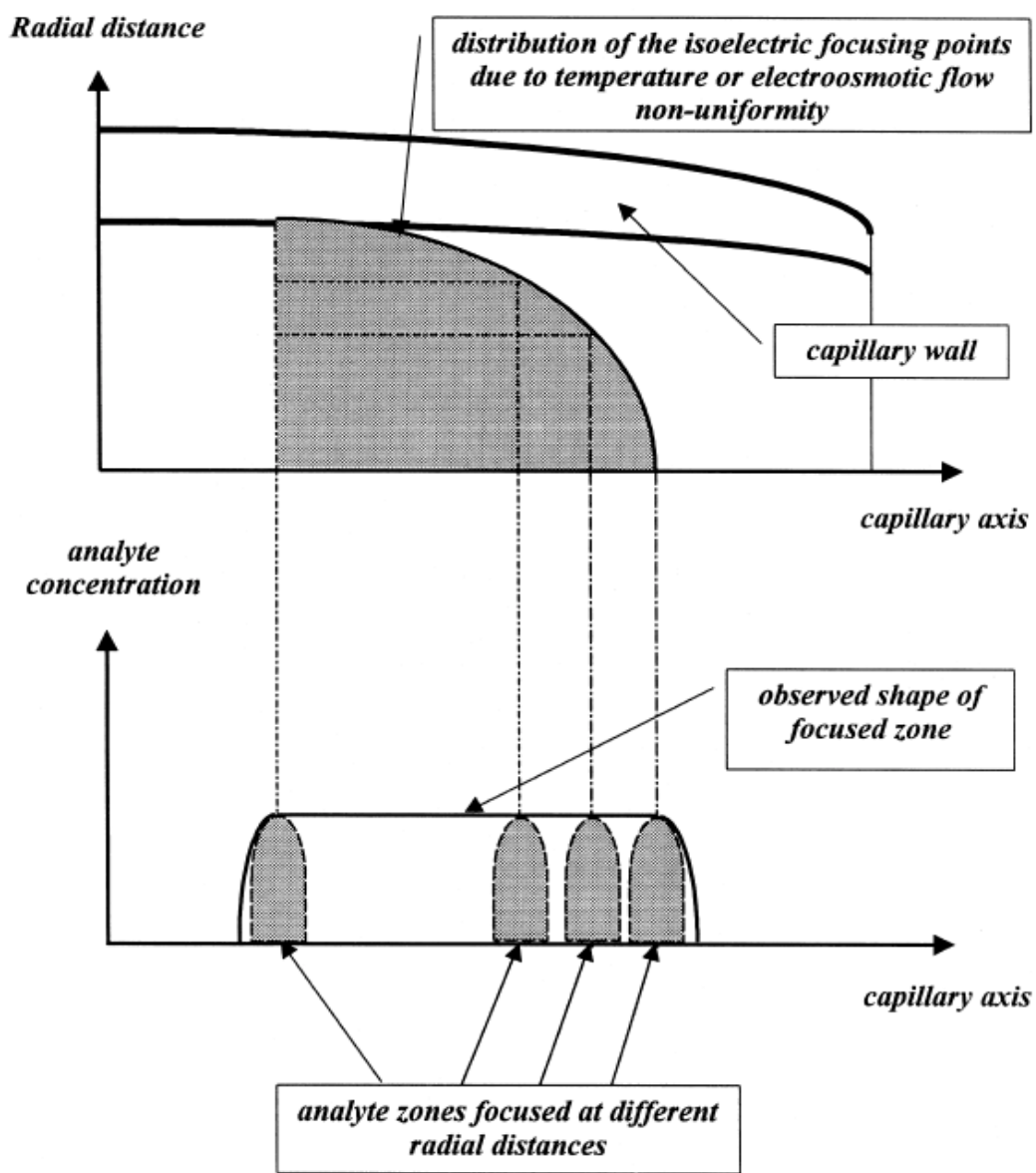
Figure 10: Broadening of focused zones.[45]

1.4.2 PRINCIPLE OF THERMALLY GENERATED pH GRADIENT

The fundamental principle for the generation of pH thermally is that most buffer solutions exhibit a noticeable dependence of pKa on temperature because the temperature coefficient of a buffer depends on its corresponding enthalpy change of ionization. The buffer solution required in a thermally generated pH gradient must have a large dependence of pKa.

Focusing protein in a thermally generated pH gradient requires that the change of the isoelectric point of the protein with temperature should be less than the change of the pH of the buffer solution. [42] The isoelectric point of the target protein usually determines the pH of buffer solution. It should be within the useful pH range of the buffer (pKa ± 1 pH unit).

The most commonly used buffer is Tris, or tris (hydroxymethyl) aminomethane because of its ideal characteristics, which are as follows:

- It is easily dissolved in water.

- It is not hydroscopic.

- It is stable in solution at room temperature for months.

- It is available in high purity.[46]

It has a large dependence of – 0.028 pKa unit per degree at 25 $^{\circ}$C.[42, 44]. This pKa unit of Tris-HCl has an approximately linear relationship with temperature form 0-80 $^{\circ}$C. [41]

The generation of pH gradient is based on the following equilibrium relation.[47]

$$K = \frac{[a_{H+}][a_{Tris}]}{[a_{TrisH+}]} \qquad (10)$$

      where k = equilibrium constant

a_{H+} = proton activity

a_{Tris} = Tris activity

a_{TrisH+} = TrisH+ activity

The dependence of a hydrogen ion activity of a buffer solution with temperature can be expressed logarithmically as shown in equation 11.

$$\frac{\partial pa_H}{\partial T} \approx -\frac{\partial \log K}{\partial T} - (2z+1)\frac{\partial \log \gamma}{\partial T} \tag{11}$$

where γ = activity of coefficient of an average ion of valence z = 1

T = absolute temperature

The first of the two terms of the equation can be obtained from the gas constant (R) and the molar heat of dissociation of an acid (ΔH^o).

$$\frac{\partial lnK}{\partial T} = \frac{\Delta H^o}{RT^2} \tag{12}$$

The second term is usually less than 10 % of the value of the first term for Tris buffer. Therefore, equation 12 can be simplified to equation 13 below:

$$\frac{\partial pa_{H^+}}{\partial T} \approx \frac{\Delta H^o}{RT^2} \tag{13}$$

The change of the pKa of Tris buffer with temperature can be more expressed more accurately by equation14. The corresponding pH gradient can be obtained by knowing the temperature inside the capillary.[44]

$$\log K_a = \frac{2984.1}{T} - 3.5888 + 0.005571T \tag{14}$$

There are different functional groups found in proteins and these groups have different molar heats of dissociation. The functional groups such as carboxyl have small molar heat of dissociation (1 kcal/mole), and thus have a small temperature coefficient while functional groups such as α-amino have large molar heat of dissociation (10 kcal/mole), and thus have large temperature coefficient.[42] Table 2 below shows the values of molar heat of dissociation, ΔH^o that are commonly found in proteins.

Functional group	ΔH^0 (kcal/mol)
Carboxyl	1
Imidazole	7
α-Amino	10
Phenolic	6
ε-Amino	12

Table 2: Molar heats of dissociation.[42]

With these different molar heats of dissociation together with the small and larger temperature coefficients, the isoelectric points of neutral and basic proteins are more sensitive to temperature changes than the isoelectric points of the acidic proteins.[42]

Therefore, a thermally generated pH gradient is expected to be advantageous for focusing acidic and neutral proteins because they have more carboxyl groups in their functional groups. However, basic proteins can be focused by using buffers with low dpH/dT.[44]

In thermally generated pH gradient, a buffer with a substantial large dependence of pKa is needed. Tris-HCl, which has a pKa value of 8.075 at 25 ^{o}C displays an exceptional large dependence of -0.028 pKa unit per degree at 25 ^{o}C. Tris buffer has a large effect of temperature on pH.[40-42, 44]

There are other buffers that have high-temperature coefficients. These buffers are glycylglycine (pK = 8.25, dpH/dT = 20.026 at 25 °C), cholamine chloride (pK = 7.1, dpH/dT = 0.027 at 20 °C), 2-amino-2-methyl-1,3-propanediol (pK = 8.79, dpH/dT = 20.029 at 25 °C). Generally, all these buffers have amino groups as the ionization groups; therefore, they possess large ΔH^o, which makes their dInK/dT larger than other types of buffers.[44]

1.4.3 EFFECT OF TEMPERATURE

An important parameter that determines the Joule heating produced inside the capillary/separation channel and the temperature gradient is the current passing through the capillary/separation channel. The amount of heat generated inside the capillary/separation channel is dependent on current applied and the higher the current, the greater the amount of heat generated inside the capillary/separation channel and the larger the temperature difference it produces.[44]

1.4.4 EFFECT OF THE SHAPE OF THE CHANNEL

The volume of the tapered channel also determines the span of the temperature gradient. There can be changes in the volume because of changes in the length, outer diameter, wide and narrow end inner diameters of the separation channel. These changes will affect the temperature gradient and the smaller the volume, the larger the temperature difference. Therefore, the temperature gradient can be generated using a uniform inside diameter channel while the outside diameter can be varied due to the fact the different amount of heat are lost through the wall of differing thickness.[44]

1.5 MICROFABRICATED CARTRIDGES (MICROFLUIDIC CHIPS)

The surface treatment of a solid material to obtain microstructures is called microfluidic systems (achieved by film deposition, photolithography, etching and bonding). The process is called microfabrication.[48] Microfabrication is a well known photolithographic technique for structures in the micrometer range, such as structures for electrophoresis, flow injection analysis, and a detector cell.[48] The process enables the realization of biomedical instrumentation that is unattainable using conventional manufacturing techniques.[49]

Microfluidic systems are devices in which analyte is transported within miniature channels. Microfluidic chips have found wide application in both genomics and proteomics. These systems have found applications ranging from reactions to separations to analyses.[50] In recent years, microfabricated devices have been widely used for electrophoretic separations because of the growth experienced in the area of micro total analysis, which is also called lab-on-a chip.[51-54] High speed analysis both chemical and biological analyses can be achieved with the use of these relatively small size microfabricated devices.[53, 55]

The first analytical miniaturized device on silicon, a gas analyzer was fabricated more than 25 years ago and it was used to separate a simple mixture in few seconds.[51, 56] Nevertheless, the response of scientific world to this device was none, despite its advantages (minute size and rapid separation capabilities) until 1990, when there was re-emergence of silicon-based analyzers. In recent years, there is a dramatic growth in this area of research.[51, 54]

The success of integrating electrophoresis in planar chips using silicon and glass substrate was demonstrated for the first time in 1992.[51] A wide varieties of analytes (DNA, neurotransmitters, explosives) and the bioassay of clinically relevant compounds have been analysed using CE in the planar format because application of the method to planar chip formats is easy.[54, 57]

Microfabricated devices have two main potential advantages, which are:

- Portability - this is a reflection of their small sizes.

- Disposability – this is a reflection of the low cost that would result from the batch fabrication.[58]

Microfabrication techniques in semiconductor industry form the basis for capillary electrophoresis on microchips. CIEF has been transferred to the microchip format in the recent years and this has reduce sample volumes, cost and analysis time.[59]

Many research have been performed mostly in the area of separation using microfluidic chips fabricated on glass, quartz and polymer substrates.[49] The common substrates for the production of microchips are glass substrates due to their good optical properties, well-developed microfabrication methods and its well-understood surface chemistry.[52] Polymeric materials have got increasing attraction because of their low manufacturing costs. The common polymers used in microfabrication include poly (dimethylsiloxane) (PDMS), poly (methyl methacrylate) (PMMA), poly (ethylene terephthalate) glycol, polycarbonate (PC), polystyrene (PS) and polycycloolefin (PCOC), and also ceramics substrate.[60-62]

There are various detection methods involved with the use of microfabricated devices which include absorbance detection, electrospray mass spectrometry, laser-induced

fluorescence, raman spectroscopy, chemiluminescence, holographic refractive detection, whole column detection and electrochemical detection. [53-56, 58-59] The most popular detection method that has been used for CE chips is LIF because of its superior selectivity, sensitivity and compatibility with the dimensions of the typical chip. [56, 63-67]

1.5.1 ADVANTAGES OF MICROFLUIDIC CHIPS

- Reliable

- Inexpensive

- Versatility in design

- Compatibility

- Portability

- Reduced analysis time

- Low consumption of sample and reagents

- High resolution

- Whole channel detection

- Reduced size of operating system

- Increased separation efficiency

- Reduced production of wastes

- Use of low voltages for highly efficient separation

- Potentiality for parallel operation and integration with other miniaturized devices

- Some studies are difficult in larger-scale devices. [57, 63, 65-78]

1.5.2 APPLICATIONS OF MICROFLUIDIC CHIPS

Microfluidic chips have displayed a great potential in the areas of medicinal chemistry, biology and environmental sciences. Various measurements and different assays can be done using microfluidic chips and these include pH, fluid viscosity, chemical binding coefficients, molecular diffusion coefficients, enzyme reaction kinetics, chemical gradient formation, DNA analysis (this is one of the leading applications), PCR amplification, cell patterning, immunoassays, capillary electrophoresis, isoelectric focusing, flow cytometry, sample injection of proteins for analysis via mass spectrometry, micellar electrokinetic chromatography(MEKC) to analyze biogenic amines, isotachophoresis- combined with raman spectroscopy detection to assay herbicides (paraquat and diquat).[65, 67-68, 77, 79-85]

1.5.3 FABRICATION PROCESS

The technique that is widely used for the fabrication of microfluidic systems is soft lithography. It is an alternative set of techniques for fabricating micro and nanostructures and it was originally developed in the laboratory of Professor George Whitesides at Harvard University, USA. The advantages of these techniques are as follow:

- They are cheap.

