

Development of Isoelectric Focusing Techniques for Protein Analyses

By
Yanwei Zhan

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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

Isoelectric focusing (IEF) is a powerful approach in separations of zwitterionic substances such as proteins, peptides and amino acids. It is important in proteomic research. Generally, in IEF, carrier ampholytes (CAs) are necessary to establish a stable pH gradient. However, CAs also bring attendant problems such as a decrease in detection sensitivity and suppression of ionization of analytes in mass spectrometry (MS) detection. It is desirable to build a pH gradient without using CAs. A simple slab based design was developed to establish a pH gradient using the electrolysis of water and the strength of free flow electrophoresis (FFE). The simple and robust CA free FFE-IEF design was applied in protein fractionation.

In capillary format, capillary isoelectric focusing (CIEF), coupled to MS is a promising hyphenated technique for biomolecular analysis based on the combination of the high separation power of CE and the high specificity of MS. Coupling of the instruments is usually achieved with a coaxial sheath liquid interface, which decreases the detection sensitivity because of the dilution of sample by the sheath liquid. In this project, nano-electrospray, a sheathless interface, was used for coupling. Additionally, another major challenge is the presence of CAs which suppresses the ionization of analytes and contaminates the MS. In order to complete this project, a microcross union was chosen to couple CIEF with MS. A makeup solution was introduced to dilute the concentration of CAs after IEF to assist the ionization for MS detection. The makeup solution could replace the sheath liquid and could be maintained at a low flow rate so that

nanoelectrospray could be performed.

Monoliths can be described as integrated continuous porous separation media for micro scale separation columns. CAs were immobilized at different positions in the column according to their pIs, generating a monolithic immobilized pH gradient (M-IPG). In this project, carrier ampholytes was immobilized in poly (GMA-co-EDMA) based monolithic capillary and poly (GMA-co-acrylamide) based monolithic capillary to form a pH gradient. Two proteins were separated by IEF, which was implemented in poly (GMA-co-acrylamide) based monolithic capillary without CAs. The interface to MS was performed following the use of a microcross union as described previously. No typical noise of CAs was observed in the MS spectrum.

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Dedication

I dedicate this thesis to my parents, my husband Jixian and my loved daughter Eliya.

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LIST OF ABBREVIATIONS

| | |
|-------|--|
| AIBN | Azobisisobutyronitrile |
| CAs | Carrier ampholytes |
| CE | Capillary electrophoresis |
| CEC | Capillary electrochromatography |
| CGC | Capillary gel electrophoresis |
| CIEF | Capillary isoelectric focusing |
| CZE | Capillary zone electrophoresis |
| DMSO | Dimethyl sulfoxide |
| EDMA | Ethylenedimethacrylate |
| EOF | Electroosmotic flow |
| ESI | Electrospray ionization |
| FFE | Free flow electrophoresis |
| GMA | Glycidyl methacrylate |
| IEF | Isoelectric focusing |
| IPG | Immobilized pH gradient |
| ITP | Isotachopheresis |
| LC/MS | Liquid chromatography coupled with mass spectrometry |
| LIF | laser induced fluorescence |
| MALDI | Matrix-assisted laser desorption/ionization |
| M-IPG | Monolithic immobilization pH gradient |

| | |
|----------------|---------------------------------------|
| MS | Mass spectrometry / Mass spectrometer |
| pI | Isoelectric point |
| PVP | polyvinylpyrrolidone |
| RPLC | Reversed-phase liquid chromatography |
| 2-D gel | 2-dimensional gel electrophoresis |
| UV | Ultraviolet |
| WCID | Whole-column imaging detection |
| γ -MAPS | 3-methacryloxypropyltrimethoxysilane |

Development of Isoelectric Focusing Techniques for Protein Analyses

Chapter 1 - Introduction

Isoelectric focusing (IEF) is an electrophoretic method in which proteins are separated based on their isoelectric points (pIs). Usually, a pH gradient is established by carrier ampholytes (CAs). When a protein is placed in a medium with a linear pH gradient and subjected to an electric field, it will initially move toward the electrode with the opposite charge. During the migration through the pH gradient, the protein will either pick up or lose protons. When the protein eventually arrives at the point in the pH gradient equivalent to its pI, it will stop migrating because it becomes uncharged. Because the pI is a unique characteristic of amphoteric substances, IEF is a powerful tool in zwitterionic separation with high resolution. Slab gel IEF has been widely used in separation and characterization of proteins and peptides in biotech laboratories.¹ In 1985, Hjerten and Zhu first reported an IEF method performed in a capillary column format.² In 1989, Chmelik reported an application of pH gradient in free-flow electrophoresis (FFE) format.³ Two kinds of IEF are applied in this project. They are FFE-IEF and CIEF.

1.1 Free flow electrophoresis (FFE)

FFE separates charged particles, which are injected continuously into a thin carrier buffer film flowing between two parallel plates. The electric field, applied perpendicular to the flow direction, leads to deflection of charged sample components according to their

mobility or pI.⁴ The sample and electrolyte used for the separation enter the separation chamber at one end and the fractionated sample and the electrolyte are collected at the other end as shown in Figure 1.1.⁵ Depending on the electrolyte system applied, FFE can be performed in three basic modes: (1) zone electrophoretic (ZE), (2) isotachopheresis (ITP), and (3) IEF. In ZE mode, a narrow sample beam is injected into a background electrolyte of a constant composition, pH and conductivity, and individual components of the sample are separated according to the charge-to-size ratios. In ITP mode, the sample is introduced between leading and terminating electrolytes having the highest and lowest mobility of their ions in respect to the mobility of analytes. From the principle of the method, it therefore follows that the sample components can be both separated and concentrated during the separation process. In IEF, the electrolyte used for the separation is created from a set of carrier ampholytes, which leads to the formation of a linear pH gradient. The sample can be introduced either in the mixture with the electrolyte or in a narrow zone. The method is aimed at the separation of amphoteric solutes, which migrate in the electric field until they reach the position where the pH of the electrolyte is equal to its pI and become immobile and focused.

FFE offers some important advantages. Firstly, FFE allows a continuous collection of fractions combined with continuous sample feeding. For example, in preparative applications, the separation is performed continuously so that potentially hundreds of milligrams of samples can be separated. Secondly, FFE can be easily applied in different formats. There are several commercial apparatus for preparative purposes. Furthermore, small-scale separation is feasible in microfabricated free flow electrophoresis (mFFE). Thirdly, analytes can be easily collected at the end of the separation area, which can decrease the cost of further

treatment following the separation.

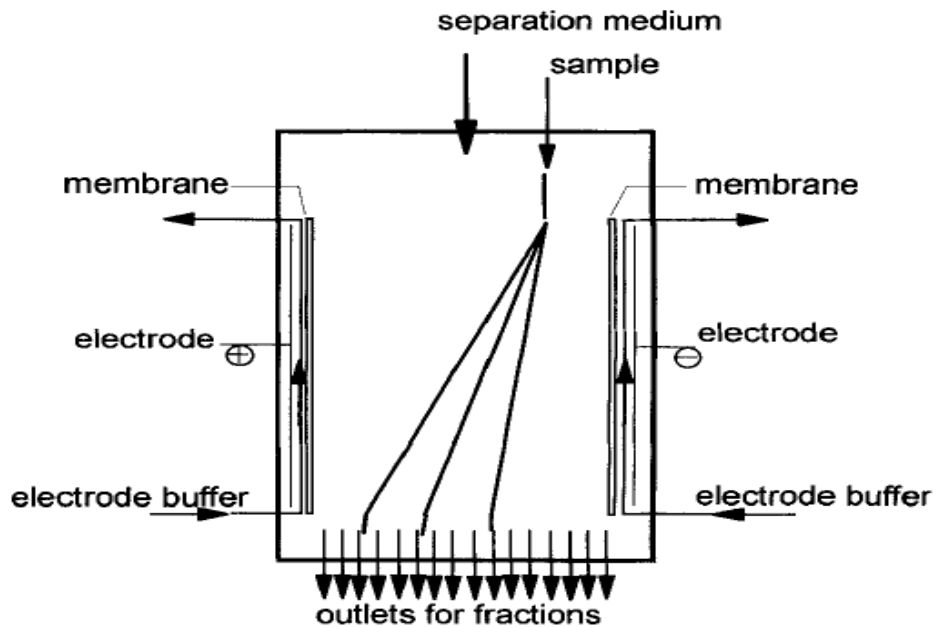


Figure 1.1: Schematic illustration of FFE⁵

FFE has been applied to a great variety of charged species, from low molecular ions up to proteins, membranes, and cells.^{6, 7} The application of FFE in proteomics has been reviewed by Chmelik.⁸

1.2 Capillary isoelectric focusing (CIEF)

When IEF is performed in capillary format, a separation column is filled with the mixture of a protein sample and carrier ampholytes (CAs). The two ends of the column are dipped into catholyte and anolyte. Under the separation voltage, a pH gradient is established along the capillary column and the proteins migrate to the point where their pI values are equivalent to the pH values and the migration stops. The proteins are focused into narrow zones at their pIs.

CIEF is expected to have both the high resolution of slab gel IEF and the advantages of a column-based separation technology that include automation and quantification. CIEF is widely applied in protein analysis. It can separate the analytes, and at same time, concentrate them. It resolves the problem of low sample loading in capillary electrophoresis. However, a major limitation is the presence of CAs, which is necessary in CIEF. High CA concentrations decrease the sensitivity of MS and UV detection.⁹

1.2.1 UV, fluorescence, and whole-column imaging detection

Usually, a conventional CIEF instrument is only equipped with a single-point, on-column detector, which is located close to one end of the column. UV and fluorescence are common detection modes.¹⁰ Therefore, a mobilization process is necessary following the focusing process. There are three ways to perform the mobilization: hydrodynamic mobilization, electrophoretic (salt) mobilization, and EOF-driven mobilization.^{11,12} Mobilization of the focused analytes requires additional time for the analysis, deforms the established pH gradient, and tends to reduce resolution and reproducibility of separation. Imaging detection systems and scanning detection systems can free CIEF from these problems. In particular, real-time whole-column imaging detectors appear to be ideal for detection of the stationary zones focused within the capillary.

WCID-CIEF was invented by Wu and Pawliszyn in 1994,¹³ and it has been commercialized by Convergent Bioscience Ltd.¹⁴ The separation column, shown in Figure 1.2, is internally coated, and its outer polyimide coating is removed to let light through. Two pieces of hollow-fibre membrane are glued to the ends of the separation column. Two

connection capillaries for sample introduction are glued to either end of the hollow-fibre membrane. The membrane here has two functions. First, the membranes in the electrolyte reservoirs allow small ions such as H^+ and OH^- to pass freely, and thus, the CIEF to occur normally. Second, the membrane can prevent the proteins from going out to the electrolyte reservoirs while proteins and CAs mixtures are injected to the column. The whole-column imaging detection system uses ultraviolet (UV) absorbance imaging detector operated at 280 nm. The IEF process in the entire separation column is monitored by the whole-column detection system, as shown in Figure 1.3. Since the whole-column detector monitors in an online fashion, all sample zones within the column are recorded by the detector simultaneously without disturbing the separation. Sample precipitation and aggregation are the two most common problems in CIEF. Different additives may be selected to improve the performance in CIEF. These features facilitate fast method development.¹⁵

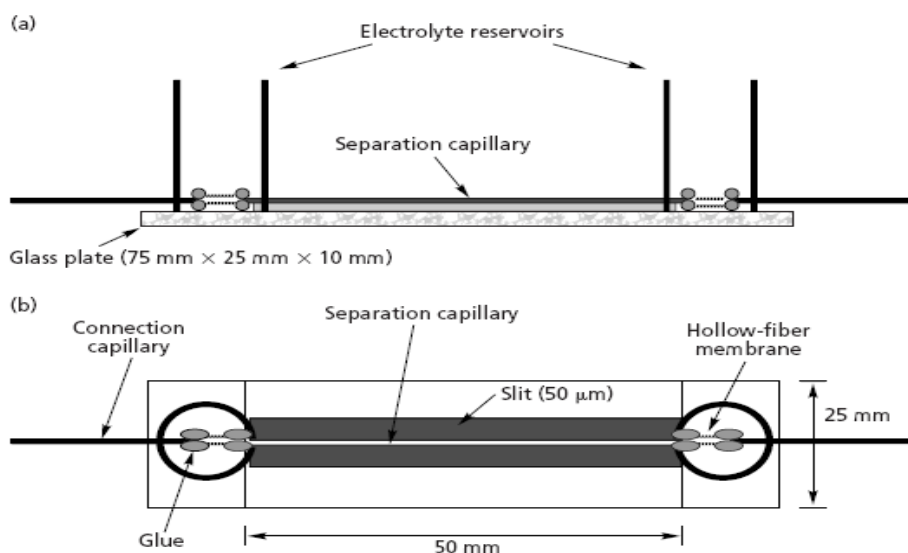


Figure 1.2 Illustrations of a capillary isoelectric focusing cartridge. (a) side view, (b) top view¹⁴.

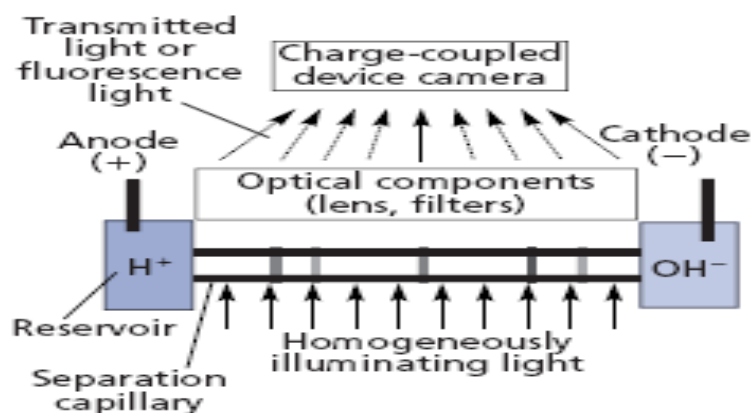


Figure 1.3: Illustration of the basic concept of the whole-column imaging detection for CIEF¹⁴.

1.2.2 Mass spectrometry

Detection in CE is usually carried out on-column using UV absorbance or laser-induced fluorescence (LIF).¹⁶ However, UV detection is not very sensitive, while LIF might require solute derivatization with a fluorescent tag. Also, UV and LIF detection do not provide the information necessary to determine directly the structure of the detected analytes.

A mass spectrometer (MS) is a device that measures the mass-to-charge ratio of ions. Mass spectrometry is not only a sensitive detection technique; it can be applied for the detection of wide range of analytes without derivatization and gives information to determine the structure of the analytes of interest as well. The use of a mass spectrometer offers unambiguous information of molecular weight and provides structural information helping with the identification of unknowns. CIEF-MS, which combines the high efficiency and resolution power of CIEF, with the high selectivity and sensitivity of MS, is an attractive analytical technique.¹⁷

1.2.3 Interfaces between CE and MS

The predominant ionization method for CE-MS is electrospray ionization (ESI).¹⁷ Matrix-assisted laser desorption ionization (MALDI) has also been used extensively.¹⁸ Another ionization method called sonic spray ionization (SSI) has also applied in coupling CE with MS.¹⁹

Creating the actual interface between the two techniques is not simple. As the capillary is held at a high voltage, there are many issues with providing a stable current for both the CE separation and the MS ionization. Another factor is that CE flow is usually not high enough to maintain stable ionization within the mass spectrometer source. The interface is critical.

Three kinds of interfaces have been developed since the first CE-MS interface was built in 1987 by Smith and his group. Coaxial liquid sheath-flow, sheathless, and liquid-junction interfaces have been constructed for coupling CE to ESI-MS, as shown in Figure 1.4.

In the coaxial sheath-flow interface configuration, the outlet of the CE capillary is simply inserted into the ESI emitters (commonly referred to as the ESI needle). The sprayer is present as a triple tube; the CE capillary in the centre surrounded by two stainless steel tubes. The inner one delivers a flow of extra solvent to make up the flow to a suitable level. The outer tube carries nebulizer gas to assist in droplet formation in the electrospray process. Electrospray is the process by which the solute ions are vaporized and ionized so that they can be separated by the MS. This kind of configuration has several advantages, including simple fabrication, reliability, and ease of implementation. The coaxial liquid sheath interface uses a makeup flow to provide stable electrospray and complete the CE electrical circuit.