- They are fast.

- The process is easy to learn.

- Their applications are straightforward.

- These techniques are accessible to a wide range of users.

- They are compatible with a wide variety of materials and surface chemistry.[86]

These techniques offer an easy, fast and cheap reproduction of micro-sized features; they enable pattern transfer metallic and non-metallic materials on curved or flexible substrate in a few or just a single simple processing step.[87]

Various materials are available for the fabrication of microfluidic chips. Most of the reported microfabrications for CE are based on the use of glass or quartz substrates employing photolithography and chemical etching. Silicon has been used for the fabrication of microfluidic chips but it is opaque in the visible/UV region of the spectrum, which makes it unfit for systems that rely on optical detection. Glass has also been widely used, although it is transparent but the vertical sidewalls are difficult to etch because it is amorphous. The use of glass has proved to be a success when applied to the separation and sequencing of DNA but adsorption can cause a problem when used with proteins.[87] The fabrication processes of these materials (glass and silicon are slow and there is also a need for a clean room for the processes. The sealing of the channels is time consuming and complicated and as well, these materials are fragile and too expensive to be treated as disposables.[88]

Due to the difficulties encountered with silicon and glass, many researchers now use polymers. Polymers exhibit very weak EOF.[89] Multiple channels can be made on a polymeric chip to run multiple CIEF at the same time. They exhibit exceptional clarity, which is good for microscopic observation processes in the microchannels.[56]

The type of material that is used to fabricate the microfluidic device must be put into consideration for the design and development of a functional device. There must be compatibility of the material with the method of detection and interfacing with the user must be easy. There should also be an allowance for the integration of functional

components. The material should not be expensive and if the devices are aimed for large-scale application, it must be compatible with micrometer-scale features and microfabrication method.[89]

Microfluidic chips fabricated with polymers can result in the development of devices from a large variety of substrates, which are suitable for a wide range of separation applications using a single replication master. Polymeric materials are less expensive and less fragile than glass and silicon.[85] Despite these strengths, polymers do present some disadvantages. Their surface chemistry has to be controlled and they are generally incompatible with high temperatures and sometimes, they are incompatible with organic solvents and low molecular weight organic solutes.[84]

Polymeric materials are now the popular materials for the fabrication of microfluidic systems because of the advantages they present over glass, silicon and quartz. The manufacturing processes are simpler and less expensive. In addition, the fabrication processes are faster. They can be tailored to specific applications because they have a range of different physical and chemical properties. These polymeric materials are more rugged mechanically than silicon and glass. This property can be applied where more brittle materials would not work.

In this research, the polymer used is polydimethylsiloxane (PDMS). PDMS displays an exceptional clarity and this makes it an excellent material for microscopic observation of processes in the microchannels.[79]

1.5.4 PDMS (Polydimethylsiloxane)

R= OH or CH$_3$
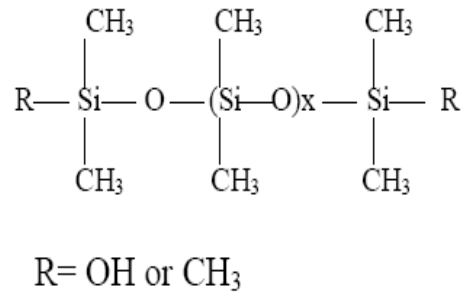
Figure 11: Chemical structure of PDMS.

The most widely used silicon-based organic polymer is PDMS.[90] Soft lithography is the technique used for the fabrication of PDMS devices by using elastomeric polymer molding. This technique is useful as a non-photolithographic technique for pattern replication and it allows rapid prototyping of microfluidic devices.[79] It has been reported that the molding process for polymer systems made of PDMS is the easiest to date among the tested fabrication methods for microstructures.[91]

PDMS is available commercially as a product of high optical quality and it is transparent above ~230 nm.[90, 92] It exhibits a high electrical bulk resistivity (>1015 Ω cm), this prevents the electrical current from flowing through the bulk material. The amount of reflected excitation light in optical detection schemes is reduced due to its relatively low refraction index (n = 1.430). The smoothness of casted PDMS surfaces in combination with its elastomeric properties ensures good adhesion on a variety of clean and flat planar or curved substrates despite its surface free energy is not high (~22 mN/m).[92]

A wide range of temperature (-100 °C to 100 °C) can be withstood by PDMS. It is not a photoresist (i.e. not photo-definable), and its patterning is made by molding methods.[93] PDMS conforms to most materials, hence PDMS devices can easily be integrated with

outside components. Objects such as polyethylene tubing, glass capillaries and sippers can be tightly and easily fitted into the holes made in PDMS by press fitting because of the elasticity of PDMS. Cellular studies can be carried out in PDMS channels because PDMS is non-toxic to proteins and cells and is also gas permeable.[79]

The general physical and chemical properties of PDMS allow its use for electrophoresis experiments using different optical detection methods.[92]

1.5.4.1 ADVANTAGES OF RAPID PROTOTYPING OF PDMS

The use of rapid prototyping of PDMS to create masters has some advantages over the conventional photolithography and micromachining of silicon, which include:

1. Production of the transparencies take less time, they can be produced in hours compared to days or weeks.

2. The production of the transparencies is less expensive than chrome masks.

3. It is easier to develop photoresist in order to create a relief on it.

4. The types of pattern that can be produced are more flexible.[88]

1.5.4.2 SURFACE CHEMISTRY AND SEALING OF PDMS

The chemical structure of PDMS has repeating units of $-O-Si\ (CH_3)_2-$ groups. This chemical structure makes the surface of PDMS to be hydrophobic. Therefore, exposing its surface to oxygen or air plasma makes it to be hydrophilic. This exposure to air plasma introduces silanol ($Si-OH$) groups and destroys the methyl groups ($Si-CH_3$). This procedure is known as plasma treatment or plasma oxidation. After the plasma treatment, the surface has to be kept exposed to water to prevent it from being exposed to air; this is

to avoid surface rearrangements, which may bring new hydrophobic groups to the surface to lower the surface free energy. [91, 90]

The sealing of PDMS channels is much simpler than sealing of channels in glass, silicon, or thermoplastics because it does not require high temperatures, high pressures and high voltages. The channels can be sealed irreversibly to other substrates such as PDMS, glass, silicon, polystyrene and polyethylene by exposing both the surfaces of the substrate and PDMS to air or oxygen plasma. Adding an excess of the monomer to one slab and an excess of the curing agent to the other is another method used to seal two slabs of PDMS irreversibly.[79] The formation of bridging, covalent Siloxane (Si-O-Si) bonds by a condensation reaction between the two PDMS substrates is responsible for the irreversible seal.[88]

1.5.5 BASIC CONCEPTS OF MICROFLUIDIC CHIPS

Microfluidic devices are usually identified by one or more channels with at least one dimension less than 1 mm.[82] Microfluidics is a field that addresses the behavior, precise control and manipulation of microliter and nanoliter volume of fluids that make up microfluidics.[94] Whole blood samples, bacterial cell suspensions, protein or antibody solutions, various buffers are the common fluids used in microfluidic devices.[82]

Laminar flow is the most important characteristic feature of fluid flow in microchannels. [82, 87, 95] This fluid flow is characterized by the Reynolds number, Re, which is defined by the following equation. [82, 87, 94- 96]

$$\mathrm{Re} = \frac{L V_{avg} \rho}{\mu} \qquad (15)$$

where L = the most relevant length scale (the cross-sectional dimension, m).

              V_{avg} = the average velocity of the fluid flow, m/s

              ρ = fluid density

              μ = the viscosity of the fluid

The Re for microchannels is usually much less than 100 (often less than 1.0) because of the small dimension of the channels. There are two common methods for moving fluid through microchannels namely pressure-driven flow, which is also called hydrodynamic flow and electrokinetic flow.[82, 95-96] There is efficient heat dissipation in small channels using electrokinetic flow and also there is flat velocity profiles because of EOF. These advantages lead to sharp peaks and high resolution in CE.[95]

1.6 ISOELECTRIC FOCUSING IN MICROFLUIDIC CHIPS

The advantages displayed by microfluidic chips have led to wide applications, especially in CE. The microchannels are fabricated in microfluidic chips using soft lithography as described earlier in this chapter. The samples are injected into the channels, separated by the application of high voltage and detected by optical detection or other methods.

The separation time usually measured in seconds or minutes are produced by small injection plugs, high fields and short separation lengths.[56] These make the use of microfluidic chips substantially faster than conventional capillary isoelectric focusing.[71]

1.7 CAPILLARY ISOELECTRIC FOCUSING-MASS SPECTROMETRY (CIEF-MS)

Coupling of CIEF with MS is an important technique as it allows the determination of the molecular masses of the analytes (proteins, peptides and others) after the separation of these analytes. The MS provides structural information and this is very useful for the identification of unknown proteins or peptides.[5] For many research, the method of choice for structural characterization of mass- and volume-limited samples is the mass spectrometer because of the advantages it displays. The commonly used detectors in CE are UV- or fluorescence-detectors. However, MS displays unsurpassed advantages, which are:

- Measurement of mass to charge ratio of both parent and fragment ions.

- Potentiality of ion isolation.

- High resolving power.

- Independence of a chromo- or fluorophor.

- Lower limit of detection (LOD) than UV (in most cases).

- Possibility of structural information.

- Easy separation of coeluting peaks using the information of the mass as second dimension.

- Structure elucidation can be done using MS/MS.

- MS/MS can also be used to reduce the chemical noise in order to gain sensitivity.

- The elemental composition of small molecules can be determined by the accurate mass and the isotopic pattern.[97, 98]

MS with its combination with CE has become an efficient instrument for postcolumn analysis of proteins, peptides and small molecules and it results in a powerful, high efficiency, information-rich analytical method. [95, 99]

The quality of the sample analyzed determines the success of protein identification by MS/MS. The level of background contamination can be reduced if care is taken in the sample preparation before mass spectrometric measurement. The type of ionization interface used also determines the sensitivity of the analysis.[100] The interface used in this research was nano-electrospray ionization.

The source of nano-electrospray uses pulled capillaries with a very small spraying orifice (1-2 μm) and a very low flow rate (20-40 nL/min). These feature lead to very small droplet sizes. Nano-electrospray operates at the flow dictated by the electrospray process itself. Due to the very small diameter of the spraying capillary, it is stable at a flow rate that seems to be lower than that, which is possible or practical in a pumped system. There are several desirable analytical properties with small droplets, which include high surface-to volume ratio, this makes most of the analyte molecules available for desorption; a long measurement time at unchanged signal level is provided by the low flow because of the concentration independence of the signal in electrospray. The typical mode of operation of this interface is to load a 0.2-2 μL volume of analyte solutions rather than loading the sample into an injector loop.[101]

In protein analysis, the advantages of this interface include the possibility of long signal averaging to obtain accurate mass measurements in protein mixtures and the possibility of desolvating glycoproteins better.[101] The sensitivity is better than conventional

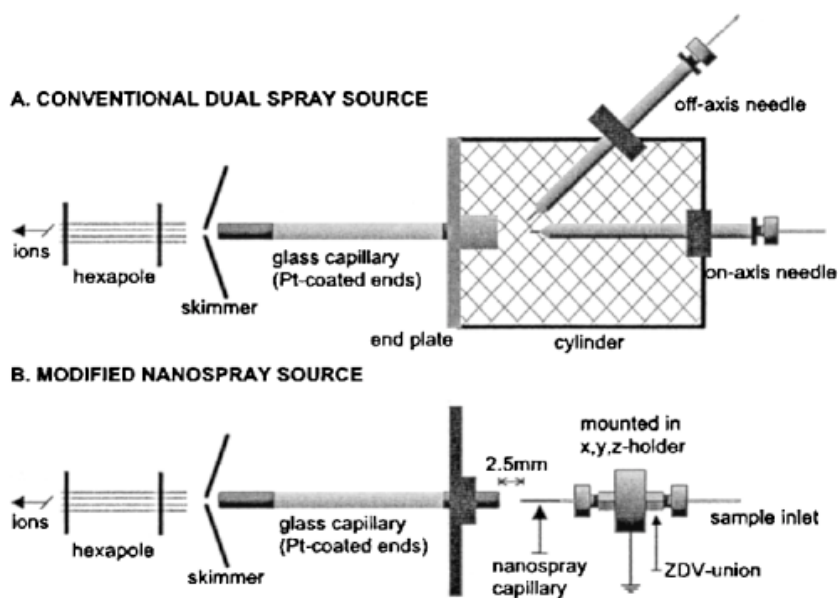electrospray needle because no drying gas is needed and the tip of the interface can be brought close to the inlet of MS.[102]

Figure 12: Electrospray Source. [103]

Figure 13: A picture of the nano-electrospray interface.

1.8 THESIS OBJECTIVE

The main objective of the project is to characterize the fabricated microfluidic chips by performing isoelectric focusing using these chips with whole column imaging detection to enhance the detection of UV-WCID. The commercial cartridge fails to provide plug-injection, which is essential to obtain high efficiency and the cartridge is expensive. Using cross-linked microchannels enhances high efficiency of separation of proteins. The use of chips displays many advantages over commercial cartridge. They are reliable, inexpensive, low sample and reagents are consumed and the analysis time is reduced.

The research focused on the use of tapered channel microfluidic chips for performing IEF using pH gradient generated thermally. Thermally generated pH gradient overcomes the limitations of using carrier ampholytes to create pH gradient for the separation of proteins. The temperature gradient is an effective way of creating pH gradient because the method is fast and cheap compared to other standard methods of pH gradient formation.