Although the interface suffers from dilution of the analytes by the sheath liquid, it is the most useful interface for stable and long lasting performance of electrospray. These advantages make coaxial sheath-flow the most common approach in connecting CE with ESI-MS. Almost all commercial CE-MS instruments have coaxial sheath-flow interfaces.^{20,21}

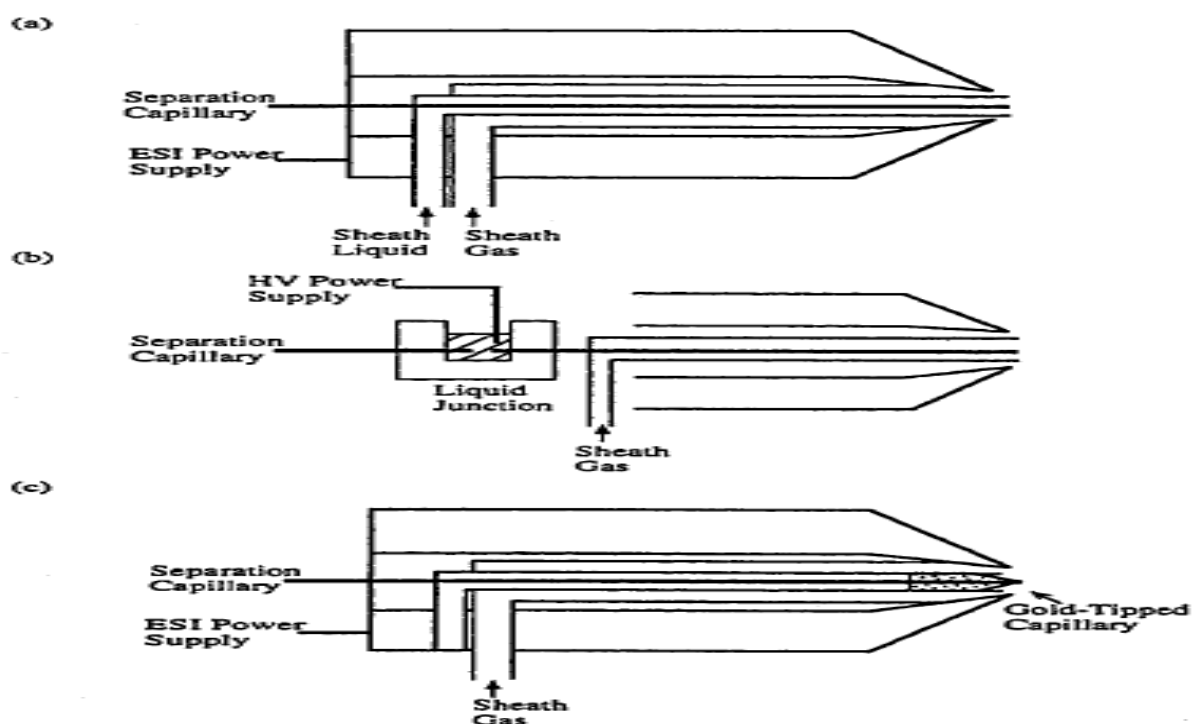


Figure 1.4: Schematic illustration of CE/MS interfaces to an ESI source. (a) Coaxial sheath-flow interface (b) liquid-junction interface (c) sheathless interface.⁵⁵

The liquid junction interface is formed by a tee junction. The tee forms a junction between the CE terminus and a makeup flow line. The tee transfers the flow to the ion source of the ESI-MS. A liquid junction is considered to have approximately 5% lower resolution than a coaxial flow interface. This may be due to the dead volume in the liquid junction between the CZE terminus and the sprayer.

The main disadvantage of sheath-flow interface is the low sensitivity of detection due

to the dilution of the analyte by the sheath liquid. To eliminate this weakness of the sheath-flow interface, a variety of sheathless interfaces have been introduced.²² Mainly two different approaches are used to fabricate sheathless interface. The first type consists of a nano-spray needle, a separation capillary and a connection unit. The second approach is using a separation capillary with a tapered end that is coated with an electrically conducting layer. The main advantage of a sheathless interface is its high sensitivity because there is no sheath liquid to dilute the CE effluent. However, sheathless interfaces also have their weaknesses. For example, the metal coating tip has a short lifetime due to electrochemical degradation. Moreover, dead volume and air bubbles make sheathless coupling probably not yet sufficiently robust for extended routine analysis.²¹⁻²³

The designation “nano-electrospray” was first introduced by Wilm and Mann because of the low nanolitre per minute flow and droplet size in the nanometre range produced by a new electrospray technique.²⁴ It is different from a conventional electrospray source by the small spray tip, the low flow rate, the small size of droplets generated and the absence of solvent pumps and inlet valves. In conventional ESI, the spray tip is around 100 μm in \O , sample flow is around 1-20 $\mu\text{L}/\text{min}$.²⁵ In nano-electrospray, the spray tip is around 1-2 μm in \O and the sample flow is around 20-40 nL/min. The diameter of droplets produced by nano-electrospray is less than 200 nm, which is about 100-1000 times smaller than those generated by a conventional ESI source.

The advantage of such small droplets is that they have a high surface-to-volume ratio resulting in high efficiency of ionization. Since no drying gas is needed and the tip can be brought close to the MS inlet, the sensitivity is better. Since the typical flow rate in CE is

about 20-200 nL/min,²⁶ the flow rate from a nano-electrospray device better matches the CE flow rate.

The Protana nano-electrospray (Odense, Denmark) is an offline device, as shown in Figure 1.5.²⁷ The sample is loaded into a metal-coated glass capillary with a tip internal diameter that ranges from 1 to 4 μm . A voltage is applied to the metal coating on the tip and an air-filled syringe provides a constant backpressure to initiate and maintain electrospray. The flow rate is dictated by the electrospray process itself. The nano-electrospray also works as an ion source, which disperses the sample solution purely by electrostatic means, without nebulizing gas and without any solvent pump. A low flow rate can be achieved by electrostatic force through the orifice of the capillary.

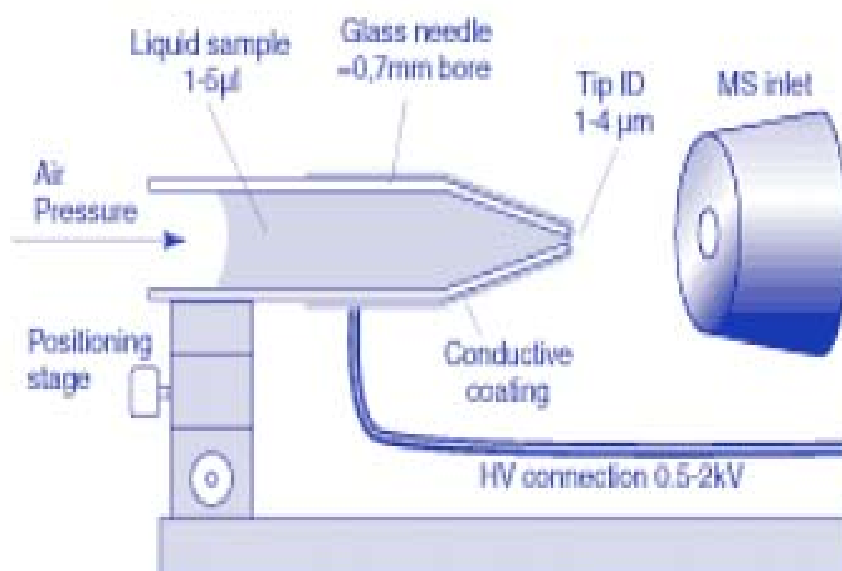


Figure 1.5: Schematic illustration of an offline nano-electrospray device.²⁷

The MS instrument, API 3000, available in our lab is equipped with an offline nano-electrospray interface. Our objective is to modify this offline interface so that online

CIEF-MS can be performed.

1.2.4 Strategies for coupling CIEF with MS

An MS is the best choice as the detector for CIEF because it can provide the information of molecular weight and structure. In other words, CIEF has the ability to separate proteins in a small volume with high efficiency and MS has the compound identification capability based on accuracy of mass determination.

One traditional bioanalytical and biochemical approach to protein characterization is two-dimensional (2-D) gel electrophoresis. Protein samples are separated first by pI and then by size in a two-dimensional gel. 2-D gel electrophoresis can resolve several thousand proteins. However, it is time-consuming, labor-intensive, and provides only semi-quantitative information. Following the 2-D idea, coupling CIEF with MS was first introduced by Tang and colleagues in 1995.²⁸ They used a commercial sheath flow CE-MS instrument for coupling CIEF to MS. A catholyte reservoir was placed in the ion source house while focusing and, after focusing, ionization was completed using a sheath flow liquid and nebulizer gas. In 1996, the same group reported a small modification in which gravity-assisted mobilization was used to compensate for moving-boundary effects caused by the sheath liquid ions in the capillary.²⁹

1.2.4.1 CAs-free system

The approaches described above have an obvious limitation. The presence of carrier ampholytes (CAs) compromises the performance of MS detection because CAs can contaminate the ionization source, suppress the analyte signal intensity, and degrade the mass resolution. To solve this problem, the second stage began with the work completed by Lamoree and colleagues in 1997.^{30,31} They devised an online microdialysis system between

the separation capillary and a transfer capillary connected to an ESI-MS as shown in Figure 1.6. They still used a sheath liquid flow to assist the ESI.

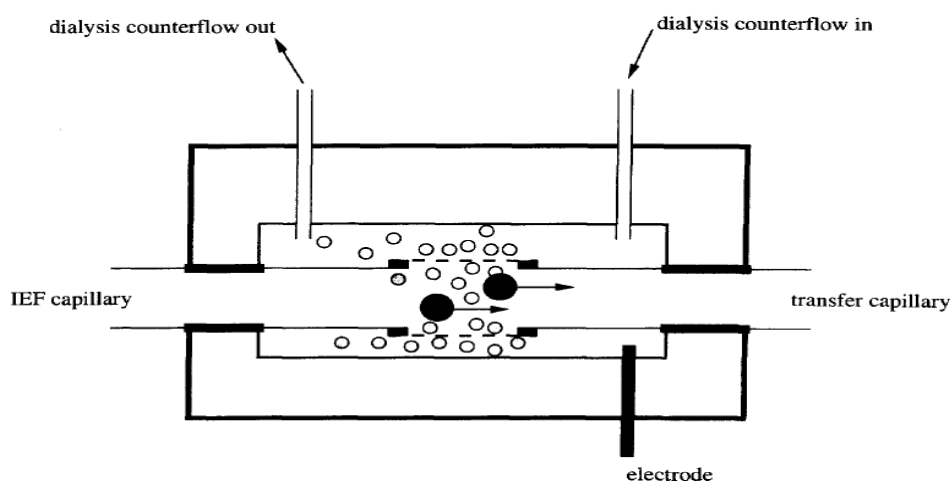


Figure 1.6: Illustration of hollow fibre-based microdialysis device for CA removal in a CIEF-MS system.³⁰

In 2000, Chartogne et al. developed a chip-based free-flow electrophoresis device to remove the carrier ampholytes.³² The device consisted of a 0.1 mm × 10 mm × 25 mm chamber with one inlet and three outlets. An electric field is applied orthogonally to the direction of buffer flow.

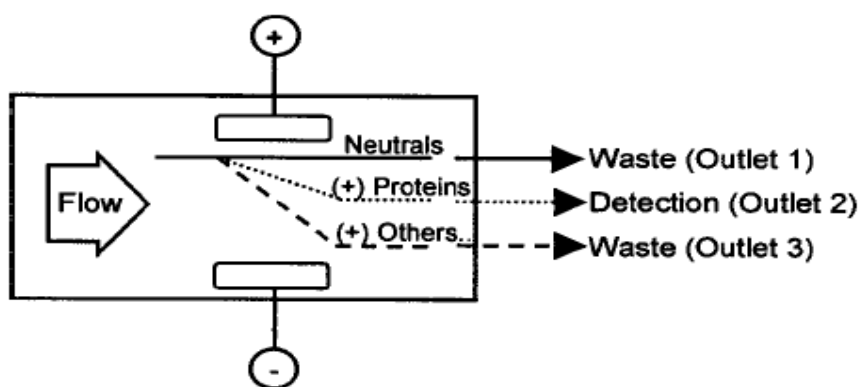


Figure 1.7 Schematic illustration of FFE for removing CAs.³²

In recent years, a chromatographic separation has been inserted after CIEF as not only a means of removing ampholytes but also means of obtaining additional resolution before MS

analysis. In 2003, Jinzhi Chen et al. developed an online combination of CIEF with capillary reversed-phase liquid chromatography (CRPLC).³³ They applied this device to the analysis of a protein and peptide mixture. In 2004, Zhou et al. also developed an online CIEF-RPLC MS approach as shown in Figure 1.8.³⁴ The advancement demonstrated with this device is the use of a microselection valve, which can control the delivery of every fraction of the CIEF capillary. Other applications reported by this method include characterization of human saliva proteins and examination of membrane proteins.³⁵

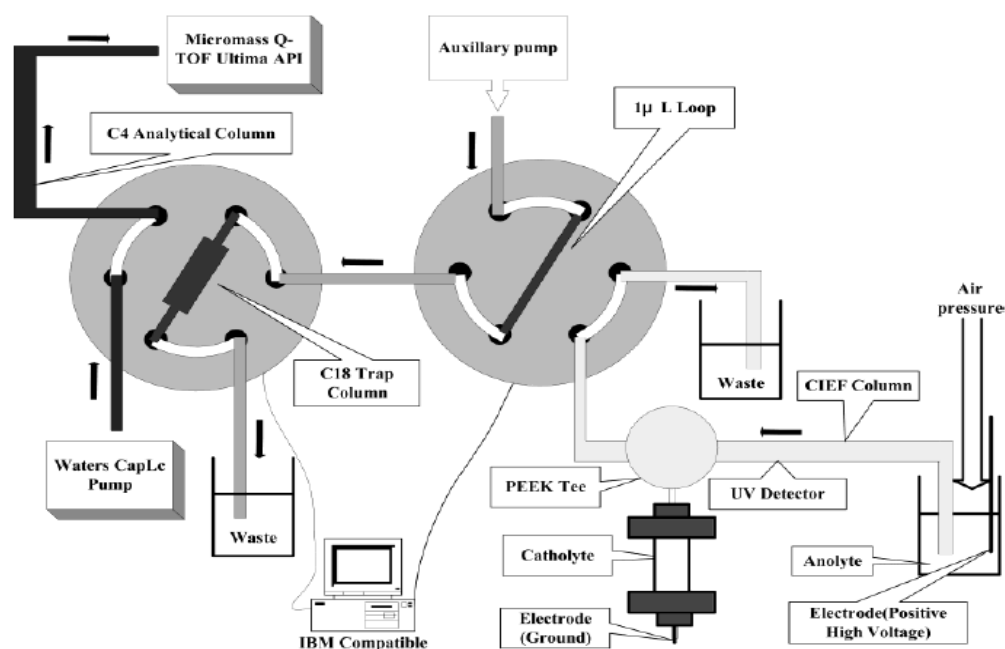


Figure 1.8: Schematic illustration of a CIEF-RPLC-MS system.³⁴

1.2.4.2 Monolithic column with immobilized pH gradient

Macroporous materials consist of an interconnected array of polymer microglobules separated by pores. Monoliths can be described as an integrated continuous porous separation media in micro-scale separation columns. The mobile phases are forced through the

separation media, which results in mass transfer between the stationary and mobile phases.³⁷ Monoliths have many advantages such as easy preparation, versatile surface modification, and high capacity. Because of these advantages, they have been widely used in scientific and industrial fields.³⁸ In this project, two simple methods are introduced to accomplish an IPG in the form of a monolith.

EDMA based M-IPG³⁶

Carrier ampholyte is a complex mixture containing thousands of different oligoamino and oligocarboxylic acids with molecular weights from about 300 to more than 1000. When CAs and glutaraldehyde are mixed together under proper conditions, CAs can react with aldehyde groups to produce Schiff bases.

A Schiff base (or azomethine), named after Hugo Schiff, is a functional group that contains a carbon-nitrogen double bond with the nitrogen atom connected to an aryl or alkyl group—but not hydrogen. Schiff bases are of the general formula $R_1R_2C=N-R_3$, where R_3 is an aryl or alkyl group that makes the Schiff base a stable imine.

This reaction is fundamental in M-IPG (Figure 1.9). Certainly the double bond $C=N$ should be reduced to stabilize the attachment. A focusing procedure fulfills the key role to achieve the immobilized pH gradient. It locates CA molecules in different positions according to their pI. This kind of immobilized pH gradient can be applied in IEF separation.

Poly acrylamide-based M-IPG^{39, 40}

The major problem encountered in protein separation is the adsorption to the inner wall or stationary phase of the column. Polyacrylamide is a neutral and hydrophilic polymer that has been widely used to solve this problem. Therefore, such a matrix was used to prepare M-IPG columns for protein analysis.

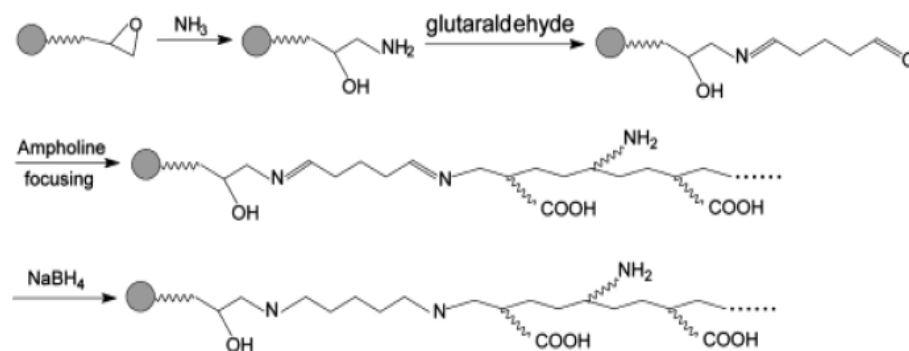


Figure 1.9: Fundamental reactions of M-IPG.³⁶

Preliminary experiments with poly GMA-EDMA, a hydrophobic monomer as the supporting material, did not produce good results. Thus, to improve the hydrophilicity of the polymer, GMA, acrylamide and *N, N'*-methylenebisacrylamide were chosen as monomers. Acrylamide and *N, N'*-methylenebisacrylamide could be dissolved in water but hardly in aliphatic alcohols, while the reverse was true for GMA. Therefore, DMSO was chosen as a porogen so that all monomers could be dissolved completely. In addition, 1, 4-butanediol proved helpful in improving the homogeneity, and a long-chain aliphatic alcohol, dodecanol, could improve the permeability of the monolith.

1.3 Objective of project

Sample preparation is often necessary to separate and concentrate various compounds prior to analysis of complex samples. In this regard, IEF is one of the best sample preparation methods. With this approach, however, CAs have to be introduced into the samples, which may result in matrix interferences. However, both gel IEF and solution IEF need CAs and some other chemicals to operate. For example, proteins are always dissolved in a solution with

high concentrations of ampholytes and often urea or detergents. These compounds have to be removed in the end because they interfere with protein analysis and they are incompatible with mass spectrometry.⁴¹ Therefore, a stable pH gradient without using CAs is considered desirable.⁴²⁻⁴⁸ Methods such as temperature gradient, concentration gradient of neutral substance, and electrochemical pH gradient are a few techniques explored in the quest for developing alternative approaches.