The research also focused on the development of compatible cartridge for nano-electrospray. MS is an important technique because it allows the determination of the molecular masses of the analytes after the separation process. The MS provides structural information and this is very useful for the identification of unknown proteins or peptides.

CHAPTER TWO

2.0 EXPERIMENTAL

In this chapter, the materials and the apparatus used for this research were discussed. The experimental procedures followed were also discussed. The sample solutions and the chemicals/reagents were prepared using deionized water and filtered with 0.2 µm Supor membrane (Acrodisc^R Syringe) filters.

2.1 SAMPLES

The samples used for this research work were majorly proteins namely Hemoglobin Ao-Ferrous Stabilized Human, Trypsin inhibitor from soybean, Human serum albumin, Hemoglobin control (AFSC Hemo control), Myoglobin from horse heart and low molecular pI markers 4.65, 8.18, 8.40 and 8.79. The proteins Hemoglobin Ao, Trypsin inhibitor from soybean, Human serum albumin, Myoglobin from horse heart were purchased from Sigma Aldrich, St. Louis, MO, USA. Hemoglobin control was obtained from Helena laboratories, Beaumont, TX, USA. The pI markers were obtained from Convergent Bioscience Ltd, Toronto, Canada. The drug, Warfarin was purchased from Sigma Aldrich, St. Louis, MO, USA.

2.2 REAGENTS

The carrier ampholyte used for the pH gradient formation was purchased from Sigma Aldrich, St. Louis, MO, USA (Pharmalyte pH 3-10). The electrolytes used were Sodium hydroxide (BDH, Toronto, Canada), Phosphoric acid (Sigma Aldrich, St. Louis, MO, USA), Tris (hydroxymethyl aminomethane) from BioRad laboratories, Hercules, CA.

Hydrochloric acid (Fisher Scientific, Nepean, Ontario, Canada) was used to adjust the pH of Tris buffer. Poly vinyl pyrrolidone, PVP was obtained from Sigma Aldrich, St. Louis, MO, USA. Methanol and Acetic acid used for preparing the sample solution for MS experiments were purchased from Fisher Scientific, Nepean, Ontario, Canada and Sigma Aldrich, St. Louis, MO, USA, respectively.

2.3 APPARATUS AND MATERIALS

The sample solutions prepared in the vials were mixed together by placing the vials on vortex (Fisher Vortex Genie 2^{TM}) to ensure that they were homogenously mixed. Mettler Toledo (MP 220) pH meter was used for pH measurement. VWR Scientific Series 400 HPS hot plate was used to heat up the Tris buffer solution for temperature measurement and the temperatures were recorded using Fluke 53II thermometer (Fluke Corporation, USA). Fisher Scientific (StereoMaster) microscope was used for the alignment of metal slits in the development of compatible cartridges for nano-electrospray mass spectrometer.

The separations of the proteins (IEF) were conducted in iCE280 instrument patented by Convergent Bioscience Ltd., Toronto Canada. The mass spectra of these proteins were obtained using nano-electrospray interface (Protana, Odense, Germany) coupled to API 3000 LC/MS/MS System (Applied Biosystems, MDS Sciex, Concord, Ontario, Canada) and kd Scientific syringe pump. The capillary with dimension (320 µm OD and 100 µm ID) used for the development of compatible cartridges for nano-electrospray mass spectrometer was purchased from Restek Corporation, USA. The hollow fiber membrane (Celgard Microporous Hollow Fiber) glued to the ends of the capillary was obtained from

Hoechst Celanese. Two types of glues were used namely Lepage Silicone Sealant (Avon, OH, USA) and Lepage Speedy Set Epoxy Syringe Glue (Henkel Canada, Mississauga, Ontario, Canada).

2.4 EXPERIMENTS

2.4.1 CIEF PROCEDURE

For the CIEF experiments, each of the samples was prepared by dissolving the proteins in 2 % Pharmalyte (pH 3-10) and 1% PVP to the desired concentration. The electrolyte reservoirs were filled with anolyte (0.1M phosphoric acid solution1% PVP) and catholyte (0.1M sodium hydroxide solution 1% PVP). The sample solution was then injected into the separation column of the cartridge and the cartridge was placed inside the iCE280 instrument. The focusing was set at 2 mins at 500 V and 20 mins at 3000 V. A steady state was attained after few minutes and this was indicated by a decrease in the current to a stable value of 3.5 µA. The data were collected at 30 seconds interval to monitor the focusing process.

The separation column was rinsed between analyses with deionized water and 1% PVP for better reproducibility.

2.4.2 FABRICATION OF THE MICROFUIDIC CHIPS

The designs of the chips are shown in figures 14 and 15, straight channel design and tapered channel design respectively. They were developed at the Microfluidic lab, University of Waterloo. The microchips were fabricated in two parts namely: Master production and Replica molding.
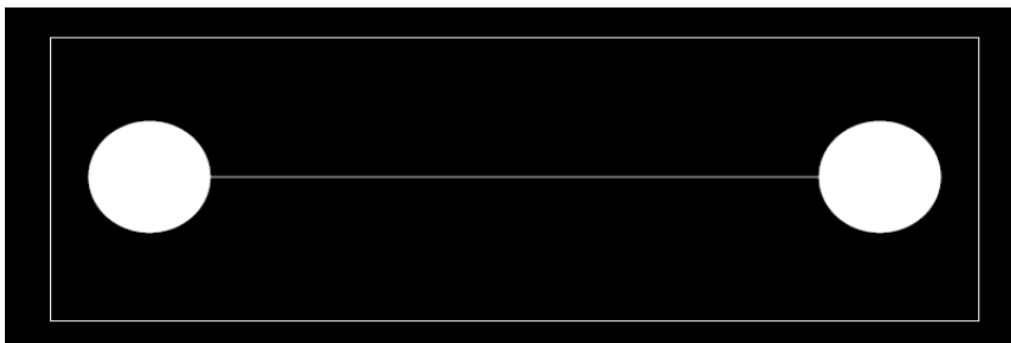
Figure 14: Design 1 (dimension 100 μm wide and 100 μm depth)

Figure 15: Design 2 (dimension 60 μm height, 400 μm narrow end and 4000μm wide end)

Photomasks of both designs were designed by using computer-aided design (CAD) program. The CAD files (the designs) were printed to transparencies using a commercial printer.

For the fabrication of the chip with straight channel design, the polymer, PDMS was weighed and mixed with curing agent in ratio 10:1. The solution was poured on the photomask. It was then placed in plasma cleaner for degassing for 45 seconds and was placed in the oven for about 1 hour to cure (the temperature was set at 80 ℃). The cured PDMS was carefully peeled from the photomask and the design of the chip was cut. The

top and the bottom layers were sealed together by placing the cured PDMS in air plasma for 1minute. The reservoirs were easily created with the aid of a hole puncher.

The straight channel chip was glued to a glass slide for better performance so that it can sit well in the iCE280 instrument. Metal slits were also glued to the chip to allow the UV light to focus only onto the separation channel (to remove stray light).

For the fabrication of the chip with tapered channel design, a masterpiece with the positive relief was made by first cleaning a glass slide in air plasma for 5 minutes and heated on a hot plate for about 8 minutes. The glass slide was spin-coated with photoresist SU-8 at a minimum rpm and was allowed to evaporate at 65 ºC by baking. Using the photomask, the photoresist was exposed to UV-light (365 nm) for 30 seconds, cured at 65ºC for 1 minute and then at 95 ºC for 3 minutes and developed in SU-8 developer for about 2 minutes. The master was taken out, rinsed with water and placed under an electric arc light for about 1 hour to make it hardened. For the replica molding, PDMS was weighed and mixed with curing agent (10:1). The mixture was degassed by placing it under slight vacuum for 30 minutes, poured over the master and cured for 3 hours under vacuum. The PDMS replica was then peeled from the master. The replica and a new glass slide were washed with ethanol, dried in a stream of argon and the surfaces cleaned in air plasma for 5 minutes. Finally, the PDMS was sealed to the glass slide and the reservoirs created with the aid of a hole puncher.

2.4.3 ISOELECTRIC FOCUSING (IEF) USING MICROFLUIDIC CHIPS

The same procedure was followed for performing IEF in straight channel chip.

For thermally generated pH gradient experiments, the relationship between the temperature gradient and the pH of the specific Tris-HCl buffer (pH 7.5) was determined. Each of the samples used was prepared by dissolving the proteins in 25 mM Tris- HCl (pH 7.5) to the desired concentration. The channel of the chip was preconditioned by first rinsing with deionized water several times and then coating it with 0.5% PVP to prevent the absorption of analyte onto the PDMS channel and afterwards rinsed with 25 mM Tris-HCl buffer solution (pH 6.86). The sample was placed at the wider end of the chip and a syringe was used to pressure-drive the sample into the channel. Two pieces of plastic tubings were cut and fixed into the two small holes on the chip. The buffer solution, 25 mM Tris- HCl (pH 6.86) was placed in the electrolyte reservoirs. The chip was then placed in the iCE280instrument for IEF. The voltage range was set at 500-1500 V and the data collected at 20 seconds interval to monitor the focusing.

2.4.5 COMPATIBLE CARTRIDGE FOR NANO-ELECTROSPRAY MASS SPECTROMETER

Compatible cartridges for nano-electrospray mass spectrometer were developed. These developed cartridges were prototypes of commercial cartridges for CIEF. The commercial cartridges are not compatible with the nano-electrospray interface due to the dimension of the capillary; the outer diameter (OD) is 200 μm and a larger OD in needed for the nano-electrospray interface.

The cartridges were built using capillary with dimension 320 µm OD and 100 µm ID (internal diameter). Two pieces of hollow fiber membrane (pore size 0.03 µm) were glued to ends of the capillary to separate the electrolytes from the sample and also to allow the passage of ions.

2.4.5.1 NANO-ELECTROSPRAY MASS SPECTROMETRY EXPERIMENTS

Each of the samples was prepared by dissolving in methanol, deionized water, acetic acid (50:49:1). The sample solution was injected into the built cartridge using syringe pump (flow rate 0.1 ml/h) and sprayed into the API 3000 LC/MS/MS System using the nano-electrospray interface. The mass spectra were detected and displayed on a PC interface of the instrument using software named Protana.

For the mass measurement of the samples separated inside the tapered channel chip, each of the sample was dissolved in methanol, deionized water, acetic acid (50:49:1). Capillaries were connected to both ends of the channel and the sample solution injected into one end of the capillary using syringe pump (flow rate 0.1 ml/h) and the other end capillary was connected to the API 3000 LC/MS/MS System using the nano-electrospray interface. The mass spectra of these proteins were detected and displayed on a PC interface of the instrument using software named Protana.

2.4.6. PROTEIN-DRUG INTERACTION EXPERIMENTS

The fabricated chips were used to investigate the interaction between human albumin serum, HSA and a drug named Warfarin. The concentrations of both HSA and Warfarin were 0.5 mM. The sample solution was prepared by dissolving the HSA and Warfarin in phosphate buffer (pH 7.3) and the sample mixture was dissolved in a 2 % Pharmalyte (pH 3-10) and 1% PVP to the desired concentration.

The same procedure for the CIEF experiments was followed.

CHAPTER THREE

3.0 RESULTS AND DISCUSSION

3.1 CIEF WITH WCID

WCID is the ideal detection method for using microfluidic chips to perform isoelectric focusing because of the advantages it displays over other detection methods. Most CE instruments and electrophoretic systems using microfluidic chips for the separation and analysis of proteins and other samples are usually equipped with a single-point, on column optical detection either UV/Vis absorption detection or fluorescence detection. With single-point detection, all the focused bands of the analytes have to be mobilized to pass the detection point, which creates another step after the focusing process. With such step, there would be distortion of the pH gradient, which results in poor reproducibility, poor resolution and the analysis time will increase. The use of uncoated capillaries or channels is an alternative for performing IEF whereby the mobilization starts during focusing but the problem with this method is that the samples may be eluted before they are focused and there may be adsorption of the samples to the surface of an uncoated capillary or separation channel. [81, 104, 105]

The most commonly used detection method for performing isoelectric focusing in microfluidic chip is laser induced fluorescence, LIF. Although, IEF using chip can be easily coupled with LIF, it is very sensitive to detect small quantity of samples. However, LIF has got some disadvantages, which include requirement of large and expensive lasers and optic systems and there is a need for derivatization because most samples are not fluorescent at usable wavelength. Mass spectrometer is another promising detector because it provides information about the molecular weight and fragmentation patterns of

samples but the size of the instrument is large, the cost is high and there is a need for high level technical expertise as well. Another promising detection method is electrochemistry but it has low sensitivity. Conductivity detection method is also widely used because it is widely applicable to a variety of analytical circumstances but there is a lack of selectivity with this method of detection.[97, 104, 106]

Performing IEF with microfluidic chips using WCID is very promising due to its advantages and there is no need for mobilization. The whole channel can be imaged by the imaging sensor CCD. WCID is a real time mode detection method, due to this, the dynamic focusing of the samples can be monitored in real time and there is also the provision of valuable information for fundamental investigations of the samples being analyzed. [81, 104] There is no need for labeling with WCID as other methods because it uses a simple procedure. Most times, the fabrication of a CE-chip system is complex but the complexity of the fabrication is reduced with WCID.[87]

Other advantages of WCID over single-point detection include speed, stability of pH gradient, high sample throughput, better resolution, better reproducibility compared with other modes of CE, accurate analysis and external factors can be ignored. There is also an opportunity for the optimization of separation conditions.[9, 14, 25-36]

WCID is equipped with simple optics as compared with other detection methods. The entire separation channel can be easily imaged without the need for mobilizing the peaks or scanning optics over the entire separation channel. This leads to faster and more sensitive analysis.[107]

Most of the polymers that are suitable for microfabrication have surfaces that are not inert to biological samples whereby attractions of the analytes to the surface by impurities or

functional group takes place. There is always loss of sample, low separation efficiency and analytical irreproducibility due to this problem.[60]

The samples are usually small with use of microfluidic chips; therefore, the ratio of the volume of the sample to the surface area shrinks significantly. The surface properties are very important because of shrinkage. This makes surface modification very important and it plays the following roles:

- Controls the functional mechanics of the chips.

- Keeps analytes from sticking to the surface of the separation channel.

- Better detection of analytes.[79, 97]

Therefore, the conditioning of the channel surface is very important in IEF so that EOF will be eliminated and also to reduce the interaction between the sample and the surface of the separation channel. There may be loss of resolution in the separation if there is interaction between the sample and the surface of the separation channel. EOF is the bulk flow of movement through the system and results from the surface charge on the inner side of the capillary wall. The sample solution can be flushed from the capillary or separation channel before the completion of the focusing. Two kinds of surface modifications are available to reduce or eliminate EOF namely chemical modification and dynamic coating. Hydrophilic molecules are chemically bound to the surface of the separation channel in chemical modification while surfactants additives are added to the sample solution in dynamic coating. The additives are dynamically bound to the surface of the separation channel.[4, 38] The additive used in this research was PVP (0.5 % and 1 % concentrations).

The fabricated microfluidic chips were used for the separation of Hemoglobin Ao-Ferrous Stabilized Human with a concentration of 0.1 mg/ml mixed with two pI markers (pIs 4.65 and 8.18). The following figure shows the electrophoregram obtained using WCID.
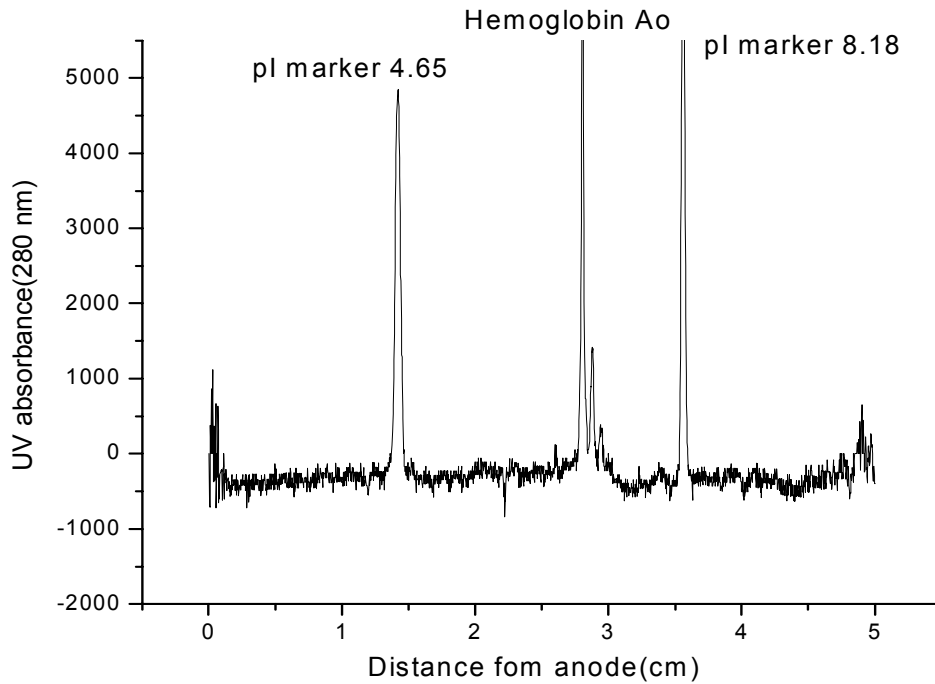

Figure 16: Separation of Hemoglobin Ao-Ferrous Stabilized Human (0.1 mg/mL) mixed with two pI markers (4.65 and 8.18) using microfluidic chip (100 μm by 100 μm).

Table 3 shows the reproducibility of peak positions for Hemoglobin Ao-Ferrous Stabilized Human (0.1 mg/mL) mixed with two pI markers (pIs: 4.65 and 8.18) in six consecutive separations. The RSD values are within 1.6%.

Table 3: Peak positions (pixel) of Hemoglobin Ao-Ferrous Stabilized Human in six consecutive separations using microfluidic chip

	pI 4.65	Hemoglobin	pI 8.18
Run	Pixel	Pixel	Pixel
1	552	1132	1441
2	567	1146	1458
3	569	1139	1449
4	570	1155	1468
5	573	1162	1478
6	579	1186	1498
Average	568.3	1153.3	1465.3
SD	8.2	17.6	18.9
RSD (%)	1.5	1.5	1.3

The data in table 3 show that the separation is reproducible. The pI markers were used to determine the pI of the sample- Hemoglobin Ao and the pI is 7.2.

The same sample solution was separated using the commercial cartridge and the following figure 17 illustrates the electrophoregram obtained using WCID.
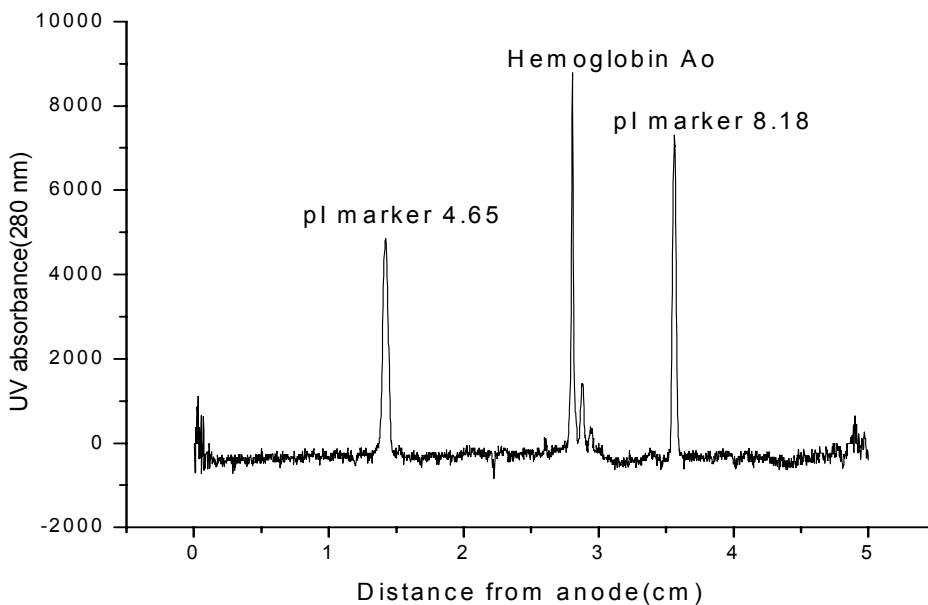

Figure 17: Separation of Hemoglobin Ao-Ferrous Stabilized Human (0.1 mg/mL) mixed with two pI markers (4.65 and 8.18) using commercial cartridge.

Table 4 shows the reproducibility of peak positions for Hemoglobin Ao-Ferrous Stabilized Human (0.1 mg/mL) mixed with two pI markers (pIs: 4.65 and 8.18) in six consecutive separations. The RSD values are within 3.0%.

Table 4: Peak positions (pixel) of Hemoglobin Ao-Ferrous Stabilized Human in six consecutive separations using commercial cartridge

	pI 4.65	Hemoglobin	pI 8.18
Run	Pixel	Pixel	Pixel
1	574	1168	1479
2	580	1187	1499
3	582	1149	1458
4	587	1174	1491
5	609	1176	1486
6	617	1206	1524
Average	591.5	1176.7	1489.5
SD	17.3	19.1	21.9
RSD (%)	2.9	1.6	1.5

The pI markers were used to determine the pI of the sample- Hemoglobin Ao and the pI is 7.0.

With data shown in tables 3 and 4, the results obtained using both microfluidic chips and commercial cartridge are comparable. The pI value obtained using microfluidic chips is close to the value obtained using commercial cartridge. The separation is reproducible with a microfluidic chip, which is an important parameter in IEF. There is improvement in the resolution due to the shortness of the separation channel because a very short plug of sample is needed to improve resolution using microfluidic chips. The separation efficiency is increased with the coating of the microchannel with PVP and also its

addition to the sample solution. Analytical irreproducibility is also reduced with PVP. Metal slits were glued to remove stray light to obtain better sensitivity.

The use of microfluidic chips is cost effective as the commercial cartridge is expensive The fabrication process is simple and fast as well and the consumption of samples and reagents is low compared with the use of commercial cartridges.

3.2 THERMALLY GENERATED pH GRADIENT

Tris buffer was chosen for the generation of the pH gradient because it has a substantial temperature coefficient of pH (dpH/dT is -0.028 K^{-1} at 25 ^{o}C), which allows the target analytes to be concentrated and separated in the separation channel along the pH gradient based on their isoelectric points. The pH of the chosen buffer solution must be slightly higher than the pI of the analyte and it is usually determined by the isoelectric point of the target proteins. The pH must be within the useful pH range of the buffer

($pK_a \pm 1$ pH unit).[39, 40, 42] The chosen analytes in this research were Myoglobin from horse heart (pI 7.35), Hemoglobin Ao- Ferrous Stabilized Human (pIs 7.0 and 7.2) and Trypsin inhibitor from soybean (pI 4.6) and the pH of the buffer solution was 7.5. The pH of the electrolyte was ensured that it was lower than the pI of the protein sample to prevent protein loss from the separation channel before the establishment of the pH gradient. [42]

The relationship between temperature gradient and the pH of the specific Tris-HCl buffer (pH 7.5) was determined. A calibration curve was obtained as illustrated in figure 18.
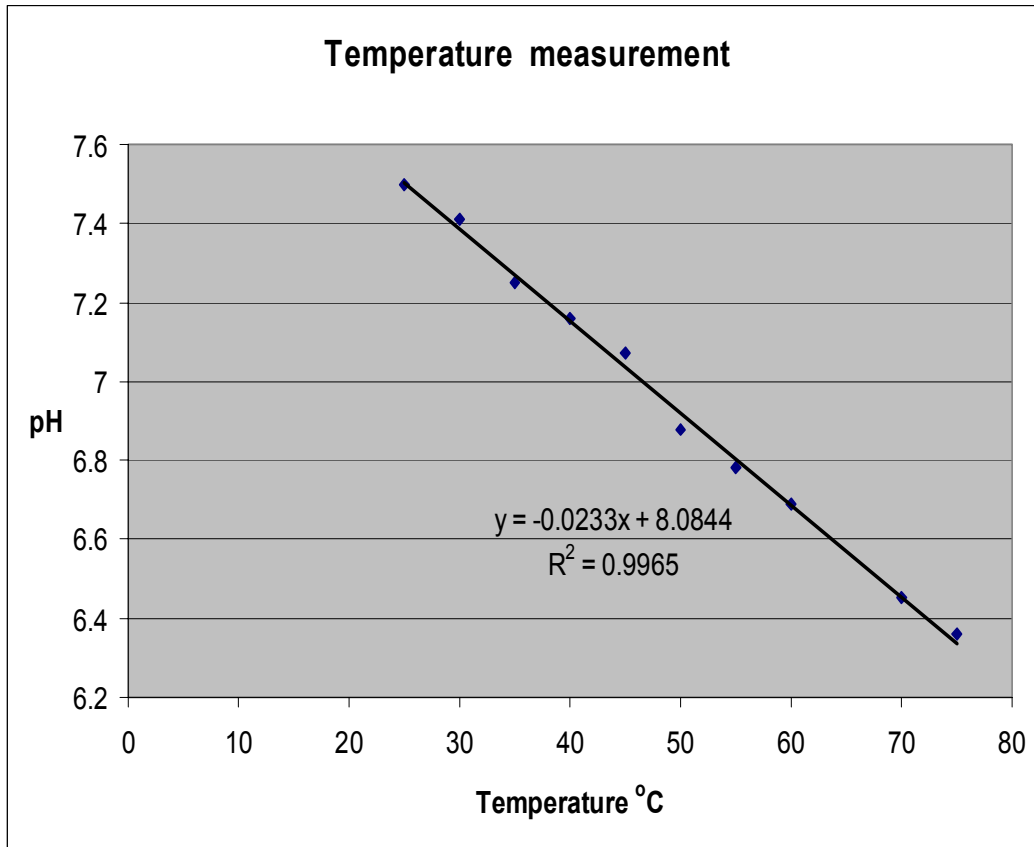
55

Figure 18: Linear relationship between the pH of the buffer (25 mM Tris-HCl, pH 7.5) and temperature

A corresponding pH gradient was produced inside the tapered channel by the generated thermal gradient. An effective way to establish a stable pH gradient in a short time is the use of a temperature gradient to create a thermally generated pH gradient. [44]