Creating pH gradients by electrolysis is not a new technique.^{46,49} The hydrogen ions and hydroxyl ions were generated by the decomposition of electrolyte at the electrodes. Under the electric field, H^+ and OH^- ions could migrate to cathode and anode, respectively, and thus create a pH gradient. The theory describing such a system was published by Hagedorn and Kuhr.⁵⁰ Huang and Pawliszyn used the electrolysis of water to create a pH gradient in a capillary for IEF of proteins.⁵¹ The pH gradient was created in a capillary without CAs. However, it was difficult to extract the separated analytes from the capillary. To solve this issue, a combination of isotachopheresis (ITP) with carrier ampholyte-free IEF was performed in a commercial isotachopheretic apparatus.^{48,52} The combination of electrolysis and FFE was also performed in a microfluidic device in Yager's group.^{53,54} In their microfluidic channel, pH gradients were electrochemically formed. The pH gradients were visible observed and quantified by using acid-base indicators. These stable pH gradients have been applied for continuous concentration and separation. In this project, a simple ampholyte-free IEF free-flow electrophoresis design was developed for the separation of proteins.

Generally, CE-MS coupling is usually achieved with a coaxial sheath liquid interface,

which decreases the detection sensitivity because of the dilution of sample by the sheath liquid. The advantages of nanospray have been introduced previously. In order to accomplish the combination of CIEF and MS in our lab, modification of an offline apparatus for online coupling has to be done first. Another challenge is how to decrease the concentration of CAs before MS detection. The approaches involve (1) optimization of the condition of CIEF for MS coupling and (2) introduction of a makeup solution to dilute the CAs and assist ESI. To complete this project, research has been divided into five stages. These five stages include: (1) offline set-up of Protana nano-electrospray (offline) with MS; (2) modification of offline device for online coupling; (3) optimization of performance of electrospray device; (4) optimization of the CIEF; and finally (5) coupling CIEF with MS.

The other approach is using M-IPG to remove the CAs. CAs were immobilized in poly-based (GMA-co-EDMA) monolithic capillary and poly-based (GMA-co acrylamide) monolithic capillary to form a pH gradient. Mixed proteins samples would be separated after the preparation of monolithic column and the immobilization of CAs. The separation efficiency of IEF in M-IPG will be proved by MS detection.

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Chapter 2 Preparation and application of ampholyte-free FFE-IEF for protein fractionation

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Development of a Simple Ampholyte-Free Isoelectric Focusing Slab Electrophoresis for Protein Fractionation

Yanwei Zhan, Tibebe Lemma, Marcel Florin Musteata, and Janusz Pawliszyn.

The contributions of Tibebe Lemma involved experimental suggestions and helping with photograph. The contributions of Marcel Florin Musteata involved experimental suggestions and manuscript revision.

2.1 Introduction

Among the most useful chromatographic and electrophoretic techniques, isoelectric focusing (IEF) is a powerful separation tool. However, the limitations of using CAs are obvious. The omitting of CAs will facilitate coupling IEF with other techniques.

As mentioned in the introduction, FFE offers some advantages. One of them is easy collection after the separation. In this chapter, a simple and robust design is developed for the separation of proteins using FFE-IEF techniques without CAs. Dialysis membranes with 2% agarose gel are used to establish a stable pH gradient and four proteins are selected as model compounds. The colour of the selected proteins helps to visually observe the separation process. Following FFE, the collected samples were analyzed by capillary isoelectric focusing with whole column image detection in order to determine the fluid composition at each outlet; this approach

gives an accurate depiction of the separation that takes place within the developed device.

2.2 Experimental Sections

2.2.1 Chemicals and materials

β -Lactoglobulin (bovine), hemoglobin (human), myoglobin (horse heart), cytochrome c (horse heart), carrier ampholytes (Pharmalytes 3.0-10.0), tris (hydroxymethyl)-aminomethane (Tris), dialysis membrane (tubing, COMW 12,000), PVP (MW 360,000) were purchased from Sigma (St. Louis, MO, USA). Agarose 15 was purchased from BDH Electran (BDH Laboratory Supplies). pI Markers (4.65, 8.40) were purchased from Convergent Bioscience (Toronto, Canada), silanized glass beads (20/30 MESH) were purchased from Chromatographic Specialties Inc., glass wool was purchased from Fisher Scientific. All solutions were prepared using deionized water (NanoPurity). Unless otherwise stated, all chemicals were of analytical grade.

2.2.2 Fabrication of the separation chamber

The schematic design of the apparatus is shown in Figure 2.1. Two microscope slides (75 mm \times 50 mm) were glued with two electrolyte reservoirs (plastic pipettes with stoppers) to form a chamber. The dimensions of this separation chamber are 75 \times 50 \times 3 mm. Dialysis membranes were mounted at the two ends of glass slides, which are in the electrolyte reservoirs. Two percent agarose gel was sealed as a thin film on the dialysis membrane. Eleven outlets (small plastic pipette tips) were glued at the bottom part of the chamber. Soft plastic tubes with water clamps were connected to the outlets (tips). The water clamp worked as a valve which could control the flow rate of the outlets. Glass wool was put on the top of outlets. The chamber was filled with silanized glass beads as support medium. Glass wool can prevent the glass beads from falling down through the outlets and blocking tubes. The whole device was placed between two holders. The chamber was vertical when electrolysis and IEF was performed.

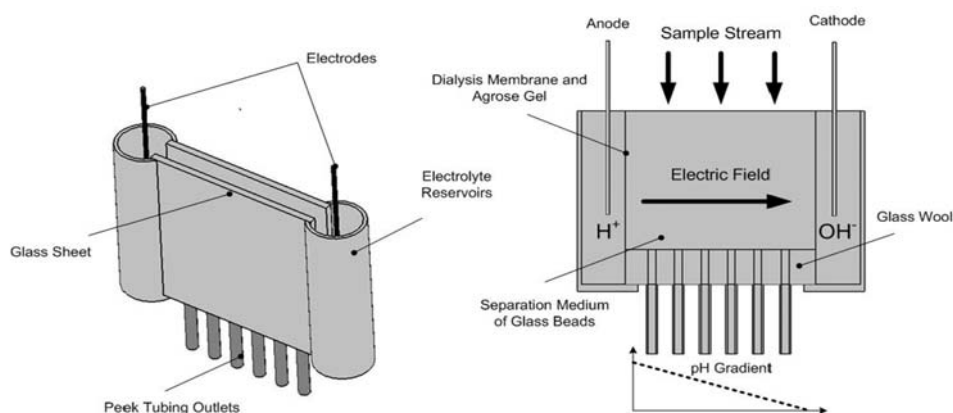


Figure 2.1: Schematic design of the apparatus for free-flow electrophoresis.

2.2.3 Sample preparation and the IEF procedure

Four proteins β -lactoglobulin (pI 5.1-5.3), hemoglobin (pI 7.0-7.2), myoglobin (pI 6.9-7.3) and cytochrome c (pI 9.6) were dissolved in 25 mmol/L Tris-HCl buffer (pH 7.25). All protein solutions were filtered using Acrodisc Syringe Filter (0.2 μm super membrane, Life Science). Sample I was a mixture of hemoglobin and β -lactoglobulin (10 mg/mL each), Sample II was a mixture of myoglobin and β -lactoglobulin (10 mg/mL each), and Sample III was a mixture of hemoglobin and cytochrome c (10 mg/mL each). Each model sample was separated using the proposed technique. The samples were directly introduced at the top of the separation chamber.

Before each run, the chamber and glass beads were rinsed with 25 mmol/L Tris-HCl (pH 7.25) buffer. Unless otherwise stated, Tris-HCl buffer was always used at this concentration and pH. Two electrolyte reservoirs were filled with the same buffer. Two platinum electrodes were inserted into the electrolyte reservoirs as anode and cathode. A constant voltage of 160 V was

applied with an EC 105 power supply (EC Apparatus Inc.). The current was also monitored by the same instrument. The temperature of the buffer in the chamber was monitored by a suitable thermometer (54 II Thermometer, FLUKE). At the beginning of the experiment, both current and temperature were higher and gradually decreased as time progressed. Twenty milliamperes (mA) was the maximum current in our FFE system and 65°C was the maximum temperature in the separation chamber. Usually, the current dropped to 1-2 mA, and the temperature decreased to 30°C after 30 minutes, then 0.2 mL mixed proteins was injected in the chamber at the top. The clamps of the outlets were opened after the sample injection. Gravity made the solution flow perpendicularly to the electric field. The flow rate of solution from outlets was controlled by clamps. Tris-HCl buffer was added continuously to the chamber from the top as make up solution in order to keep the chamber filled with liquid. Fractions from each outlet were collected in small tubes. Subsequently, each collected fraction was mixed with CAs (3-10) and PVP solution, and all the fractions were tested by iCE280 instrument to identify the sample components that eluted at each outlet. The pH value of each collected fraction in small tubes was tested by a MP 200 pH meter (Melter-Toledo, Sonnerbergstrasse, Switzerland).

2.2.4 CIEF analysis of fractions

CIEF was carried out using a whole column detection system (iCE280 analyzer, Convergent Bioscience, Toronto, Canada). The detection system consisted of a whole column optical absorption imaging detector operated at 280 nm. The light source of the whole-column UV absorption detector was a deuterium (D₂) lamp. The separation cartridge was a silica capillary (50-mm long with 100 µm i.d. and 200 µm o.d. from Convergent Bioscience) and the external polyimide coating was removed. The inner wall of the capillary was coated with fluorocarbon (Restek, Bellefonte, PA, USA) to suppress the EOF and to prevent protein adsorption onto the inner wall of the capillary. The two ends of the column were connected to

two pieces of dialysis hollow fibre membrane to separate the protein sample from the external electrolytes. The two sections of the hollow membrane were inserted into the electrolyte reservoirs. The further details about the commercial iCE280 instrument have been well described elsewhere.^{1,2} Protein samples mixed with CAs could then be continuously injected into the separation column without changing the electrolytes in the two electrolyte tanks. 100 mmol·L⁻¹ phosphoric acid and sodium hydroxide were used as anolyte and catholyte, respectively.

2.3 Result and Discussion

2.3.1 Preparation of chamber for free-flow electrophoresis

In FFE system, the electric field is applied perpendicularly to the direction of flow. High resolution separation depends on stable voltage, stable laminar flow and effective chamber cooling system. Furthermore, the generation of O₂ and H₂ by electrolysis requires the segregation of electrolytes and separation chamber. In order to accomplish this task, the chamber was designed as in Figure 2.1.

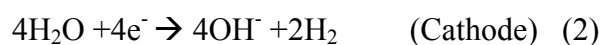
Usually, stable laminar flow is achieved with a precision pump. In this design, the pump was replaced by gravity and flow controlling valves. When the chamber was in a vertical position, the solution flowed downstream to the outlets because of gravity. The flow rate was controlled by the outlet valve, which simplified the design. The electrolyte segregation was achieved by the dialysis membrane and agarose gel. A dialysis membrane and agarose gel was used to prevent the diffusion of the protein mixtures into the electrolyte reservoirs and to prevent air bubbles from heading into the separation chamber. Another reason for using agarose gel was to decrease the conductivity of the chamber. Low conductivity could slow down the electrolysis reaction, which is very necessary in this system. Otherwise, it takes a long time to cool down the chamber, as no cooling system was attached to this chamber. At the beginning, the joule heat

resulted in high temperature in the electrolyte reservoirs and separation chamber. As time went on, the electrolysis slowed down and the whole system cooled. In order to avoid the effect of joule heat on separation, samples were injected after the system cooled down.

The chamber was filled with silanized glass beads, which worked as support medium and slowed down the diffusion of proteins. In order to prevent the glass beads from falling down through the outlets, glass wool was put on the top of outlets. The flow rate in this system was controlled by the water clamp working as a valve. When the electric field was applied, the outlets were closed. The electrolysis and the migration of cations and anions can last about 30 minutes without the perpendicular laminar flow. The pH gradient was created during this period. The samples can be injected into the chamber after the formation of the pH gradient. When the sample injection was finished, the outlets were opened. The samples were driven by two forces. One was the gravity, which leads the samples going down vertically. Another one was electrophoretic mobility under the electric field, which caused the analytes to move to cathode or anode and focusing due to their different charges. Driven by these two forces, analytes can focus on a certain position where the pH gradient is pre-established. A suitable flow rate was optimized by using hemoglobin as an analyte. Hemoglobin could be observed focused in a narrow line before it went out of the chamber through the outlets.

2.3.2 Formation of the pH gradient

In the presence of an electric field, redox reactions occur at the anode and cathode.^{3,4}



The production rate at the electrodes is high at beginning. Currents are high and a lot of gas bubbles come off the electrodes. Joule heat leads to high temperatures in the electrolyte reservoirs and separation chamber. As the electrolysis goes on, more and more H^+ and OH^- ions

collect in the electrolyte reservoirs, respectively. The anode becomes acidic and the cathode becomes basic. A continuous pH gradient in the separation chamber is established by allowing the H^+ and OH^- to migrate into the separation chamber from opposite ends, via diffusion and electromigration. The pH gradient produced by electrolysis during these experiments was measured by testing the pH value of samples from each outlet one by one. As indicated in Figure 2.2, the pH values of outlet 1 to 11 were 2.3, 3.1, 3.7, 5.4, 6.6, 7.6, 8.1, 8.4, 8.5, 8.8 and 8.9, respectively. The slope is steep in the acidic area and is even in the basic area. The generation of the nonlinear pH gradient is due to several reasons. One possible reason is that the electrophoretic mobility of protons is different from that of hydroxyl ions. Another important influence on pH comes from the Tris-HCl buffer. This buffer has strong buffer capacity in pH range 7 to 9 because the pK_a of Tris is 8.05. It can be estimated that different buffer solutions will generate different pH gradient slope which can be utilized to separate different proteins. The generated pH gradient was applied to separate mixed proteins, based on their pI points.

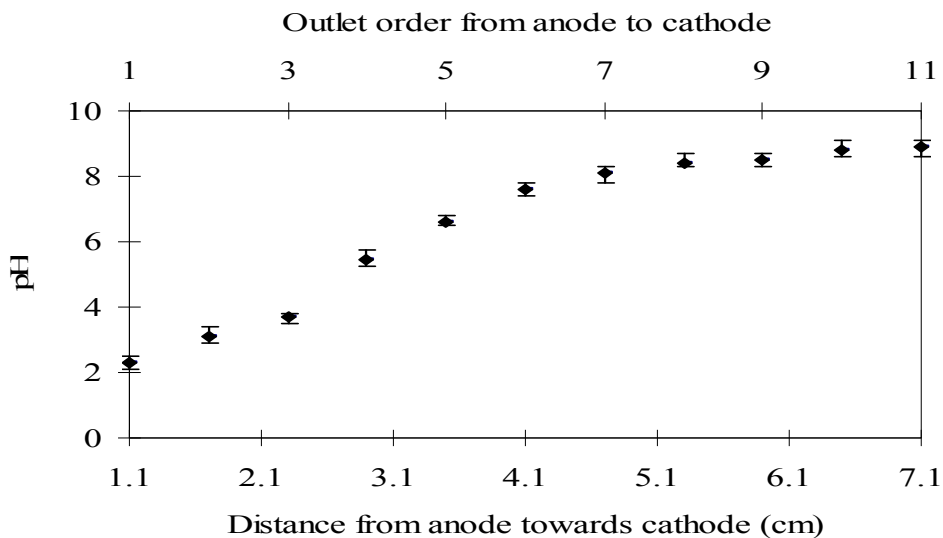


Figure 2.2: Measured pH values of the sample fractions in each outlet.

2.3.3 Application for protein separation

Due to the nature of the samples (brown colour or red colour), the focusing dynamics could be observed visually as the focusing proceeded. The whole separation process was recorded by taking photos at different intervals. Four photos that were marked (A), (B), (C) and (D) in Figures 2.3, 2.4 and 2.5 showed the different phenomena that occurred in electrophoresis as time went on. As shown in Figure 2.3, when mixed samples of β -lactoglobulin and hemoglobin were introduced into the separation chamber, the diffusion of proteins after injection could be clearly observed in Figure 2.3 A. After about 3 minutes, hemoglobin began to focus, as shown in Figure 2.3 B (a dark dot could be seen). Because not all the hemoglobin focused at this time, the coloured mass around the dark dot was still brown hemoglobin. As the electrophoresis went on, it was very clear that the hemoglobin focused into a line without any coloured mass shown in the Figure 2.3 C. Figure 2.3 D shows the elution of focused hemoglobin into outlet 6. The focusing process of β -lactoglobulin could not be seen because this protein is colourless.

Similar to Figure 2.3, the process of myoglobin focusing is clearly shown in Figure 2.4.

The process of focusing and separation of Sample III (hemoglobin and cytochrome c) is presented in Figure 2.5. Hemoglobin has brown colour and cytochrome c has red colour. Figure 2.5 A recorded the diffusion of proteins after the sample injection. After about 3 minutes, hemoglobin began to gradually focus into a dark line while cytochrome c did not focus. Figure 2.5 B shows the dark line in a red coloured mass. Because the pH gradient formed in this chamber was from 2.3 to 8.9, the pI of hemoglobin was covered and its focusing could be observed. However, the pI of cytochrome c (9.5) was not in the range of the pH gradient so that the focusing of cytochrome c could not be seen. Nevertheless, cytochrome c was positively charged when it was located in the middle of chamber as expected, as the process of

electrophoresis continued, hemoglobin remained in its focusing place while cytochrome c moved gradually to the cathode. Figure 2.5C recorded the trend clearly. Finally, hemoglobin and cytochrome c could be separated completely and eluted from different outlets as shown in Figure 2.5D. Although the generated pH gradient was out of range for cytochrome c to focus (as explained in the discussion part), the photos still showed complete separation of the two proteins. The separation can be confirmed further by testing all the collected fractions from different outlets using CIEF. Figure 2.6A exhibits the whole electropherogram of hemoglobin and β -lactoglobulin with two pI markers by CIEF-WCID. It can be seen from Figure 2.6B that β -lactoglobulin comes out of outlet 4. It is also clear that a lot of hemoglobin and some β -lactoglobulin come out of outlet 6 as shown in Figure 2.6D. Hemoglobin and β -lactoglobulin are still mixed in outlet 5 as shown in Figure 2.6C. The other samples collected from the rest of outlets such as 1 to 3 and 6 to 11 have not been shown in Figure 2.6 because no protein peak could be observed. The same situation can be observed in Figure 2.7. Myoglobin and β -lactoglobulin can be separated in this chamber. In Figure 2.8, hemoglobin comes out of outlet 6 and cytochrome c comes out of outlet 8. All these results did match the pH gradient. The pH value of outlet 4 is around 5.4 which is close to the pI of β -lactoglobulin. The pH value of outlet 6 is around 7.6, which is close to pI of hemoglobin. Cytochrome c went out of outlet 8; the pH value was 8.1, which did not match the pI of cytochrome c. This was due to the fast flow rate of samples.

A stable pH gradient was established only after electrolysis. If the sample was injected while the electric field was applied, no focus phenomena could be observed, and the coloured samples gradually went out to the outlets. The pH gradient created in chamber could last for some time. When the proteins from the first injected sample went out from the outlets, a new

protein sample was injected again into the chamber, and focusing of proteins could still be observed.

The pH resolution of the chamber is limited by its width. This resolution of the presently described chamber was not high enough to separate hemoglobin from myoglobin. Once the mixture of hemoglobin and myoglobin was injected into the chamber, only one focusing line was observed.

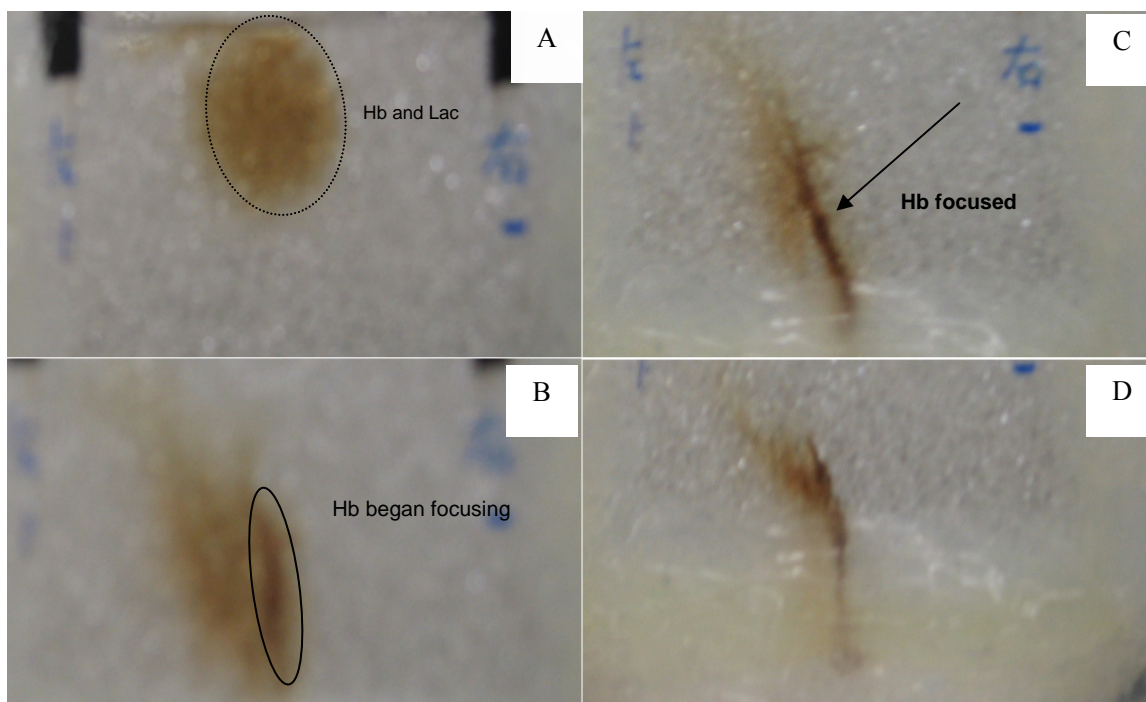


Figure 2.3: Photographic images show the isoelectric focusing process of hemoglobin and β -lactoglobulin at different periods. Photo A shows the diffusion of protein samples after the injection. Photo B shows the beginning of focusing. Photo C shows the clear focusing of hemoglobin as time goes on and Photo D shows the elution of focused hemoglobin into outlet 6.

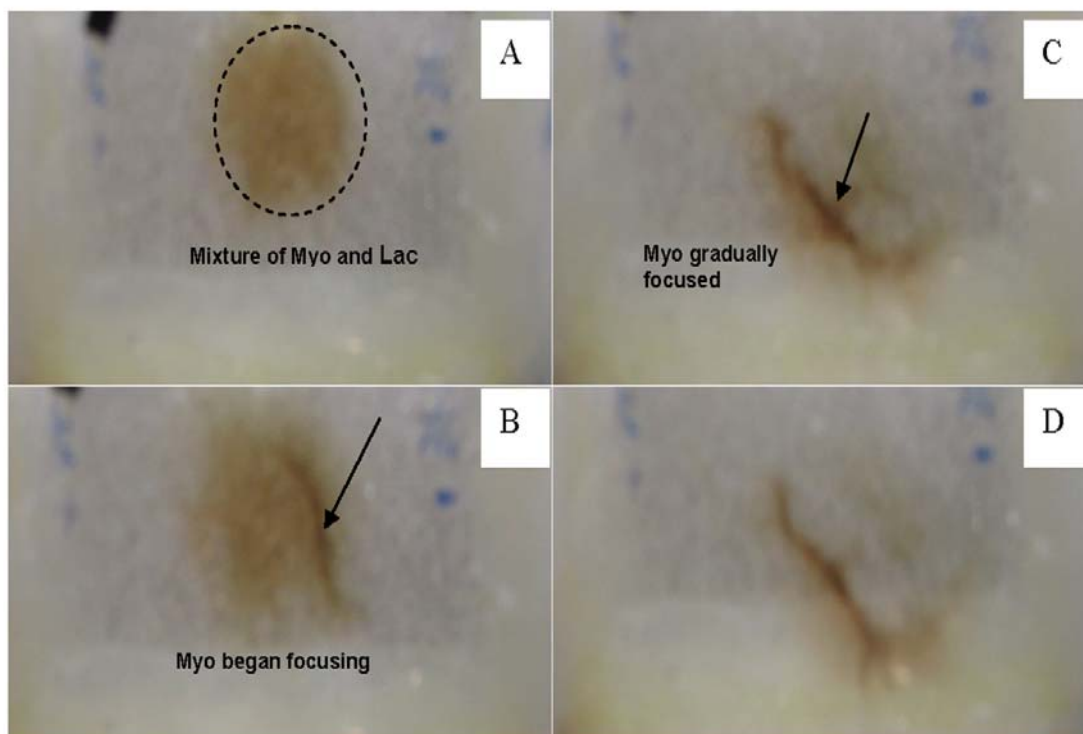


Figure 2.4: Photographic images show the isoelectric focusing process of myoglobin and β -lactoglobulin at different periods. Photo A shows the diffusion of protein samples after the injection. Photo B shows the beginning of focusing. Photo C shows the clear focusing of myoglobin as time goes on and Photo D shows the elution of focused myoglobin into outlet 6.

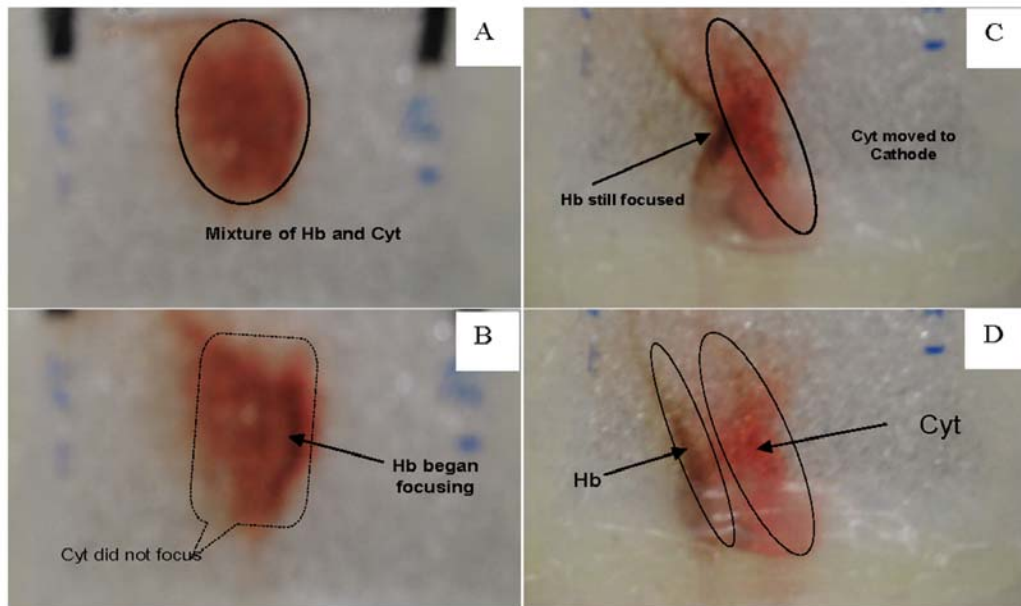


Figure 2.5: Photographic images show the isoelectric focusing process of hemoglobin and cytochrome c at different periods. Photo A shows the diffusion of protein samples after the injection. In Photo B, hemoglobin began its focus (dark line) while cytochrome c did not focus. In Photo C, as the electrophoresis time increased, hemoglobin still stayed at its focusing place while cytochrome c moved gradually to the cathode. Finally, hemoglobin and cytochrome c could be separated completely and eluted from different outlets as shown in Photo D.

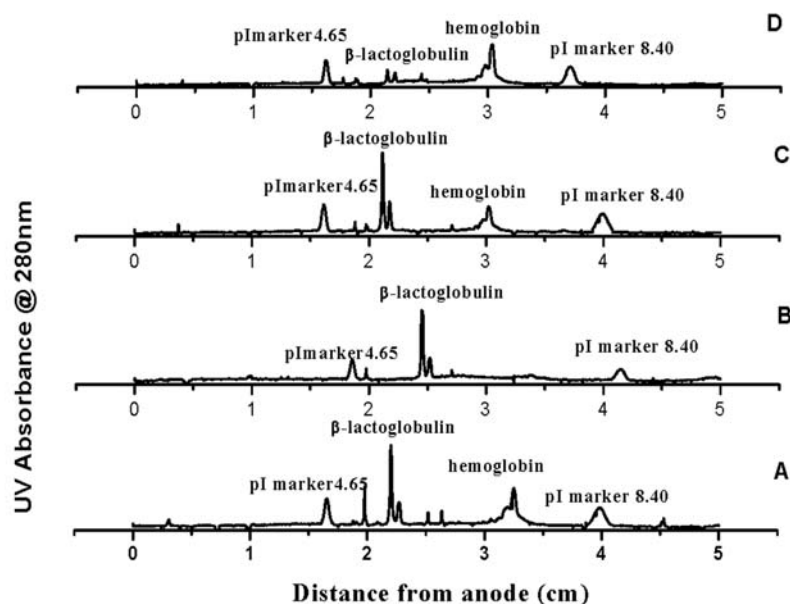


Figure 2.6: CIEF electropherograms of hemoglobin and β -lactoglobulin separation: (A) hemoglobin and β -lactoglobulin and two pI markers (control) (B) collected sample from outlet 4 (C) collected samples from outlet 5 and (D) collected sample from outlet 6. In profile A, Hb and Lac (0.2mg/mL each) were mixed with ampholyte buffer containing 2% carrier ampholyte pH 3-10, 0.5% pvp and 2 μ L of pI markers (pH 4.65 and 8.40). In profile B, sample fraction from outlet 4 was mixed with ampholyte buffer containing 2% carrier ampholyte pH 3-10, 0.5% pvp and 2 μ L of pI markers (pH 4.65 and 8.40). Profile C and D are for outlets 5 and 6, respectively. The profile A is used as a reference for profile B, C and D. The voltage used for focusing was 500V for the first 2 minutes and 3000V for the next 15 minutes. Peaks in the electropherograms are labelled.

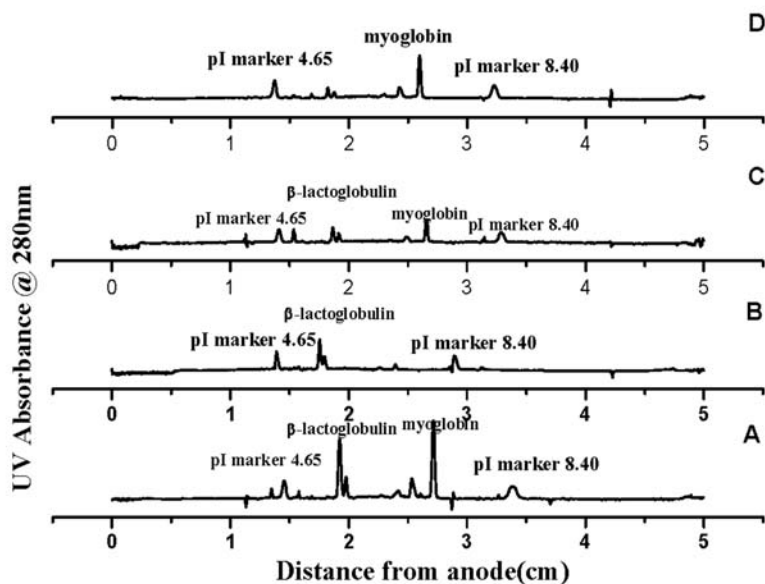


Figure 2.7: CIEF electropherograms of myoglobin and β -lactoglobulin separation: (A) myoglobin and β -lactoglobulin and two pI markers (control) (B) collected sample from outlet 4 (C) collected sample from outlet 5 and (D) collected sample from outlet 6. In profile A, Myo and Lac (0.2mg/mL each) were mixed with ampholyte buffer containing 2% carrier ampholyte pH 3-10, 0.5% pvp and 2 μ L of pI markers (pH 4.65 and 8.40). Other conditions are the same as in Figure2.6.

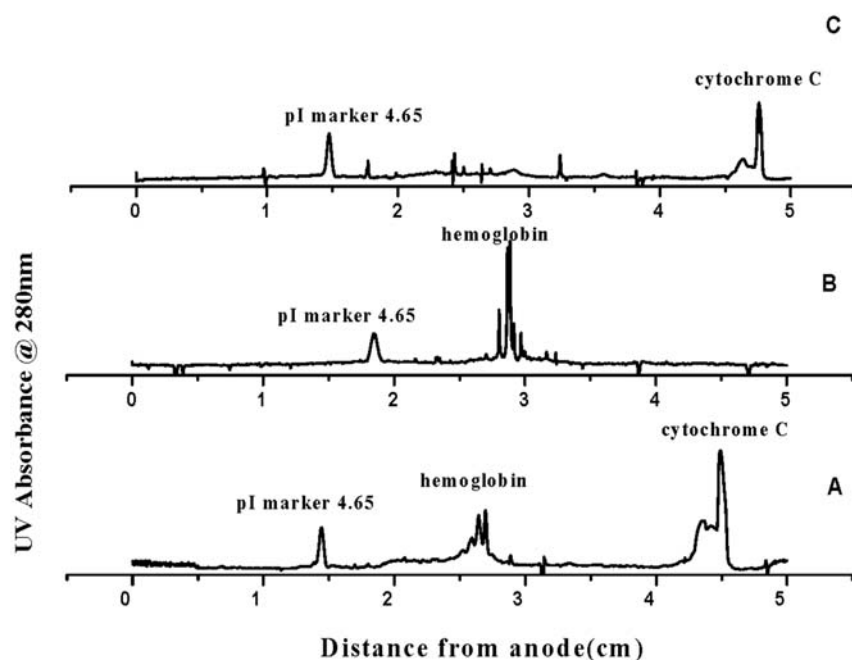


Figure 2.8: CIEF electropherograms of hemoglobin and cytochrome c: (A) hemoglobin and cytochrome c and one pI marker (control) (B) collected sample from outlet 6 (C) collected samples from outlet 8. In profile A, Hb and Cyt (0.2mg/mL each) were mixed with ampholyte buffer containing 2% carrier ampholyte pH 3-10, 0.5% pvp and 2 μ L of pI marker (pH 4.65). In profile B, sample fraction from outlet 6 was mixed with ampholyte buffer containing 2% carrier ampholyte pH 3-10, 0.5% pvp and 2 μ L of pI marker (pH 4.65). Profile C is for outlet 8. The profile A is used as a reference for profile B and C. Other conditions are the same as in Figure 2.6.

2.4 Conclusions

A simple and practical apparatus that can generate a pH gradient capable of separating proteins according to their pI has been developed. Even though the pH gradients have no buffer capacity and are not linear, our design still shows potential for easy and rapid sample fractionation or preliminary fractionation. In addition, samples can be withdrawn from the

appropriate outlets for further investigations (characterization) after the separation. This can save the cost of CAs and resolve the problem of having to remove the ampholytes before MS analysis. Therefore, it is ideal for coupling with MS.