Figures 19, 20, 21 and 22 show the electrophoregrams obtained from the separation of proteins in thermally generated pH gradient using microfluidic chips. The peaks of these proteins appeared at 26 seconds of the separation time, which displays one of the advantages of microfluidic chips (fast analysis). Heat dissipation allows the application of high voltage and rapid focusing of analytes because of the separation channel (tapered shape). The amount of heat generated inside the uniform thickness channel is inversely

proportional to the width of that cross section upon the application of an electric field as explained in Chapter 1. The amount of heat generated at the narrower cross section is more than the other end. The temperature increases toward the narrow end of the channel when Tris-HCl buffer is used and this results in the formation of a lower pH gradient at the narrower end of the channel (the current density of the narrow end is higher than it is in the wider end of the channel). The temperature increase is because of Joule heating effect from the applied electric field. [38, 42, 108]
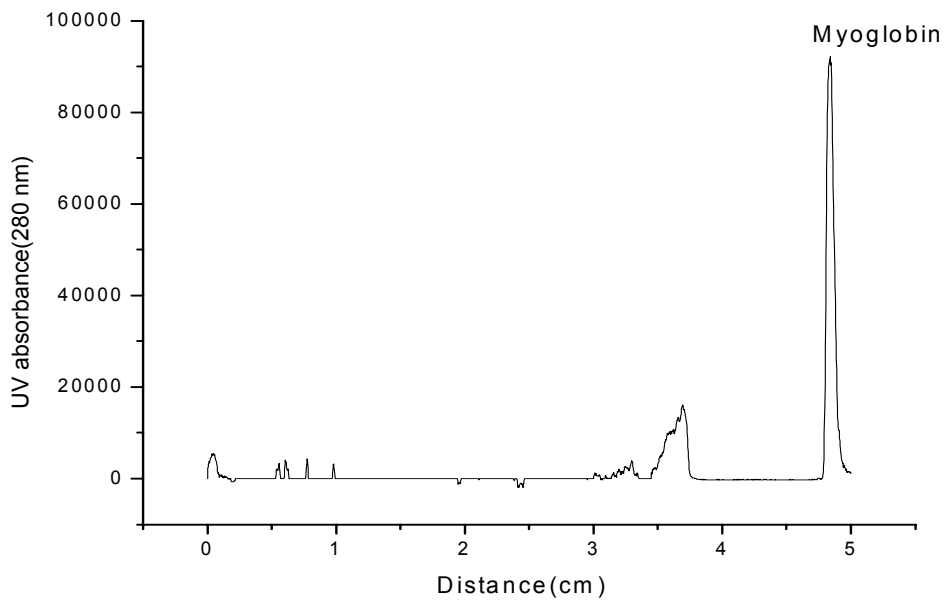

Figure 19: Separation of Myoglobin (0.1 mg/mL) in a thermally generated gradient pH using microfluidic chips.

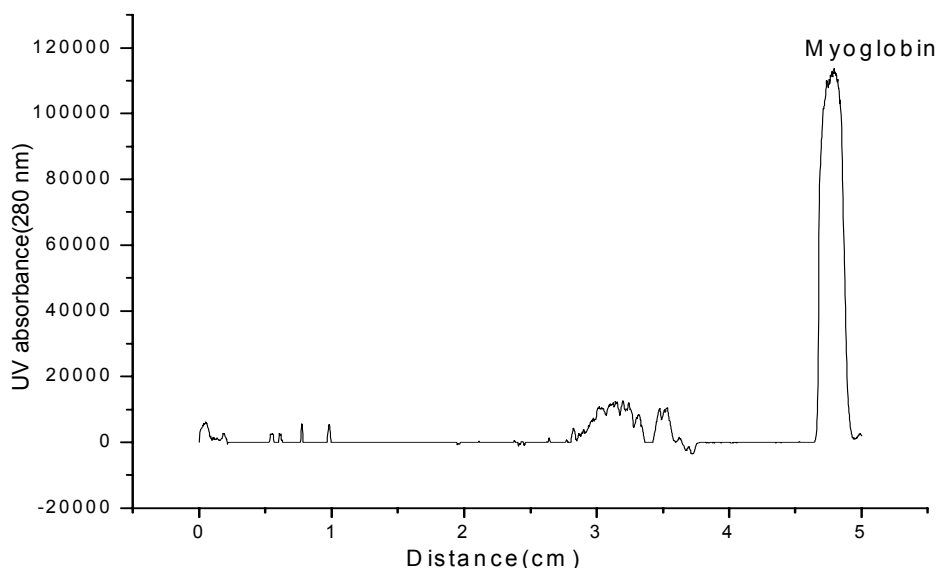The peak becomes broadened after 5 mins of separation as shown in figure 20.

Figure 20: Separation of Myoglobin (0.1 mg/mL) in a thermally generated gradient pH using microfluidic chips after 5 mins of separation.

The broadening of the focused band might be due to EOF. The wall properties of a tapered channel are non-uniform and the EOF, which has a uniform profile leads to change in the flow rate with distance along the channel axis. A non-uniform velocity distribution results due to this change and can lead to focused zone broadening.[45]

Figures 21 and 22 illustrate the separations of Hemoglobin Ao- Ferrous Stabilized Human and Trypsin inhibitor from soybean, respectively.
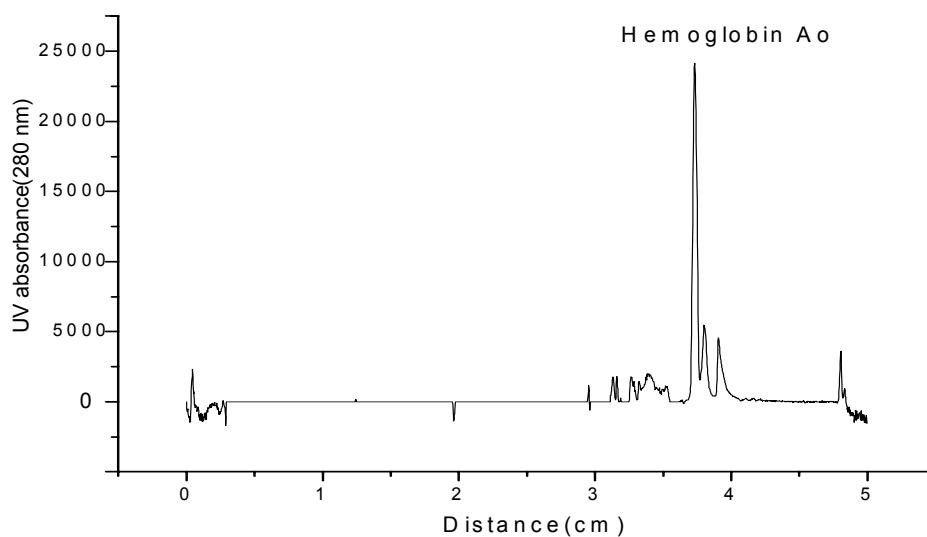
Figure 21: Separation of Hemoglobin Ao- Ferrous Stabilized Human (0.1 mg/mL) in a thermally generated gradient pH using microfluidic chips.

The protein, Hemoglobin Ao- Ferrous Stabilized Human consists of two major isoforms namely methemoglobin (75 %) and oxyhemoglobin (25 %). The Methemoglobin (pI 7.20) is balanced primarily with oxyhemoglobin (pI 7.0)
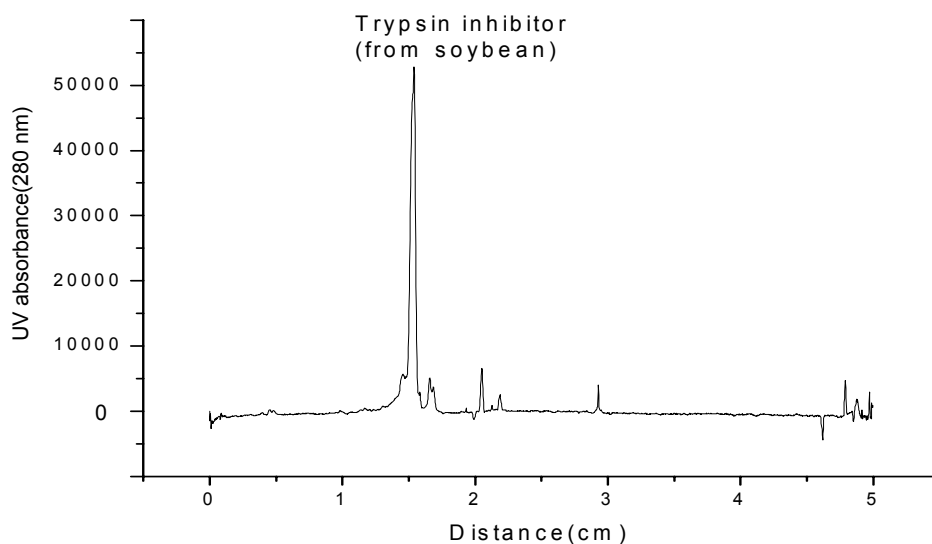
Figure 22: Separation of Trypsin inhibitor from soybean, 0.2 mg/mL (pI 4.6) in a thermally generated gradient pH using microfluidic chips.

3.3 IEF-MS

MS is very useful because it can give the structural information about the separated proteins using isoelectric focusing. MS detector simplifies the mass spectrum of proteins and as well facilitates the identification of these proteins.[38, 109]

The developed compatible cartridges for nano-electrospray mass spectrometry were used to carry out CIEF experiments using pI markers and Hemoglobin Ao- Ferrous Stabilized Human. The electrophoregrams obtained from the CIEF experiments are similar to the ones obtained the commercial cartridges. These electrophoregrams are shown in figures 23 and 24.
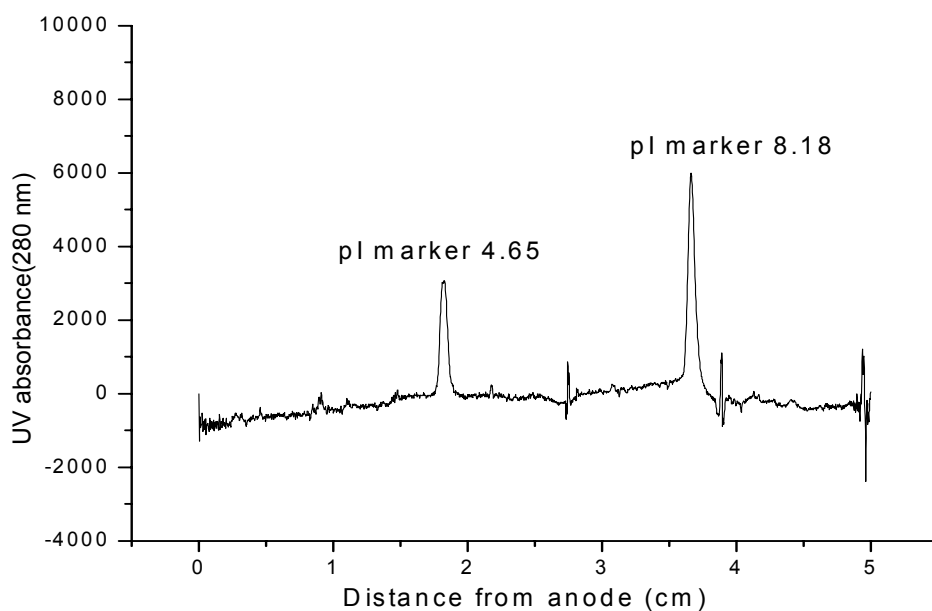
Figure 23: Separation of two pI markers (pI 4.65 and pI 8.18) in a developed cartridge for nano-electrospray mass spectrometry.
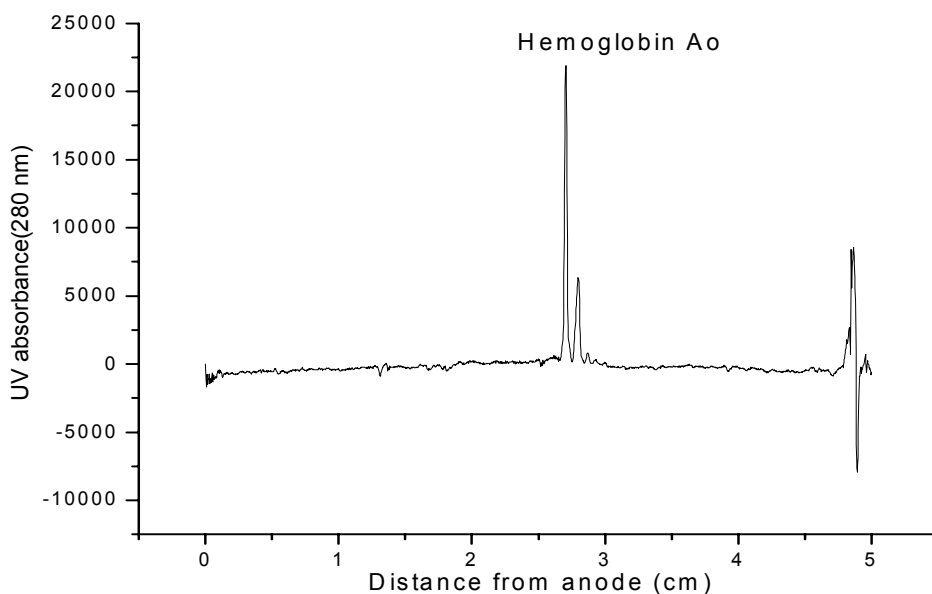

Figure 24: Separation of Hemoglobin Ao- Ferrous Stabilized Human (0.1 mg/mL) in a developed cartridge for nano-electrospray mass spectrometry.