Several factors can influence the pH gradient generated by the device, such as composition of buffer, time of electrolysis and the length of chamber.^{5,6} After further research on these factors is finished, it is anticipated that this approach will be very practical for industrial applications. The medium can be reused and the design can be easily automated. It is hoped that this method will be useful in designing microchip devices for micro-preparative separation of proteins as well as preparative-scale separation.

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Chapter 3 Coupling CIEF with mass spectrometry

3.1 Introduction

The objective of this project is to integrate CIEF with MS using a nano-electrospray interface for protein analysis. Modification of an offline interface for the online coupling of CIEF-MS is the main task of this project. Because a high concentration of CAs and most additives used in CIEF are not compatible with MS,^{1,2} decreasing the concentration of CAs is another task. The approaches involve (1) optimization of the condition of CIEF for MS coupling and (2) introduction of a makeup solution to dilute the CAs and assist ESI.

The advantage of a liquid-junction interface is the independent control of the separation and spray capillaries.³ Therefore, a microcross union, which is similar to liquid-junction interface, was selected to perform the coupling.

3.2 Experiments

3.2.1 Instrument: Protana nano-electrospray (Odense, Denmark), API 3000 (Applied Biosystems, MDS Sciex), iCE 280 (Convergent Bioscience), Syringe pump (Kd Scientific 780100 USA).

3.2.2 Chemicals and materials: Pharmalytes, cytochrome c (horse heart), myoglobin (horse heart) were obtained from Sigma Aldrich (Canada), testosterone in acetonitrile was obtained from Cerilliant (USA), ammonia; methanol, acetic acid and ethanol were obtained from Fisher Scientific (Canada).

Nano ES spray Capillary and modified upchurch microcross union was obtained from Proxeon

Biosystems (USA), Genuine Eppendorf® Geloader™ Tips (1-10µL) was obtained from Eppendorf (Germany). Spray capillary for modified union (Pico Tip™) was obtained from New Objective (USA). Capillary with 100 µm i.d. × 360 µm o.d. for monolithic column and connection was obtained from Polymicro Technologies (USA).

All solutions were prepared using deionized water (NanoPurity). Unless otherwise stated, all chemicals were of analytical grade.

3.2.3 Sample preparation: 1mg/mL testosterone standard solution was diluted to 1µg/mL testosterone in acetonitrile:water (50:50) solution,

Myoglobin and cytochrome c were dissolved in deionized water (1 mg/mL). Concentrated samples were diluted to desired concentration using methanol:water:acetic acid (50:49:1) solution or water.

All solutions were filtered using an Acrodisc syringe filter (0.2 µm super membrane, Life Science).

3.2.4 Methods

3.2.4.1 CIEF-WCID method: Please see Chapter 2 Experimental Section 2.2.4

3.2.4.2 MS optimization

The MS used was an API 3000 (Sciex Canada) triple quadrupole equipped with a Protana nano-electrospray ionization source. A home-modified electrospray interface was used to perform the direct infusion experiment and also to couple CIEF with MS. To obtain a stable spray, some parameters had to be carefully adjusted and optimized. Samples were pushed through the capillary and spray tip to MS by syringe pump. The quadrupole was scanned from m/z 500 to 1500 at a scan rate of 3 second/scan.

Some important parameters such as composition of background solution, spray voltage,

spray tip position, and concentration of analytes were studied.

3.2.4.3 Coupling CIEF with MS

When CIEF was completed with an iCE instrument, the cartridge was taken out. One connecting capillary next to the anode was connected to a syringe pump using a capillary adapter and a sleeve, another connecting a capillary next to the cathode was connected to one end of the microcross union using a capillary adapter and sleeve. The makeup solution (methanol, water, acetic acid) was introduced from the other end by capillary and syringe pump. The mixture was pushed to the spray tip for ESI. Figure 3.1 shows the schematic connection. The MS instrument can record spectra of the whole process. From the total ion current spectrum, any time point, and any time range can be chosen to check the mass spectrum of analyte at that time point or check the average mass spectrum within that time range.

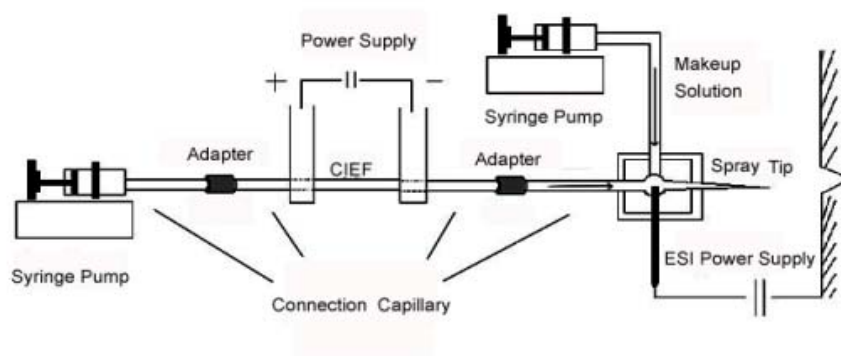


Figure 3.1 Schematic connection of CIEF with MS via pressure from syringe.

3.3 Results and Discussion

3.3.1 Offline nano-electrospray with MS



Figure 3.2: Picture of Protana interface with API 3000.

The Protana nano-electrospray interface system (Figure 3.2) consists of three major components: the ion source, microscope and monitors, and spray tip holder. The holder has an arm for mounting the spray tip. The arm is controlled by an X-Y-Z manipulator. This manipulator and monitors are used for fine adjustment of the position of spray tip in front of the orifice of a MS

Loading samples into spray tips without introducing any air bubbles is not easy and requires practice.

Testosterone, cytochrome c, and myoglobin were tested with the Protana nano-electrospray device. In positive mode, the strongest signals for testosterone were obtained at m/z 289.1 and m/z 289.4 (shown in Figure 3.3) in the conventional ESI and nano-electrospray, respectively. It clearly shows that the nano-electrospray source has been successfully set up with the MS.

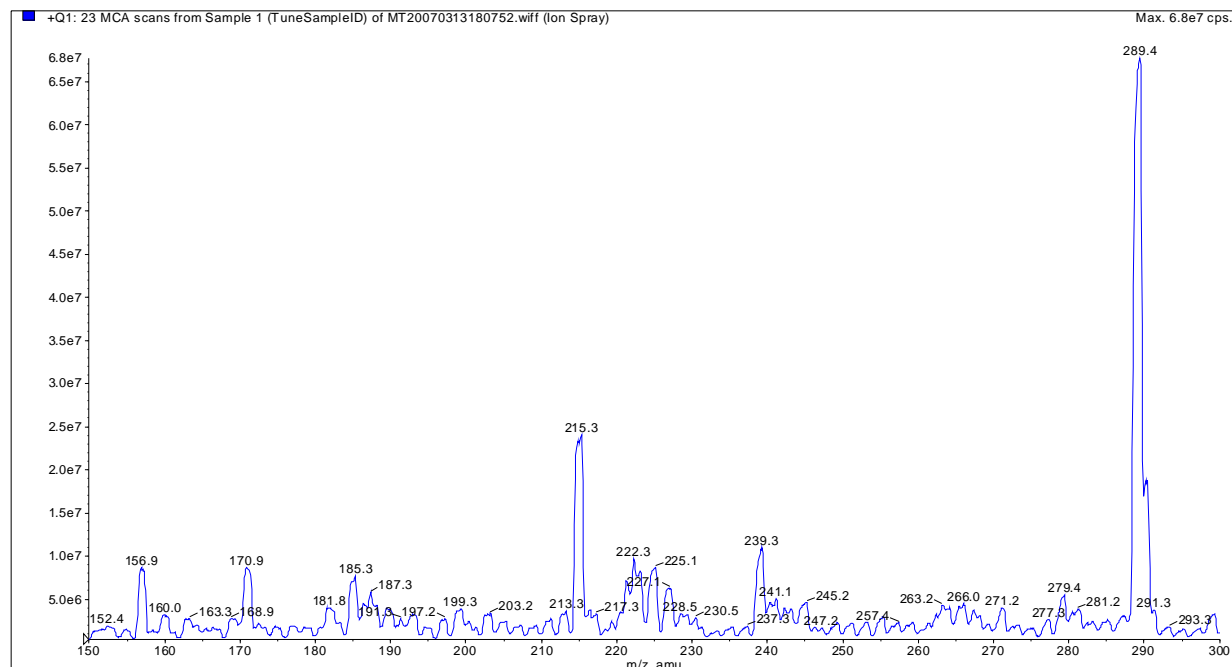


Figure 3.3: Positive nano-electrospray ionization mass spectrum of testosterone.

Electrospray produces multiple charged ions for proteins. There are several equations to calculate the number of charges and the molecular weight.⁴ For adjacent peaks, assume $N_2 = N_1 + 1$, where N is number of charges (raw value is rounded to the nearest integer). The detected M (m/z) is given by

$$M_1 = (MW + N_1) / N_1 \tag{2}$$

where MW is the actual mass. The measured M is the sum of the mass plus the mass of the

protons forming the positive ion. Then

$$N_2 = (M_1 - 1) / (M_2 - M_1) \quad (3)$$

And

$$MW = N_2 (M_2 - 1) \quad (4)$$

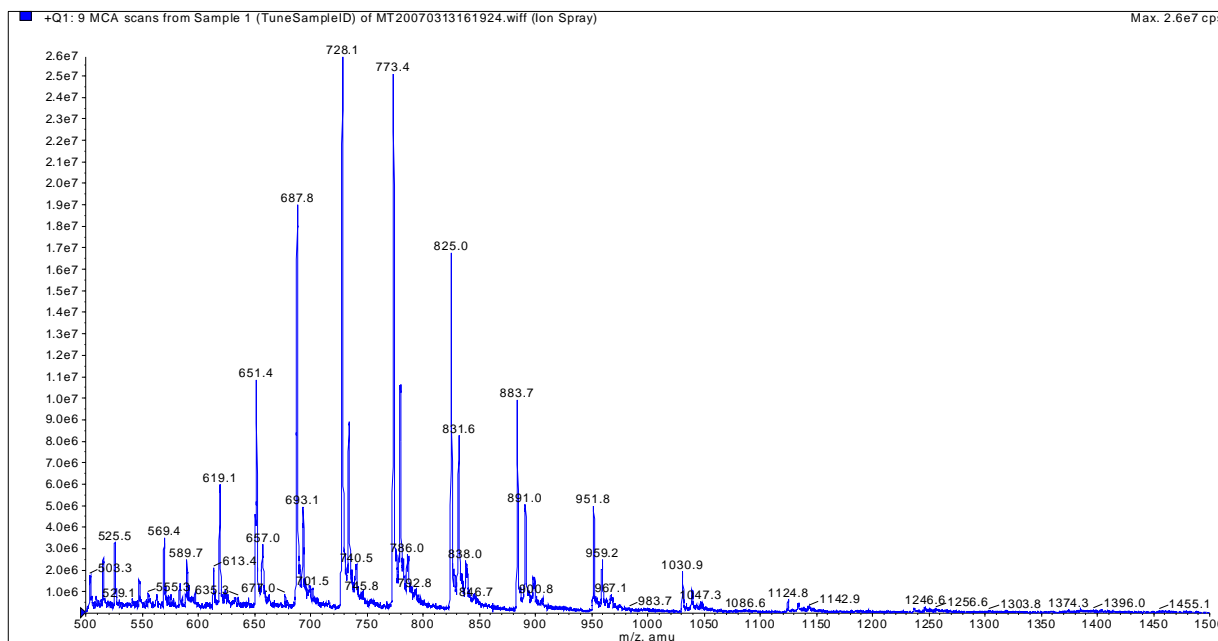


Figure 3.4: Positive nano-electrospray ionization mass spectrum of cytochrome c.

From these equations, in Figure 3.4, it can be calculated that m/z of 1030.9 is the result of 12 positive charges on cytochrome c. MW of cytochrome c is 12,359 Dalton. The charge range is from 12-21. From Figure 3.5, it can be calculated that m/z of 1305.1 is the result of 13 positive charges on myoglobin. MW of myoglobin is about 16,953 Dalton. These results acquired with the Protana nano-electrospray are in good agreement of related reports in the literature. This indicates that off line Protana nano-electrospray is working properly.

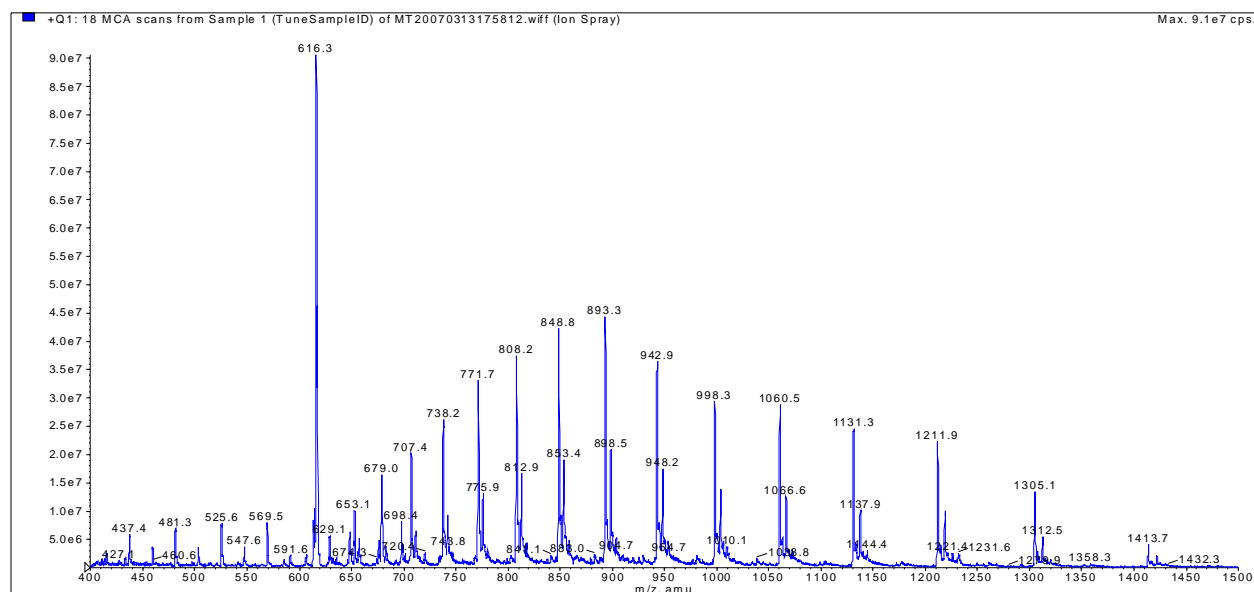


Figure 3.5: Positive nano-electrospray ionization mass spectrum of myoglobin

3.3.2 Modification of offline device for online coupling

In chromatography ESI-MS coupling, a sufficient flow of the eluent is assured and electrospray high voltage can be selected independently of the separation. However, in CE-ESI-MS, coupling requires finding the proper balance between the liquid flow, separation voltage, and the electrospray conditions. A variety of ESI interfaces have been previously developed for the CE-MS including coaxial sheath flow, sheathless, and liquid junction arrangements. The coaxial sheath flow interface is the only design available on commercial instruments. It is robust yet its sensitivity is usually lower due to the dilution of the solutes by the makeup liquid.

In our lab, only this offline design is available. To be compatible with the MS instrument, the electro power provided by the MS for ESI has to be utilized. WCID-CIEF has its own electric power system. After focusing, analytes are mobilized toward to the MS by pressure application via a syringe pump. It is similar to LC-ESI-MS. Based on the above description; a liquid-junction interface was chosen to replace the offline interface as shown in Figures 3.6 and 3.7.

This kind of union has four ends. One end has an integrated platinum electrode, which the electricity from MS for the ESI can be connected to. One end can be used for inserting the spray tip with the sleeve and the opposite end can be used for connecting the CIEF capillary. The fourth end is flexible. This flexibility is the advantage of this design, especially for coupling CIEF with MS.

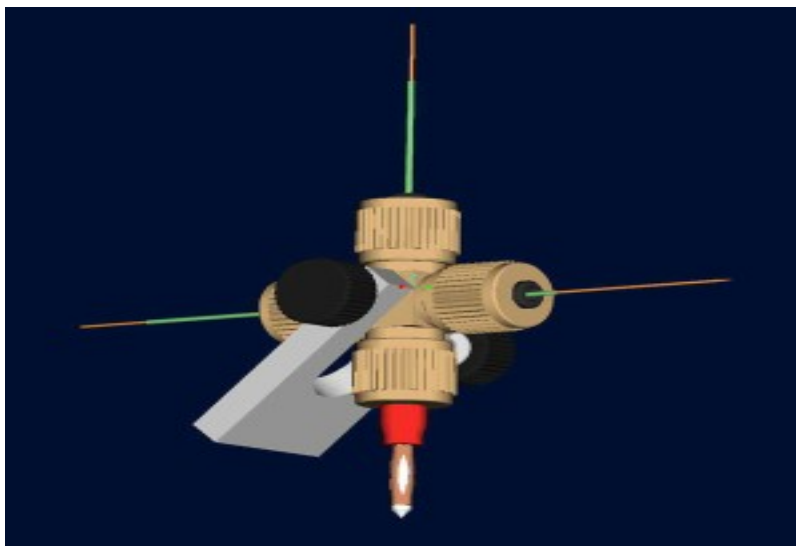


Figure 3.6: Picture of modified upchurch microcross union.

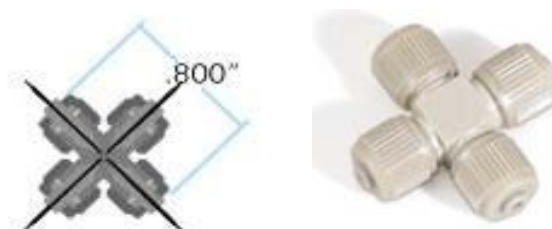


Figure 3.7: Picture and detailed structure of inside of microcross union.

After the union is chosen, the adapter for mounting the union on the arm of the Protana is

designed and fabricated in the machine shop of University of Waterloo as shown in Figure 3.8

3.3.3 Optimization of electrospray device

To obtain stable electrospray, some parameters have to be carefully adjusted and optimized. An infusion method was used. Samples were pushed through the capillary and spray tip to the MS by a syringe pump. Some important parameters such as composition of background solution, spray voltage, spray tip position, and concentration of analytes have been studied.

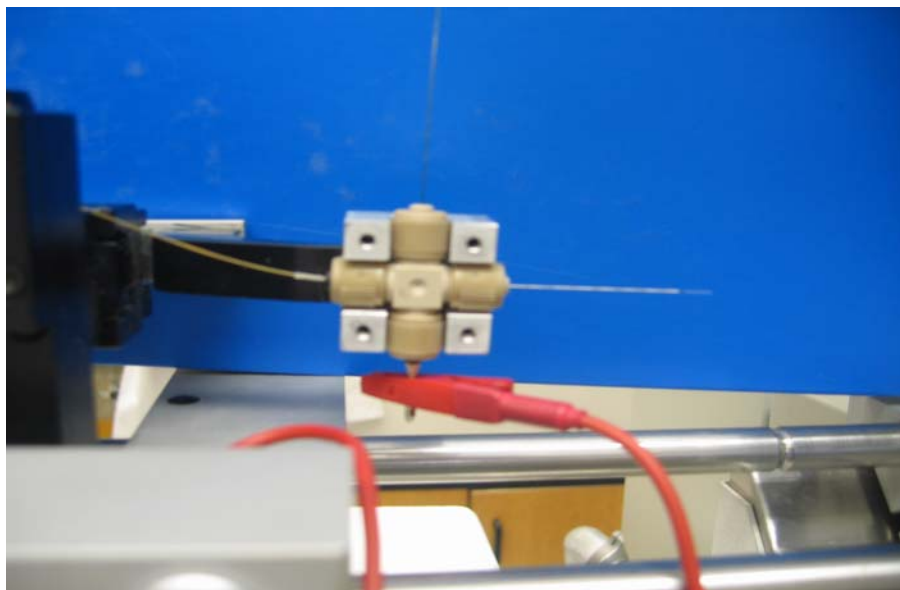


Figure 3.8: Picture of adapter for mounting the spray union.

Generally, the composition of background solution should depend on the analyte of interest. There are still some general rules. Our results confirm the general rule: moderately polar organic solvent is often required to achieve a stable spray. For example, the ESI of myoglobin under the condition of 99% water with 1% acetic acid is fairly poor. In the mass spectrum (Figure 3.9), no fingerprint of myoglobin can be obtained. This is likely because the surface tension of water is large so that it is difficult to get fine spray under regular voltage. The ESI of myoglobin in 99% methanol with 1% acetic acid shows some improvement because the surface

tension is decreased due to the organic solvent. Some of the fingerprint of myoglobin such as m/z 998 and 1060 can be recognized from Figure 3.10. The slight improvement is due to the fact that the efficiency of ionization of acetic acid in pure methanol is poor so that the protonation of myoglobin is not sufficient. In contrast, ESI of myoglobin under 50% methanol 49% water 1% acetic acid is very stable. The fingerprint of myoglobin is very clear in Figure 3.11.

The spray voltage is also important. Usually, there is a critical voltage below which no stable ESI can be obtained. When the spray voltage is higher than the critical, stable ESI can be obtained. However, higher voltage will give higher noise.

The position of spray tip is very important. From experience, if the tip is too far from the MS orifice, too few ions can enter the MS for detection. If the tip is too close to the orifice, the curtain gas will directly block the ions from getting into MS. There is a best position where best total ion current (TIC) and mass spectra of analytes are obtained.

After the optimization of position of spray tip and spray voltage, different concentrations of myoglobin, such as 100 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, and 1 $\mu\text{g/mL}$ in 50% methanol, 49% water, and 1% acetic acid, were tested. The mass spectrum of myoglobin at 100 $\mu\text{g/mL}$ is similar to the mass spectrum of myoglobin at 10 $\mu\text{g/mL}$ (Figure 3.11). The only difference is the peak intensity. When the concentration of myoglobin decreases to 1 $\mu\text{g/mL}$, the mass spectrum still showed the fingerprint of myoglobin, but the multiple charge characterization is not obvious as shown in Figure 3.12.

Stable ESI only can be acquired from a solution which has both organic solvent and water. This means that the protein sample after IEF has to be in solution with methanol:water:acetic acid. Therefore, it is necessary to introduce makeup solution after CIEF.

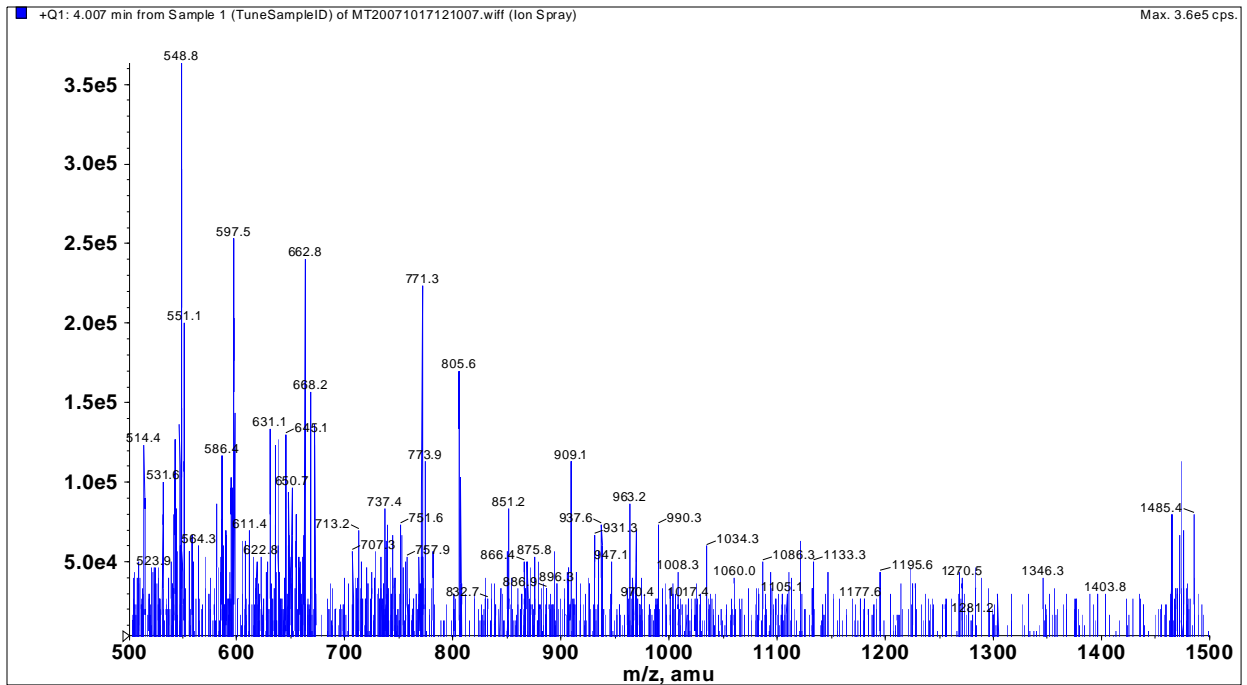


Figure 3.9: Mass spectrum of 10 $\mu\text{g}/\text{mL}$ myoglobin in 99% water with 1% acetic acid.

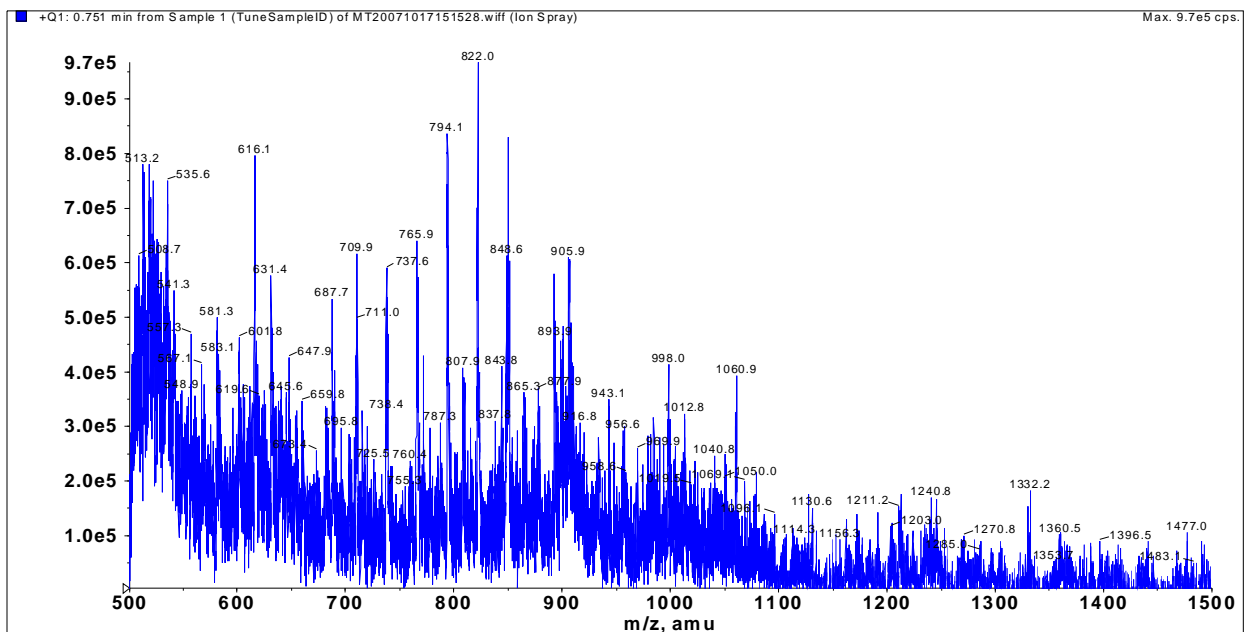


Figure 3.10: Mass spectrum of 10 $\mu\text{g}/\text{mL}$ myoglobin in 99% methanol with 1% acetic acid.

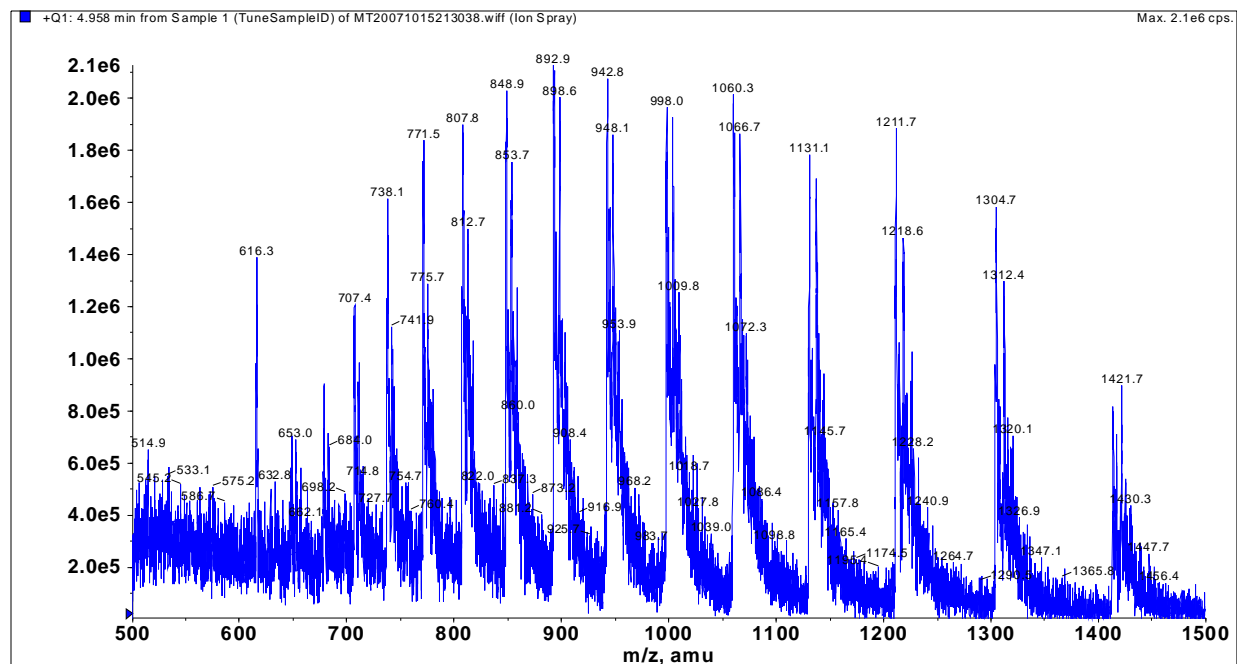


Figure 3.11: Mass spectrum of 10 µg/mL myoglobin in 50% methanol, 49% water, and 1% acetic acid.

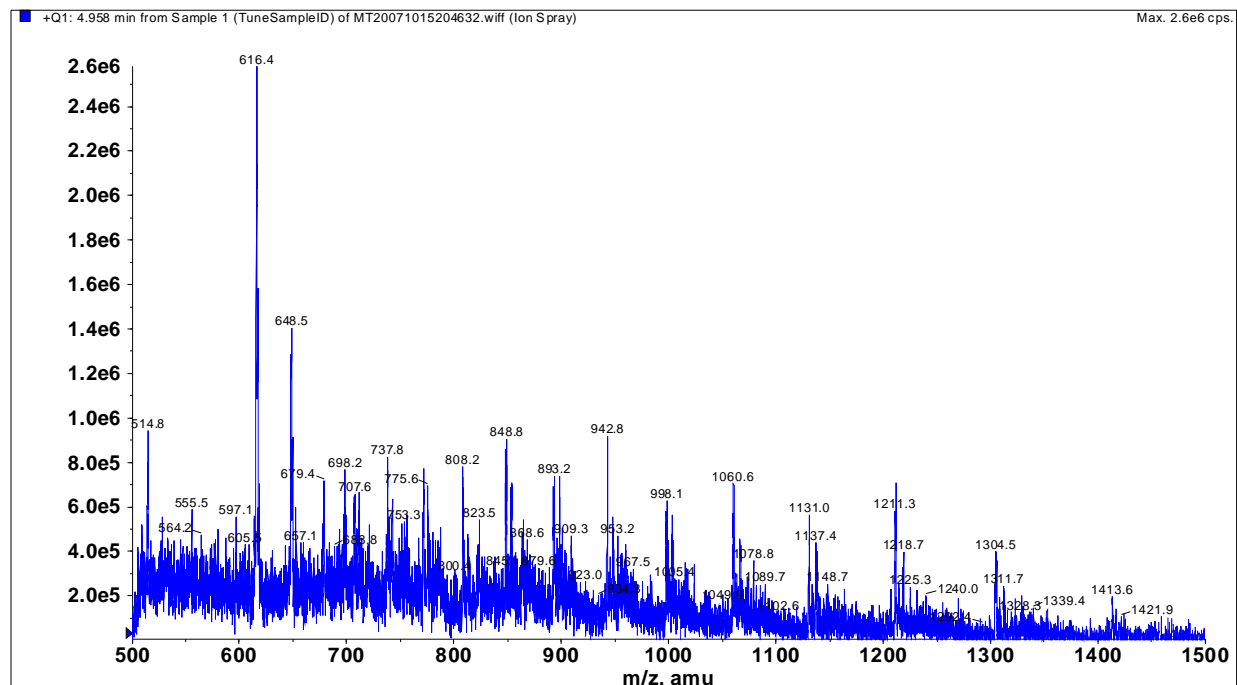


Figure 3.12: Mass spectrum of 1 µg/mL myoglobin in 50% methanol, 49% water, and 1% acetic acid.

3.3.4 Optimization of CIEF conditions for its coupling with MS

Coupling CIEF with MS can combine the high separation resolution from CIEF and the structure information from MS. However, the major difficulty in coupling is that high concentration of CAs and most of the additives used in CIEF are not compatible with MS. Usually, the buffer capacity of CAs is determined by their concentration. However, CAs suppress the ionization of analytes in ESI. There should be a trade-off between the concentration required for CIEF and the concentration tolerable for MS. A workable compromise can be found.

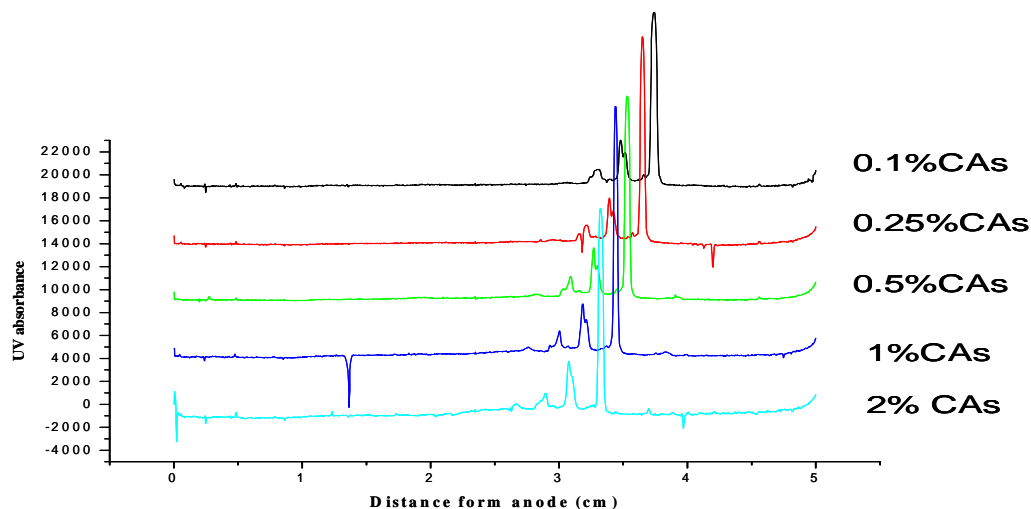


Figure 3.13: CIEF electropherograms of myoglobin in different concentration of CAs

Some proteins are easily precipitated and aggregated at their pI value. Many chemicals can be used as additives to reduce the chance of protein precipitation and aggregation during the CIEF. In CIEF methods, polymer solutions are usually added into the samples, such as methyl

cellulose (MC) and polyvinylpyrrolidone (PVP). The purpose of the polymer is to modify the surface of the separation column and to enhance the resolution of the separation by decreasing sample diffusion. In this research, they were omitted because their high viscosity can contaminate the MS. After taking all this into consideration, myoglobin was used as a standard analyte to test the effects of CAs with different concentrations without any additives and polymers. A total five of different concentrations of CAs (2%, 1% 0.5%, 0.25%, and 0.1 %) were evaluated with the same concentration of myoglobin added to each cartridge. The CIEF electropherograms are shown in Figure 3.13. From Figure 3.13, it can be found that when the concentration of carrier ampholytes decrease to 0.1%, the separation (three peaks of myoglobin) is still acceptable. There is no significant change of resolution and peak shape.