The spike observed on the peak of Hemoglobin Ao- Ferrous Stabilized Human is because of protein precipitation.[28]

The developed cartridges were used to inject samples into the API 3000 MS by connecting the cartridge to the MS using the nano-electrospray interface. The sample solutions were dispersed by the nano-electrospray with electrostatic without nebulizer gas and solvent pump into the mass spectrometer to obtain their mass to charge ratios. The mass spectra shown in figures 25 and 26 were the spectra observed for Hemoglobin Ao and Myoglobin
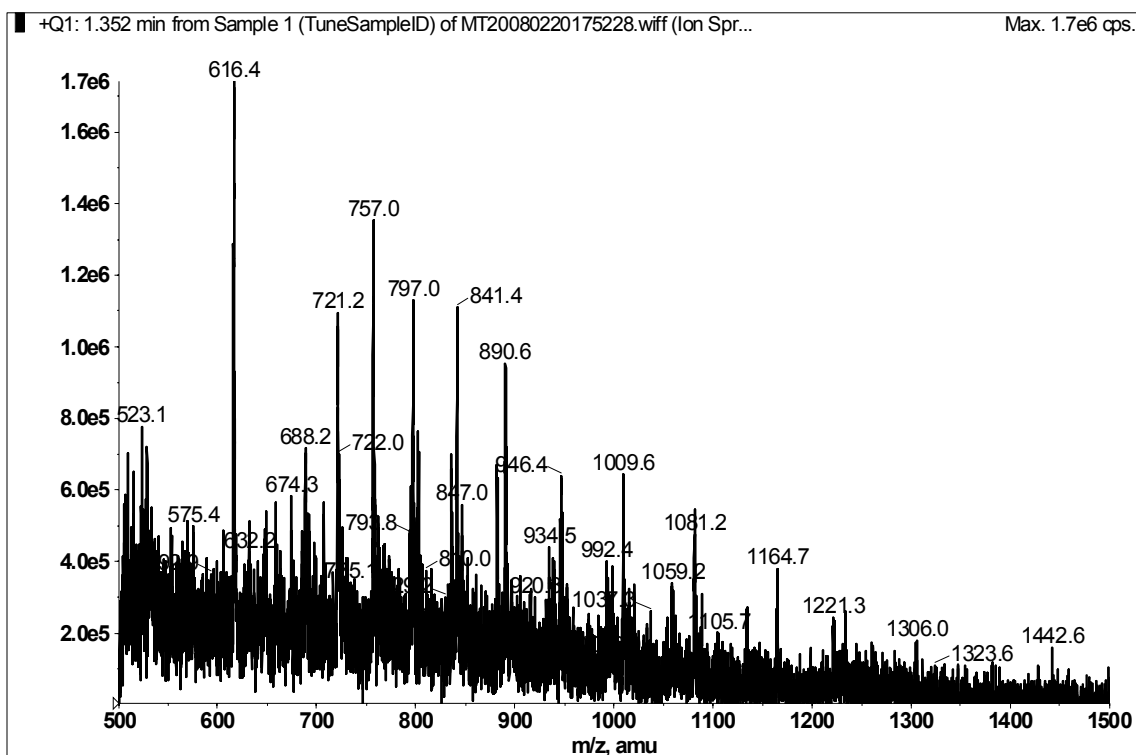

Figure 25: Mass spectrum of Hemoglobin Ao- Ferrous Stabilized Human using the developed cartridge for nano-electrospray mass spectrometry.
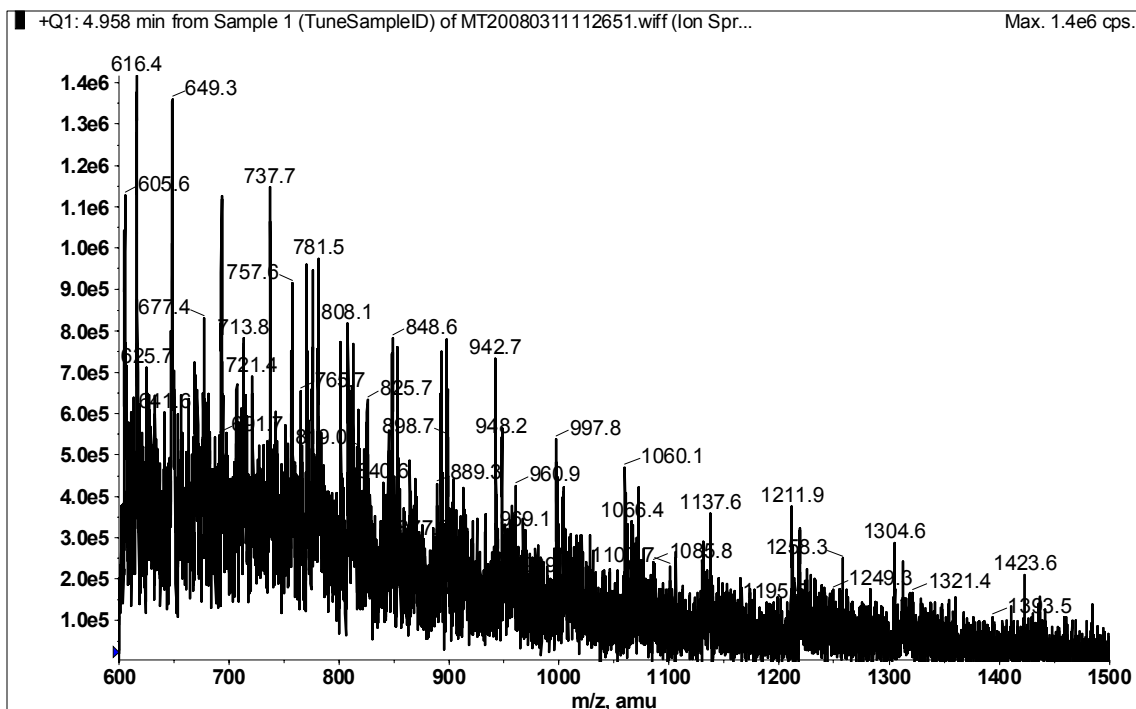
Figure 26: Mass spectrum of Myoglobin using the developed cartridge for nano-electrospray mass spectrometry.

The coupling of microfluidic chips (with tapered channel design) to MS looks promising because the separation of proteins using tapered channel are performed without the use of carrier ampholytes. The performance of MS is affected with the presence of CA because there may be contamination with the ionization source if they are present in sample solution and the analyte signal intensity and the resolution can be reduced.[110]

The proteins separated in the microfluidic chips using the thermally generated pH gradient were injected into the nano-electrospray mass spectrometer to obtain their molecular masses. The mass spectra obtained are shown in figures 27, 28 and 29.
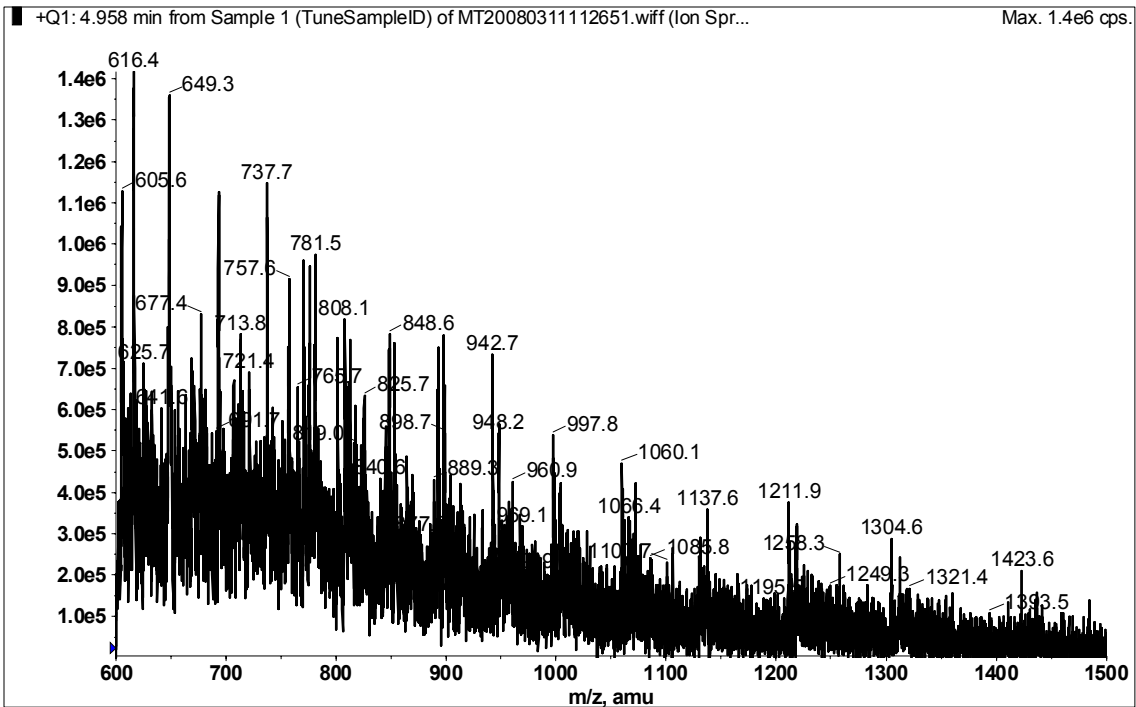
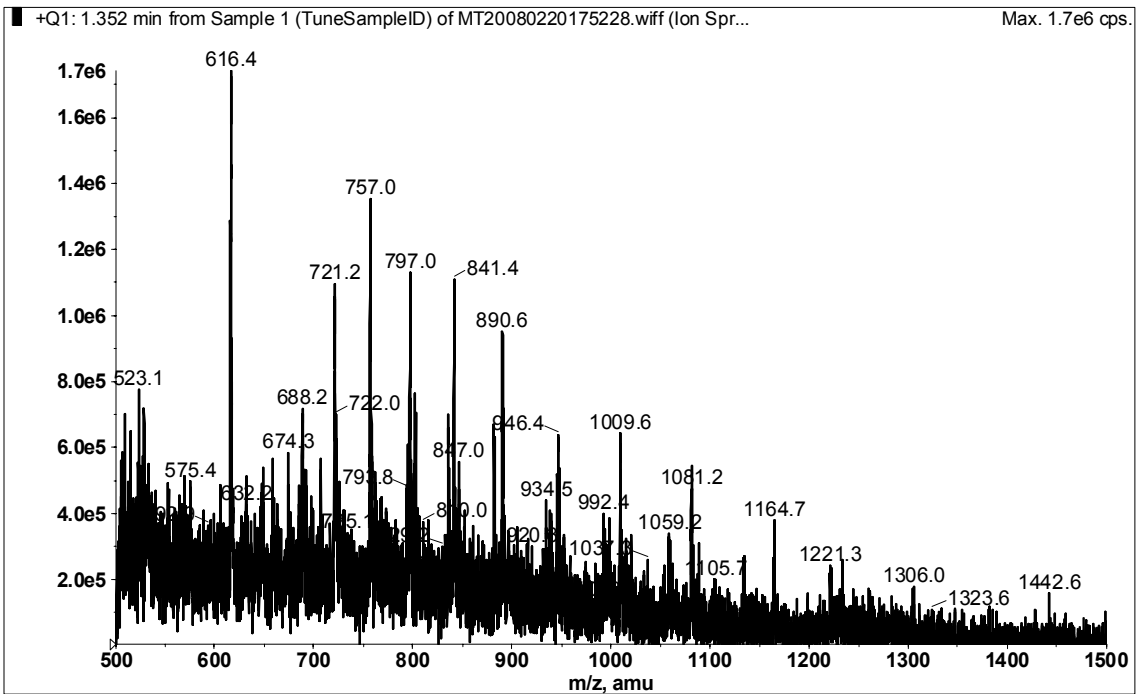**Figure 27**: Mass spectrum of Myoglobin from horse heart.

Figure 28: Mass spectrum of Hemoglobin Ao- Ferrous Stabilized Human.
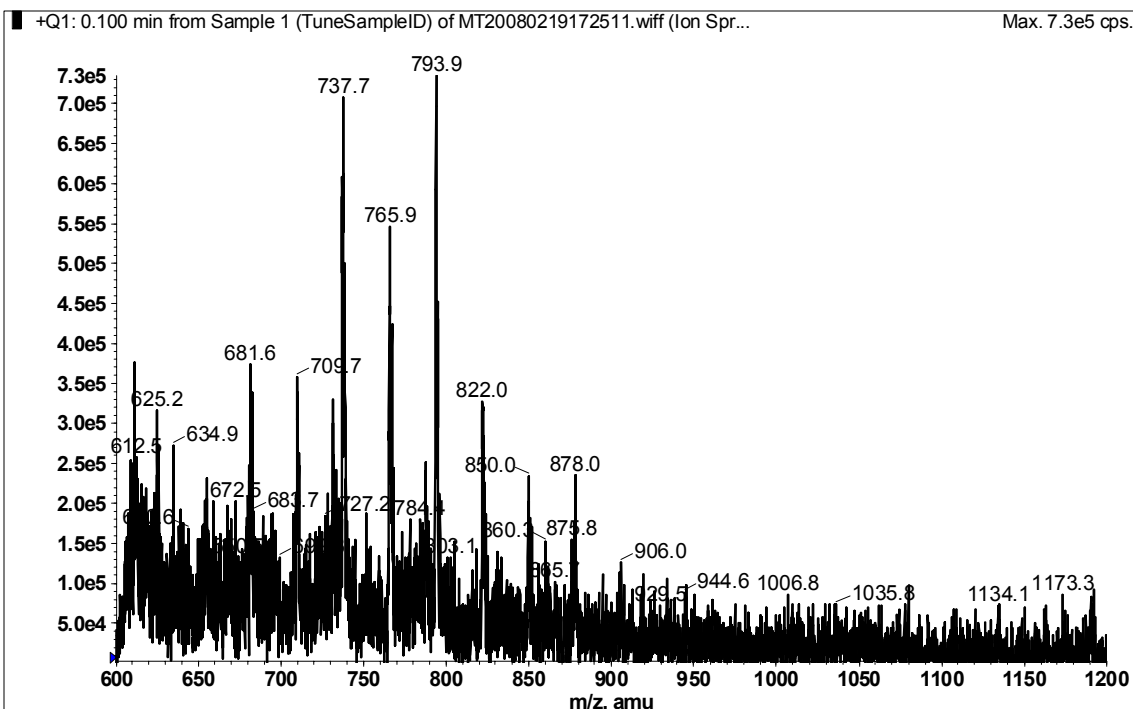
Figure 29: Mass spectrum of Trypsin inhibitor from soybean.