From Figure 3.13, some interesting phenomenon can be found. When the concentration of CAs gradually decreased, the peaks of myoglobin moved away from the anode. One reason that might cause the shift is because the inner coating is not strong enough to completely suppress the EOF. Another reason is that no polymer was added into the mixture of analyte and CAs. It shows the carrier ampholytes function not only to form the pH gradient and control the capacity of buffer. It also might have the capability of suppress the EOF. To test this hypothesis, solutions with three different concentration of CAs (0.01%, 0.5%, and 1%) and the same concentration of myoglobin were applied to CIEF using the same cartridge. A total of 30 minutes of focusing electropherograms were recorded. Four different times, 5, 10, 20, and 30 minutes, were chosen to demonstrate the whole process. Figures 3.14, 3.15, and 3.16 show the electropherograms of myoglobin with 0.01%, 0.5%, and 1% concentration of CAs.

From Figure 3.14, the electropherogram of myoglobin at 30 minutes has almost disappeared from the range of detection. In Figure 3.15, the electropherogram of myoglobin at

30 minutes was still in the detection range. It shows that higher concentration of CAs has high capability to suppress EOF.

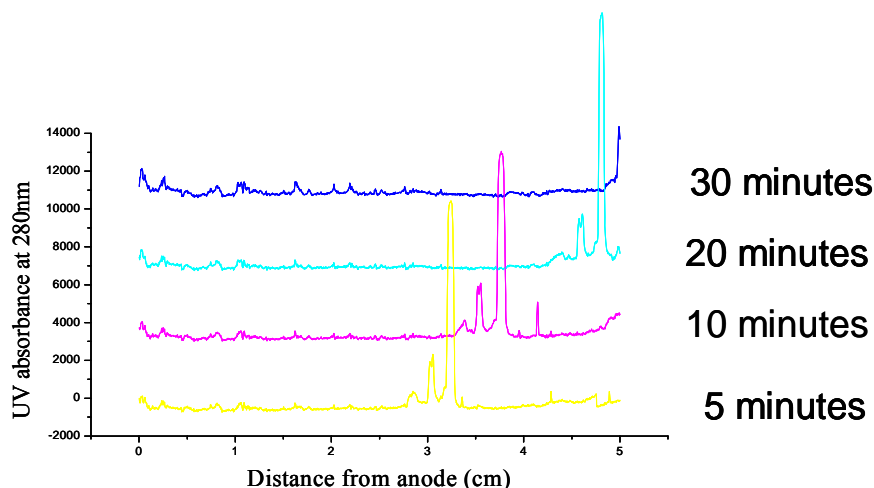


Figure 3.14: CIEF electropherograms of myoglobin in 0.01% of CAs.

3.3.5 Optimization of MS conditions for coupling CIEF

To prepare sample solutions with different concentration of CAs, different amounts of CAs were added to a series of solutions with the same concentration of myoglobin (20 $\mu\text{g/mL}$). An infusion method was used. The effects of CAs on ESI were tested by trying different CAs concentration such as 1%, 0.5%, 0.4%, 0.2%, 0.1%, and 0.01%.

From Figure 3.17, it is apparent that the ionization of myoglobin is totally suppressed in 1% of CAs. It showed that when the concentration of CAs decreased to 0.5%, the ionization of myoglobin can be clearly observed as shown is Figure 3.18. The ratio of signal-to-noise is not high enough. The ratio of signal-to-noise is improved when the concentration of CAs was decreased to 0.1% as shown in Figure 3.19.

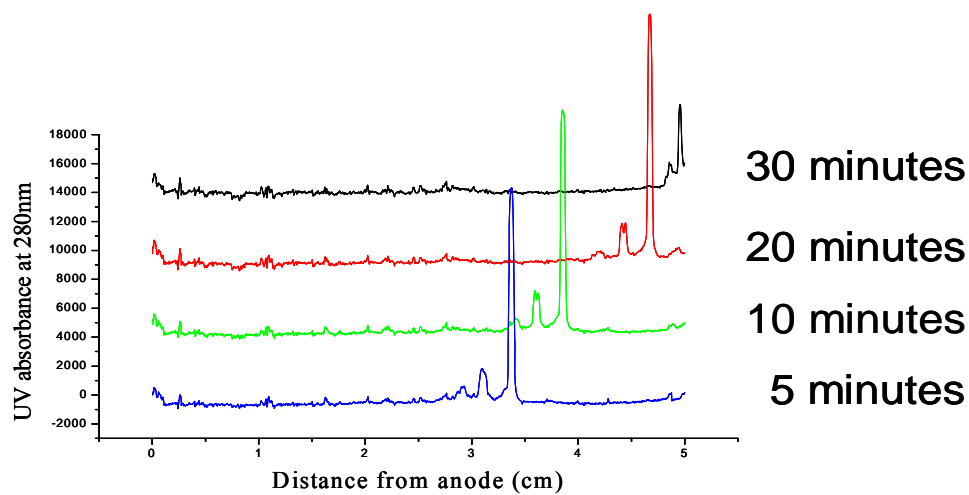


Figure 3.15: CIEF electropherograms of myoglobin in 0.5% of CAs.

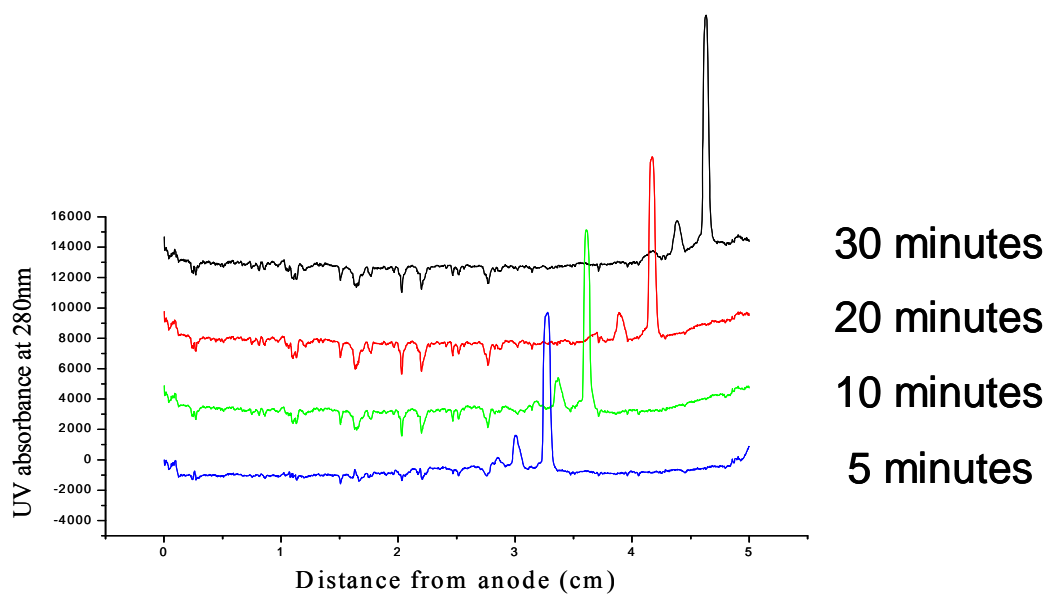


Figure 3.16: CIEF electropherograms of myoglobin in 1% of CAs.

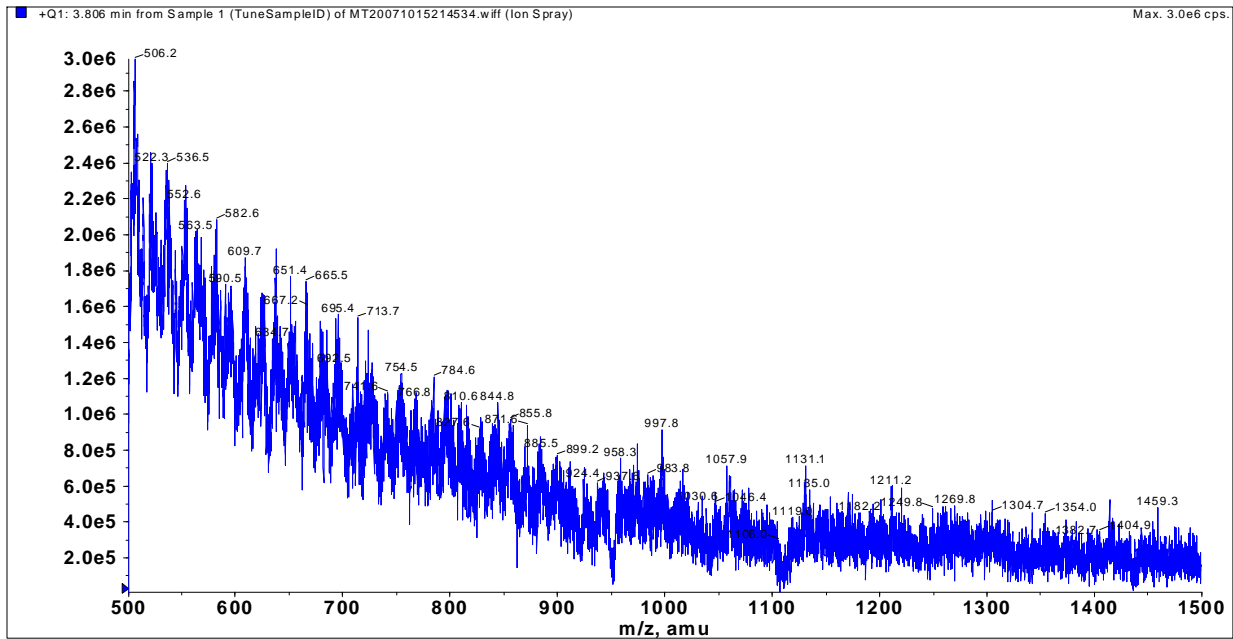


Figure 3.17: Mass spectrum of 20 µg/mL myoglobin in 50% methanol, 49% water, and 1% acetic acid with 1% CAs

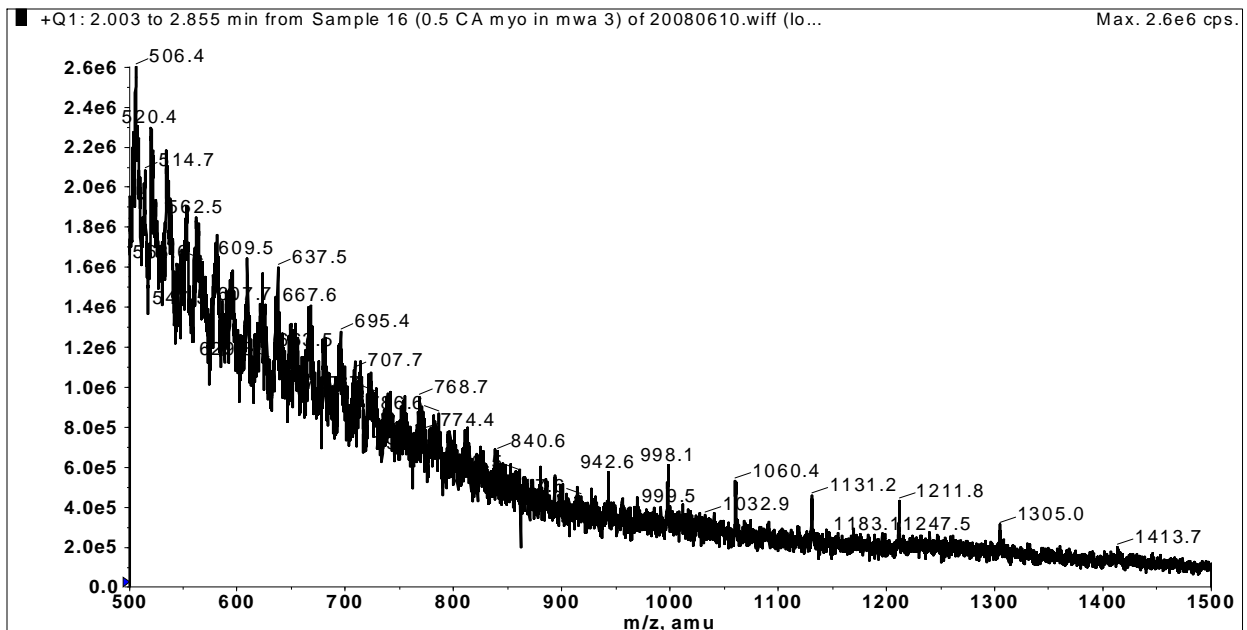


Figure 3.18: Mass spectrum of 20 µg/mL myoglobin in 50% methanol, 49% water, and 1% acetic acid with 0.5% CAs.

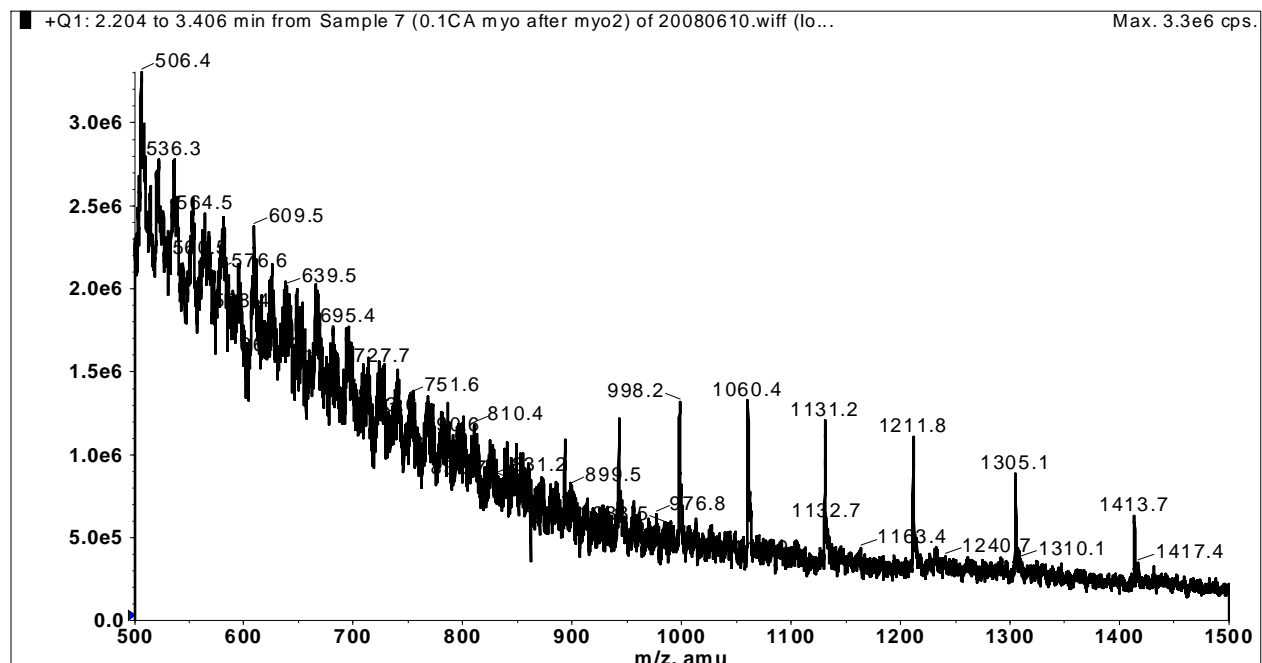


Figure 3.19: Mass spectrum of 20 µg/mL myoglobin in 50% methanol, 49% water, and 1% acetic acid with 0.1% CAs.