The number of charges and the molecular weight of each of the samples were calculated using the following equations. They were calculated from the mass to charge ratios.

Assume $N_2 = N_1 + 1$, for adjacent peaks (where N is number of charges).

$$M_1 = \frac{(M_w + N_1)}{N_1} \tag{16}$$

$$N_2 = \frac{(M_2 - 1)}{(M_2 - M_1)} \tag{17}$$

$$Mw = N_2(M_2 - 1) \tag{18}$$

where M_1 and M_2 are detected mass and Mw is the actual mass.

The calculated mass for Myoglobin was 16,952 Daltons, for Hemoglobin Ao, the calculated masses were α – 15,128 Daltons and β – 15,871 Daltons Hemoglobin Ao has

65

two subunits) and the calculated mass for Trypsin inhibitor from soybean was 19,966 Daltons. The values obtained are in good agreement with the figures reported in the literature and the figures reported in some journals.[111-114]

3.4 PROTEIN AND DRUG INTERACTION

The investigation of the interactions between protein and drugs is very important in pharmaceutical field. The microfluidic chips were used to perform IEF to observe the interaction between HSA and Warfarin. Warfarin is an anticoagulant that is administered to people orally and by injection in rare cases to treat thrombosis (to stop blood from clotting).[115-116] This drug is widely used and it binds with high affinity to site I (one of the two major binding sites) of HSA.[116-119] It has been reported that the structural details of the interaction between Warfarin and HSA are not known.[117]

Figures 30 and 31 show the separation of HSA and pI markers using both commercial cartridge and microfluidic chips. These electrophoregrams are used as controls.

Figure 30: Separation of HSA and pI markers using commercial cartridge.
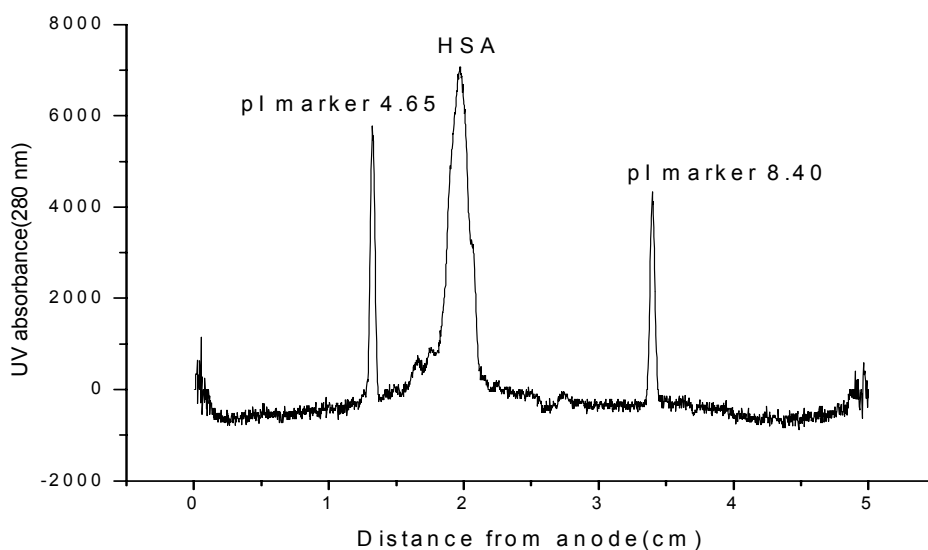
Figure 31: Separation of HSA and pI markers using microfluidic chips.

The electrophoregram obtained using microfluidic chip is similar to the one obtained using commercial cartridge as shown in figures 28 and 29. The pI markers were used to determine the pI of HSA. The calculated pI value of HSA using the commercial cartridge was 5.7 and the value obtained for microfluidic chip was 5.8, which is in agreement with the one obtained using commercial cartridge.

Figures 32 and 33 show the interaction between Warfarin and HSA using both commercial cartridges and microfluidic chips.
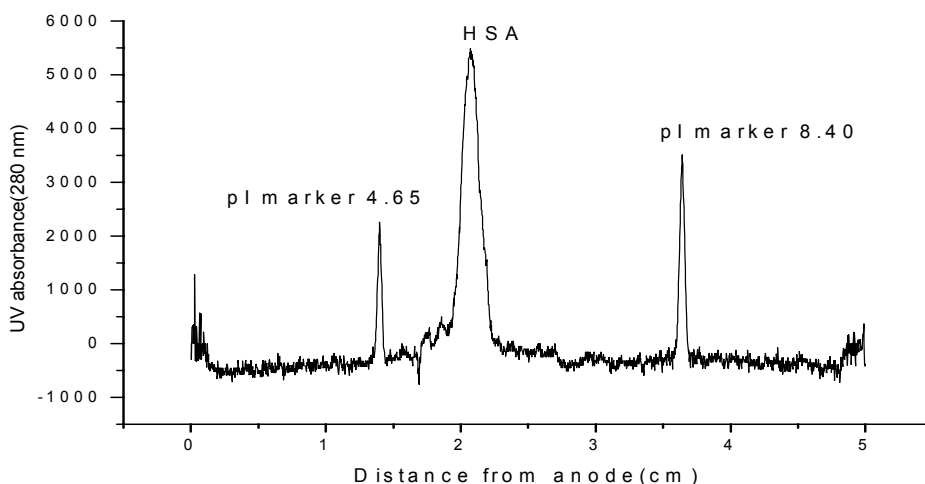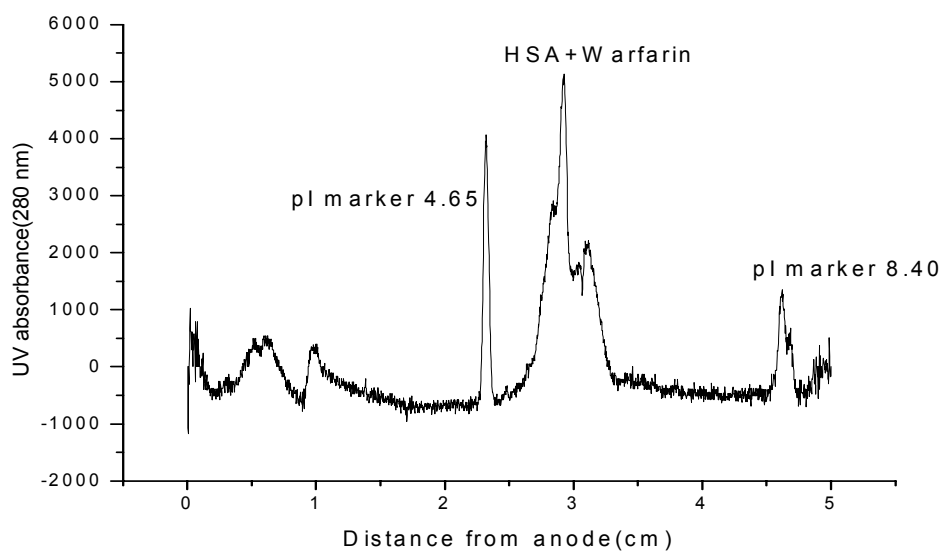
Figure 32: Separation of HSA and pI markers using commercial cartridge.
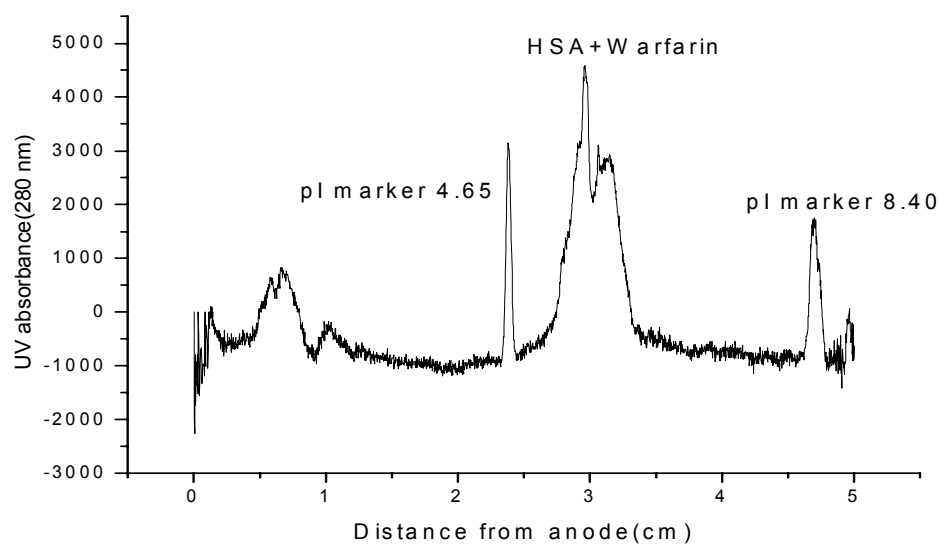
Figure 33: Separation of HSA and pI markers using microfluidic chips.

Similar results were obtained using both commercial cartridge and microfluidic chips for the investigation of interaction between Warfarin and HSA. A shift was observed with

68

the interaction between the drug and the protein and this led to a change in the pI of HSA because there was a strong interaction between the protein and the drug. This results in the conformational change in the protein as shown in the peak of HSA (illustrated in the electrophoregrams above). The calculated pI values were 5.9 and 6.0 using commercial cartridge and microfluidic chips, respectively. There was also a shift in the peaks of the pI markers, which might be due to EOF.

However, the investigation of the interaction between drugs and proteins using microfluidic chips has to be studied the more as the results presented in this research are preliminary results.

CHAPTER FOUR

4.0 CONCLUSION

Whole column imaging detection is the ideal detection method for isoelectric focusing of analytes using microfluidic chips. There is dynamic monitoring of the focusing of the analytes in real time and there is no need of mobilization step, which leads to a faster and more sensitive analysis. WCID does not need any labeling of analytes as other methods because it uses a simple procedure. The complexity of chip fabrication is reduced with WCID compared with other methods, which involve complex fabrication of a CE-chip system. There is also an opportunity for the optimization of separation conditions.

The use of tapered channel for performing isoelectric focusing in which the separation is carried out without the use of carrier ampholytes (due to the problems caused by these ampholytes) is a promising method for coupling isoelectric focusing with mass spectrometer to give further information about the analytes, especially their molecular masses.

The fabrication process of the microfluidic chips is simple and fast. The design of the chips is versatile. Small amounts of samples and reagents are consumed with the use of microfluidic chips to perform isoelectric focusing of proteins. The use of microfluidic chips gives room to high throughput analysis. High throughput analysis and the low consumption of samples and reagents are the two major advantages of microfluidic chips. The investigation of the interaction between drugs and proteins looks very promising with microfluidic chips.

There is a need for the optimization of the fabrication process and the separation conditions to avoid variations from chip to chip. The fabrication procedure, run-run

performance and also surface modification can lead to variations if these parameters are not taken care of properly.

REFERENCES

1. http://en.wikipedia.org/wiki/Capillary_electrophoresis

2. P.D. Grossman, J.C. Colburn, Capillary Electrophoresis-Theory and Practice, Academic Press Inc, San Diego, California, **1992.**

3. X. Wu, T. Huang, J. Wu, J. Pawliszyn, LCGC, 19 **(2001)** 526-545.

4. P.G. Righetti, Isoelectric focusing: theory, methodology and application, Elsevier Biomedical Press, Amsterdam, **1983**.

5. www.chemsoc.org.

6. Z. Liu, A.P. Drabovich, S.N. Krylov, J. Pawlisyn, Anal. Chem. 79 **(2007)** 1097-1100.

7. J. Wu, S.C Li, A. Watson, J.Chromatogr. A, 817 **(1998)** 163-171.

8. X. Fang, C. Tragas, J. Wu, Q. Mao, J. Pawlisyzn, Electrophoresis, 19 **(1998)** 2290-2295.

9. Z. Liu, T. Lemma, J. Pawlisyn, Journal of Proteome Research, 5 **(2006)** 1246-1251.

10. J. Wu, T. Huang, Electrophoresis, 27 **(2006)** 3584-3590.

11. Y. Shen, S.J. Berger, G.A. Anderson, R.D. Smith, Anal. Chem. 72 **(2000)** 2154-2159.

12. B. Yao, H. Yang, Q. Liang, G. Luo, L. Wang, K. Run, Y. Gao, Y. Wang, Y. Qiu, Anal. Chem. 78 **(2006)** 5845-5850.

13. J. Wu, C.Tragas, A. Watson, J. Pawlisyn, Analytica Chimica Acta, 383 **(1999)** 67-78.

14. A.A. Mohammad, A.O. Okorodudu, B.M.G. Bissell, P. Dow, G. Reger, A. Meier, P. Guodagno, J.R. Peterson, Clinical Chemistry, 43 **(1997)** 1798-1799.

15. C.F Poole, The Essence of Chromatography, Elsevier, Amsterdam, **2003**.

16. E. Jaspers, J. Overmann, App. Environ. Microbiol. 63 **(1997)** 3176-3181.

17. Y. Shen, S.J Berger, R.D. Smith, Anal. Chem., 72 **(2000)** 4603-4607.

18. L.M. Villar, P. González-Porqué, J. C. Masjuán Allvarez-Cermeño, A. Bootello, G. Keir. J. Immunol. Methods, 258 **(2001)** 151-155.

19. P. Glukhovskiy, G. Vigh. Anal. Chem., 71 **(1999)** 3814-3820.

20. P.K. Jensen, A, Kamel Harrata, C.S. Lee, Anal. Chem., 70 **(1998)** 2044-2049

21. T. Watanabe, A. Yamamoto, S. Nagai, Bioeng. 85 **(1998)** 451-453.

22. R. Weinberger, Practical Electrophoresis. Academic Press, New York, **2000**.

23. J.P Landers, Handbook of Capillary Electrophoresis CRC Press, **1994**.

24. L.A. Holland, N.P. Chetwyn, M.D. Perkins, S.M. Lunte, Pharmaceutical Research, 14 **(1997)** 4, 372-387.

25. X. Wu, T. Huang, Z. Liu, J. Pawliszyn, Trends in Analytical Chemistry, 24 **(2005)** 369-382

26. J. Wu, J. Pawliszyn, Journal of Liquid Chromatography, 16 **(1993)** 1892-1902.

27. F. Kilár, Electrophoresis, 24 **(2003)** 3908-3916.

28. X. Wu, J. Pawliszyn, Analytical Sciences, 17 **(2001)** 1189-1192.

29. L. Goodridge, C. Goodridge, J. Wu, M. Griffiths, J. Pawliszyn, Anal. Chem. 76 **(2004)** 48-52.

30. J. Wu, J. Pawliszyn, Am. Lab., 26 **(1994)** 48-52.

31. J. Wu, J. Pawliszyn, Anal. Chem., 67 **(1995**) 2010-2014.

32. Q. Mao, J. Pawliszyn, J. Biochem. Biophys. Methods, 39 **(1999)** 93-100.

33. Q. Mao, J. Pawliszyn, W. Thormann, Anal. Chem., 72 **(2000)** 5493-5502.

34. T. Huang, J. Pawliszyn, The Analyst Communication **(2000)**.

35. J. Wu, J. Pawliszyn, Analyst, 120 **(1995)** 1567-1571.

36. Q. Mao, J. Pawliszyn, Journal of Chromatography B, 729 **(1999)** 355-359.

37. Z. Liu, J. Pawliszyn, Anal. Chem., 77 **(2005)** 165-171.

38. T. Huang, X.-Z. Wu, J. Pawliszyn, Anal. Chem., 72 **(2000)** 4758-4761.

39. J. Pawliszyn, J. Wu, J. Microcol. Sep. 5 **(1993)** 397-401.

40. C.H. Lochmüller, Analytica Chimica Acta, 249 **(1991)** 297-302.

41. C.H. Lochmüller, S.J. Breiner, Journal of Chromatography, 480 **(1989)** 293-300.

42. T. Huang, J. Pawliszyn, Electrophoresis, 23 **(2002)** 3504-3510.

43. B. Kates, C.L. Ren, Electrophoresis, 27 **(2006)** 0000-0000.

44. X.-H. Fang, M. Adams, J. Pawliszyn, Analyst, 124 **(1999)** 335-341.

45. J. Pawliszyn, Y.-P. Ciu, S.N. Semonov, Instrumentation Science & Technology, 28 **(2000)** 4, 281-301.

46. www.broadleyjames.com

47. S.J. Luner, A. Kolin, Proceedings of the National Academy of Sciences, 66 **(1970)** 3, 893-903.

48. A. Manz, J.C Fettinger, E. Verpoote, H. Ludi, H.M Widmer, D.J. Harrison, Trends in Analytical Chemistry, 10 **(1991)** 144-149.

49. J.P. Choudhury, Handbook of Microlithography, Micromachining and Microfabrication, **1997**, 2.

50. G.H.W. Sanders, A. Manz, Trends in Analytical Chemistry, 19 **(2000)** 6, 364-378.

51. D.R. Reyes, D. Iossifidis, P.-A. Auroux, A. Manz, Anal. Chem. 74 **(2002)** 2623-2636.

52. Q. Fang, G.-M. Xu, Z.-L. Fang, Anal. Chem. 74 **(2002)** 1223-123**1.**

53. J. Monahan, A.A. Gerwirth, R.G. Nuzzo, Electrophoresis, 23 **(2002)** 2347-2345.

54. U. Backofen, F.-M. Matysik, C.E. Lunte, Anal. Chem. 74 **(2002)** 4054-4059.

55. J. Wang, M. Pumera, M.P. Chatrathi, A. Escarpa, M. Musameh, Anal. Chem. 74 **(2002)** 1187-1191.

56. V. Dolnik, S. Lui, S. Jovanovich, Electrophoresis, 21 **(2000)** 41-54.

57. A.P. O'Neill, P. O'Brien, J. Alderman, D. Hoffman, M. McEnery, J.Murrihy, J.D. Glennon, Journal of Chromatography A, 924 **(2001)** 259-263.

58. M.L. Chabinyc, D.T. Chiu, J. C. McDonald, A.D. Stroock, J.F. Christian, A.M. Karger, G.M. Whitesides, Anal. Chem. 73 **(2001)** 4491-4498.

59. C. Guillo, J.M. Karlinsey, J.P. Landers, Lab Chip, 7 **(2007)** 112-118.

60. J. Liu, X. Sun, M.L. Lee, Analytical Chemistry **(2005).**

61. B.W. Wenclawiak, R.J. Püschl, Analytical Letters, 39 **(2006)** 3-16.

62. L. Székely, A. Guttman, Electrophoresis, 26 **(2005)** 4590-4604.

63. S. Götz, U. Karst, Anal. Bioanal. Chem. 387 **(2007)** 183-192.

64. O. Hofmann, X. Wang, A. Cornwell, S. Beecher, A. Raja, D.D.C. Bradley, A.J. deMello, J.C. deMello, Lab Chip, 6 **(2006)** 981-987.

65. J.-L. Fu, Q. Fang, T. Zhang, X.-H. Jin, Z.-L. Fang, Analytical Chemistry **(2006)**.

66. M. Gong, K. R. Wehmeyer, P. A. Limbach, F. Arias, W.R. Heineman, Analytical Chemistry **(2006)**.

67. H.-F. Li, J.-M. Lin, R.-G. Su, K. Uchiyama, Electrophoresis, 25 **(2004)** 1907-1915.

68. J. Liu, C. Hansen, S.R. Quake, Anal. Chem. 75 **(2003)** 4718-4723.

69. S.-H. Chen, Y.-H Lin, L.-Y. Wang, C.-C. Lin, G.-B. Lee, Anal. Chem. 74 **(2002)** 5146-5153.

70. T. Kamei, B.M. Paegel, J.R. Scherer, A.M. Skelley, R.A. Street, R.A. Mathies, Anal. Chem. 75 **(2003)** 5300-5305.

71. X. Huang, J. Ren, Electrophoresis, 26 **(2005)** 3595-3601.

72. M. Pumera, Talanta, 66 **(2005)** 1048-1062.

73. H. Cui, K. Horiuchi, P. Dutta, C.F. Ivory, Anal. Chem. 77 **(2005)** 1303-1309.

74. H. Cui, K. Horiuchi, P. Dutta, C.F. Ivory, Anal. Chem. 77 **(2005)** 7878-7886.

75. R.F. Renzi, J. Stamps, B.A. Horn, S. Ferko, V.A. VanderNoot, J.A.A. West, R. Crocker, B. Wiedenman, D. Yee, J.A. Fruetel, Anal. Chem. 77 **(2005)** 435-441.

76. C. Li, Y. Yang, H. G. Craighead, K.H. Lee, Electrophoresis, 26 **(2005)** 1800-1806.

77. H. Shadpour, S.A. Soper, Analytical Chemistry **(2006)**.

78. G. Xu, J. Wang, Y. Chen, L. Zhang, D. Wang, G. Chen, Lab Chip, 6 **(2006)** 145-148.

79. J. M.K. Ng, I. Gentling, A.D. Stroock, G.M. Whitesides, Electrophoresis, 23 **(2002)** 3461-3473.

80. R.S. Ramsey, J.M. Ramsey, Anal. Chem. 69 **(1997)** 1174-1178.

81. Q. Mao, J. Pawliszyn, Analyst, 124 **(1999)** 637-641.

82. www.faculty.washington.edu.

83. H. Choi, K.-B. Lee, K.R. Yoon, I.S. Choi, S. I. Woo, K. Choi, S.C. Lee, Y. Kim, Bull. Korean Chem. Soc. 25 **(2004)**, 4, 560-562.

84. C.-H. Tsai, C.-H. Tai, L.-M. Fu, F.-B. Wu, J. Micromech. Microeng. 15 **(2005)** 377-385.

85. M.W. Toepke, D.J. Beebe, Lab Chip, 6 **(2006)** 1484-1486.

86. Y. Xia, G. M. Whitesides, Annu. Rev. Mater. Sci. 28 **(1998)** 153–84

87. J.C. McDonald, D.C. Duffy, J.R. Anderson, D.T. Chiu, H. Wu, O.J.A. Schueller, G.M. Whitesides, Electrophoresis, 21 **(2000)** 27-40.

88. D.C. Duffy, J.C. McDonald, O.J.A. Schueller, G.M. Whitesides, Anal. Chem. 70 **(1998)** 4974-4984.

89. M.L.-S. Mok, L. Hua, J.B.-C. Phua, M.K.-T. Wee, N.S.-K. Sze, Analyst, 129 **(2004)** 109-110.

90. http://en.wikipedia.org/wiki/Polydimethysiloxane

91. G.J.M. Bruin, Electrophoresis, 21 **(2000)** 3931-3951.

92. C.S. Effenhauser, G.J.M. Bruin, A. Paulus, M. Ehrat, Anal. Chem. 69 **(1997)** 3451-3457.

93. A. Govindaraju, A. Chakraborty, C. Luo, J.Micromech.Microeng **(2005)** 15, 1303-1309.

94. http://en.wikipedia.org/wiki/Microfluidics

95. S.K. Sia, G.M. Whitesides, Electrophoresis, 24 **(2003)** 3563-3576.

96. B.H. Weigl, R.L. Bardell, C.R. Cabrera, Advanced Drug Delivery Reviews 55 **(2003)** 349-377.

97. C.S Henry, Methods in Molecular Biology, Microchip Capillary Electrophoresis; methods and Protocols, 339 **(2006)** Humana Press Inc., Totowa, NJ.

98. J. Ohnesorge, C. Neusüß, H.Wätzig, Electrophoresis, 26 **(2005)** 3973–3987.

99. M.R.N. Norton, S. Terabe, Analytical Sciences, 21 **(2005)** 5-13.

100. J.H. Chan, A.T. Timperman, D. Qin, R. Aebersold, Anal. Chem. 71 **(1999)** 4437-4444.

101. M. Wilm, M. Mann, Anal. Chem. 68 **(1996)** 1-8.

102. M. Jussila, K. Sinervo, S.P. Porras, M-L. Riekkola, Electrophoresis, 21 **(2000)** 3311-3317.

103. J. Jänis, J. Rouvinen, M. Leisola, O. Turunen, P. Vainiotalo, J. Biochem. 356 **(2001)** 453–460.

104. M. Vlčkova, F. Kalman, M.A. Schwarz, Journal of Chromatography A, 1181, **(2008)** 145-152.

105. N. Li, K. Kessler, L. Bass, D. Zeng, Journal of Pharmaceutical and Biomedical Analysis, **(2006).**

106. M. Hashimoto, K. Tsukagoshi, R. Nakajima, K. Kondo, A. Arai, Journal of Chromatography A, 867, **(2000)** 271-279.

107. J. Han, A.K. Singh, Journal of Chromatography A, 1049 **(2004)** 205-209.

108. S.M. Kim, G.J. Sommer, M.A. Burns, E.F. Hasselbrink, Anal. Chem. **(2006).**

109. W. Tan, Z. H. Fan, C.X. Qui, A.J. Ricco, I. Gibbons, Electrophoresis, 23 **(2002)** 3638-3645.

110. M. H. Lamoree, U. R. Tjaden, J. van der Greef, J. Chromatogr. A. 777 **(1997)** 31.

111. N.J. Clarke, S. Naylor, Biomed. Chromatogr. 16, **(2002)** 287-297.

112. Q. Tang, A. K. Harrata, C.S. Lee, Anal. Chem. 67 **(1995)** 3515 – 3519.

113. L. Yang, C.S. Lee, S.A. Hofstadler, R.D. Smith, Anal. Chem. 70 **(1998)** 4945 – 4950.

114. R. Weinberger, Practical Electrophoresis. Academic Press, Boston, **1993.**

115. http;//en.wikipedia.org/wiki/Warfarin.

116. C. Bertucci, A. Canepa., G.A. Ascoli, I.F. Lopes Guimaraes, Chirality, 11 **(1999)** 675-679.

117. H. Watanabe, U. Kragh-Hansen, S. Tanase, K. Nakajou, M. Mitarai, Y. Iwao, T. Maruyama, M. Otagiri, J. Biochem. 357 **(2001)** 269–274.

118. J. Wilting, W. F. van der Giesen, L.H.M. Janssen, M.M. Weideman, M. Otagiri, The Journal of Biological Chemistry, 255 **(1980)** 3032-3037.

119. G. Zilberman, L. Smith, Analyst, 130 **(2005)** 1483-1489.