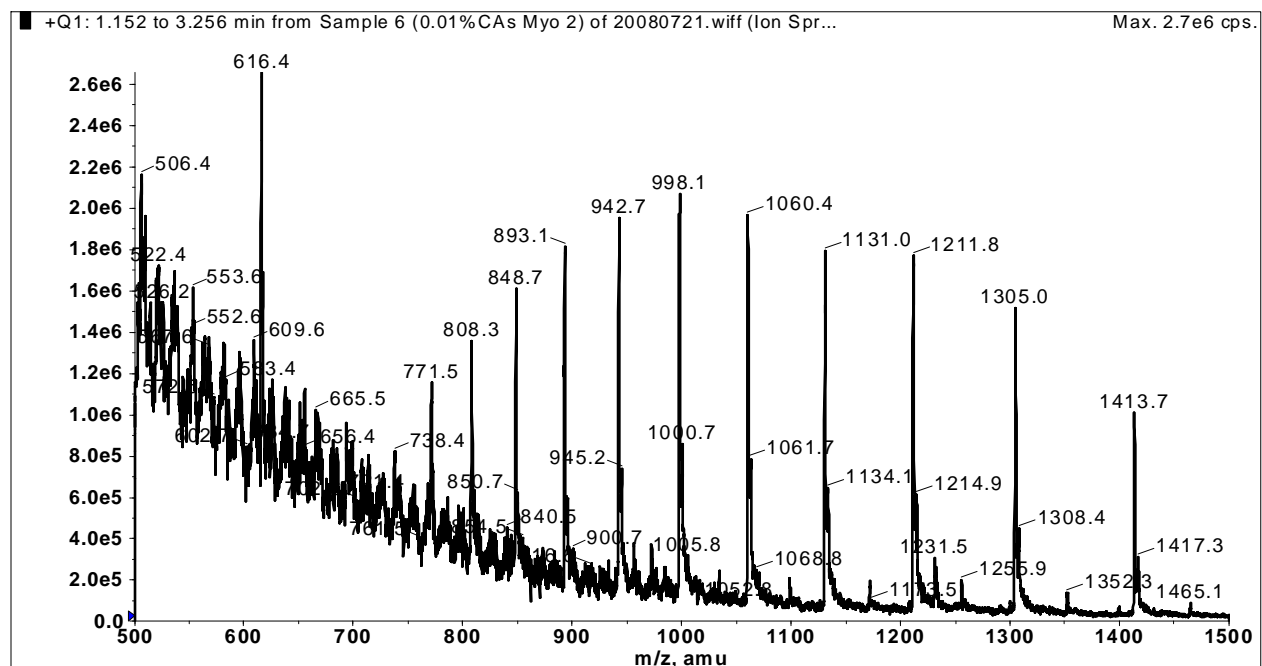


Figure 3.20: Mass spectrum of 20 µg/mL myoglobin in 50% methanol, 49% water, and 1% acetic acid with 0.01% CAs.

It is a very interesting observation that when the concentration of CAs was decreased to 0.01%, the effect of suppression was still observed from the mass spectrum and Figure 3.20 shows the effect of suppression is still obvious. This result argued against the merit of coupling using a dialysis system, therefore, the introduction of methanol, water, and acetic acid solution to dilute to concentration of CAs after focusing was evaluated. As mentioned previously, the methanol, water, and acetic acid solution can assist with ionization. The following experiments proved introducing makeup solution could end with better results.

A CIEF solution, 20 $\mu\text{g/mL}$ myoglobin in water with 0.1% CAs, was prepared. This solution was infused through the spray tip. The acquired mass spectrum of myoglobin, shown as Figure 3.21, is quite different. The fingerprint of myoglobin could not be observed. It proved again that the ESI effect in water solution without methanol and acid was poor.

Another CIEF solution, 40 $\mu\text{g/mL}$ myoglobin in water with 0.5% CAs, was infused from one end, the makeup solution (methanol, water, and acetic acid) was introduced from the other end. Both of them were mixed together in the cross section before they were being eluted through the spray tip for ESI. From Figure 3.22, the acquired mass spectrum of myoglobin clearly showed the fingerprint of myoglobin. It shows that ESI is good. From this result, it can be proved that the microcross union design is a proper design for coupling CIEF with MS.

3.3.6 Coupling CIEF with MS

After all the optimization was done, the WCID-CIEF separation was performed using 0.5% CAs without any additives and polymers. β -Lactoglobulin and myoglobin were selected as model analytes. After the focusing in iCE280 instrument, the cartridge was taken out. One connecting capillary next to the anode was connected to syringe pump using capillary adapter

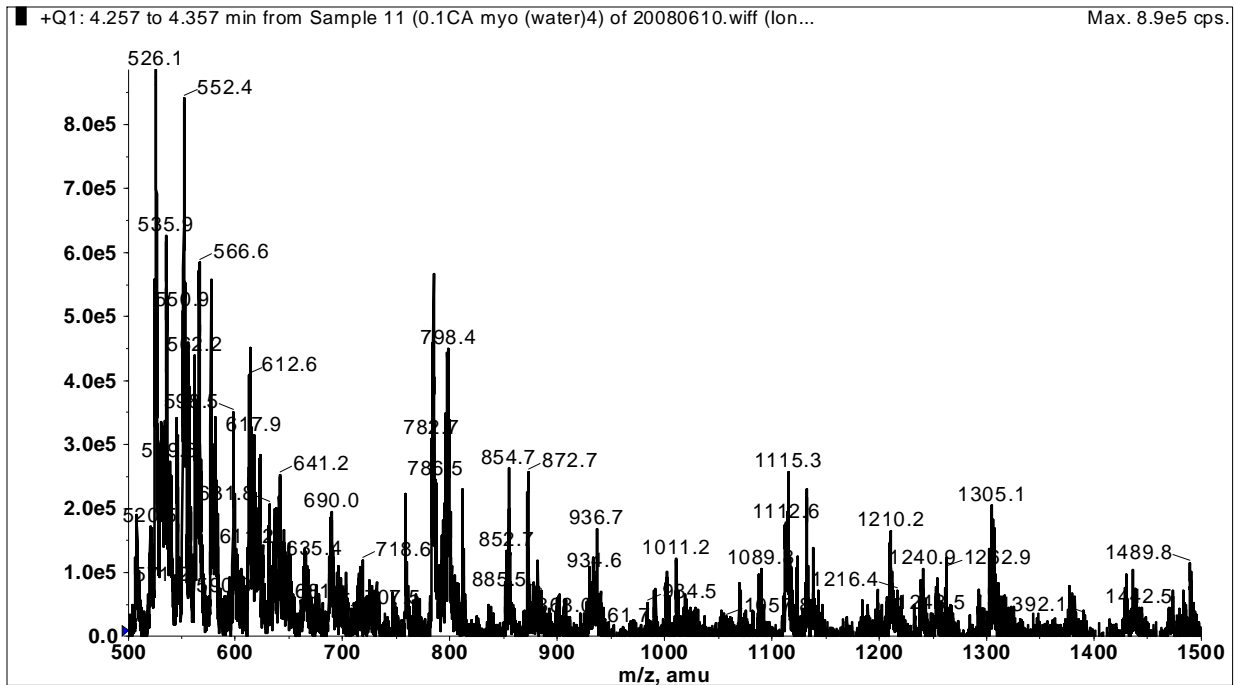


Figure 3.21: Mass spectrum of 20 µg/mL myoglobin in water with 0.1% CAs.

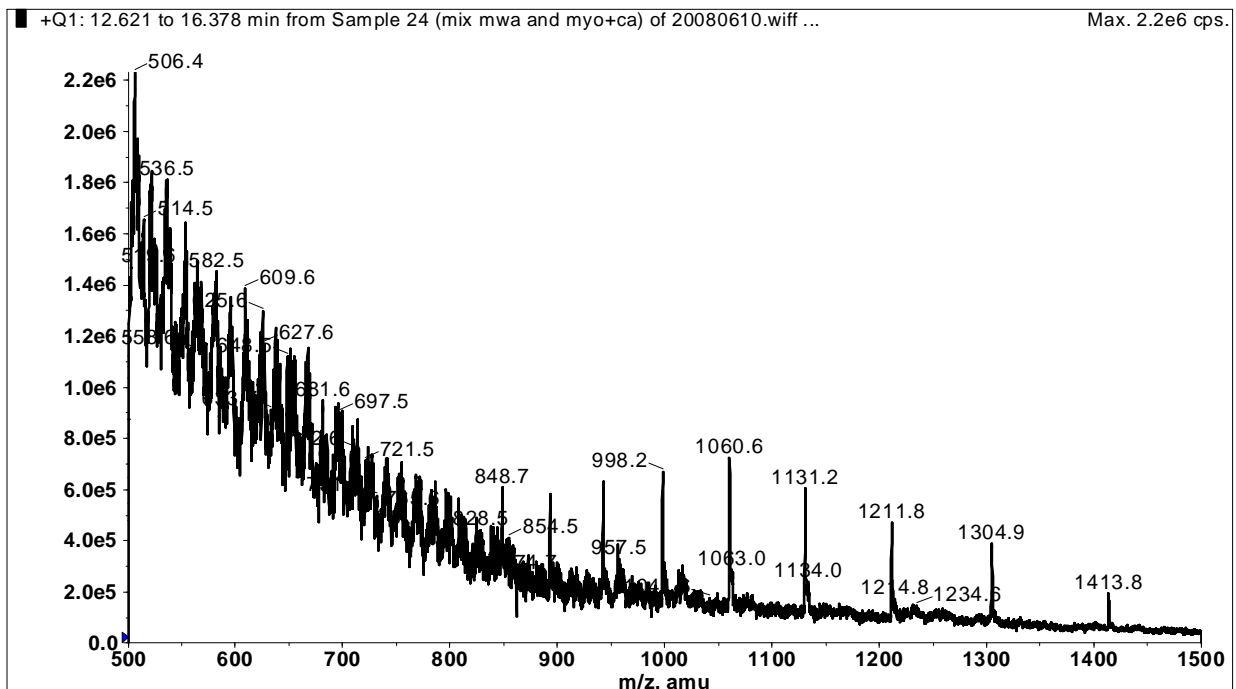


Figure 3.22: 0.5% CAs, 40 µg/mL myoglobin in water mixed with 50% methanol, 49% water, and 1% acetic acid solution.

and sleeves, capillary next to the cathode was connected to one end of the microcross union using capillary adapter and sleeves too. The MWA (methanol, water, and acetic acid) was introduced by capillary and syringe pump. The mixture was pushed to the spray tip for ESI. MS can record the spectra of the whole process. From the total ion current spectrum, any time point and any time range can be chosen to check the mass spectrum of analyte at that time point or check the average mass spectrum within that time range. Figure 3.23 is the TIC spectrum of β -lactoglobulin and myoglobin which were focused in WCID-CIEF before they were sent to MS for detection. Theoretically, myoglobin will be closer to the cathode than β -lactoglobulin because pI of myoglobin is 6.8-7.2 while the pI of β -lactoglobulin is 5.1 to 5.3. Accordingly, the myoglobin will be eluted before the β -lactoglobulin. From Figure 3.23, three different three time intervals which were marked as A, B, and C were chosen to check the mass spectrum for identification. Before the identification, the fingerprint of β -lactoglobulin should be tested and shown.

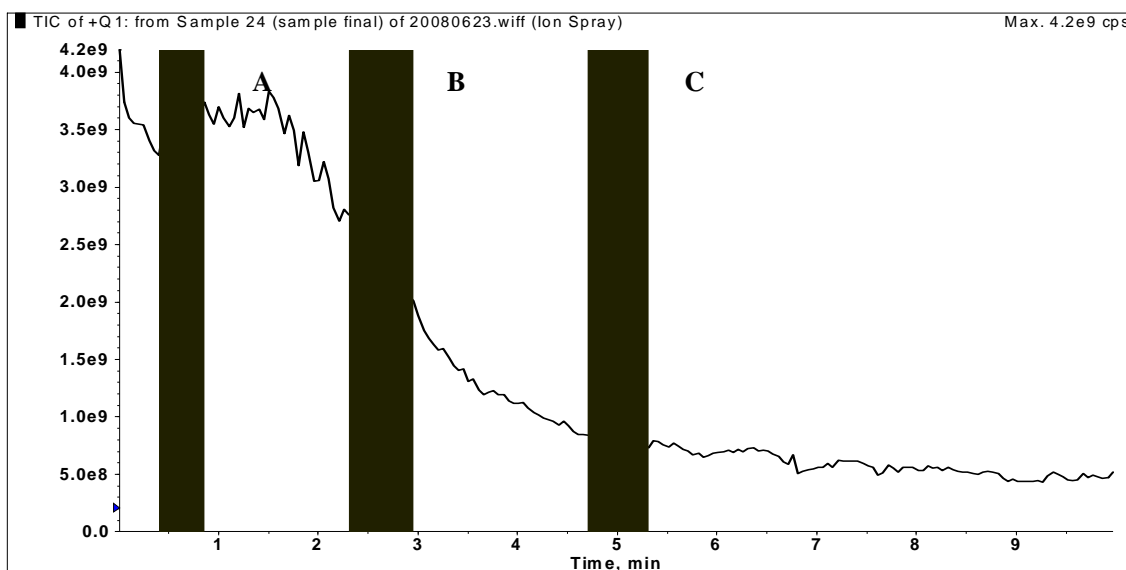


Figure 3.23: MS total ion current of samples after the separation in CIEF.

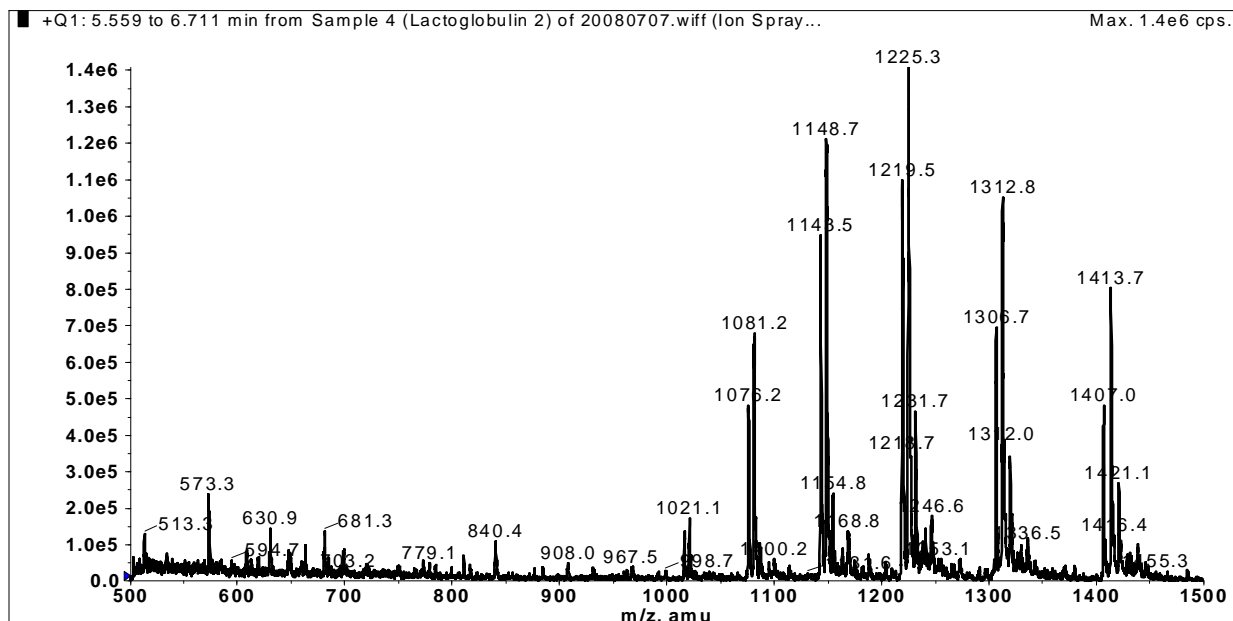


Figure 3.24: Mass spectrum of 10 µg/mL β-lactoglobulin in 50% methanol, 49% water, and 1% acetic acid.

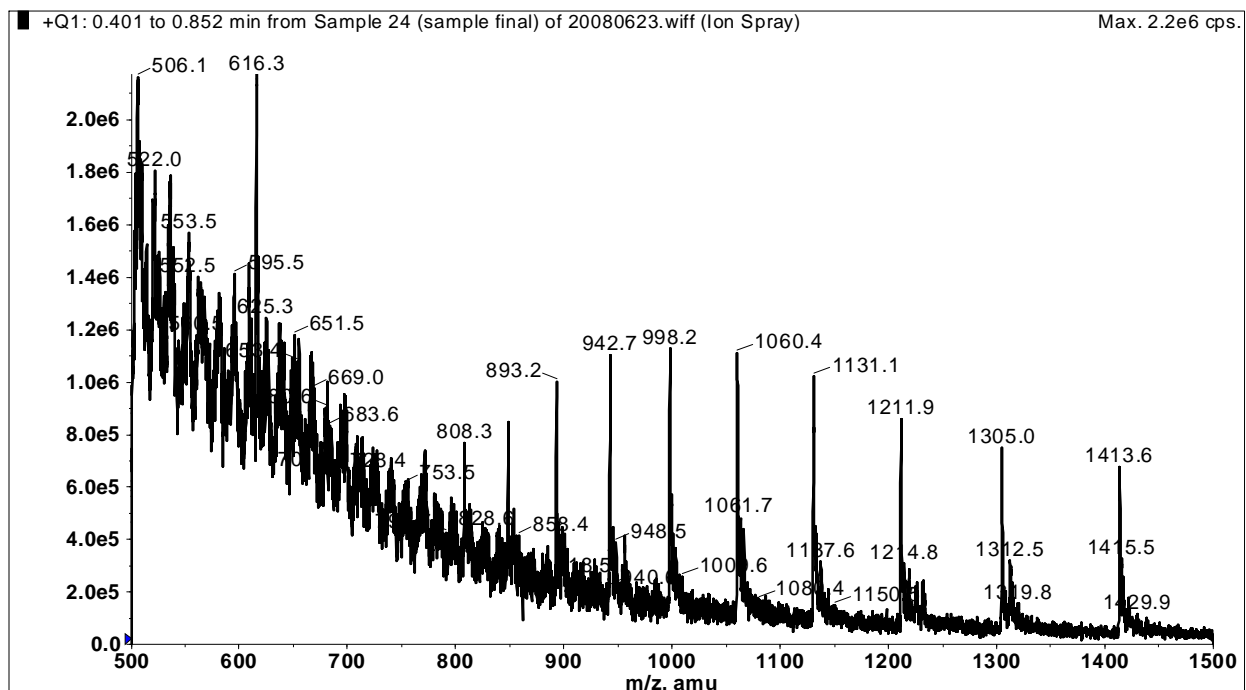


Figure 3.25: Average mass spectrum of time A.

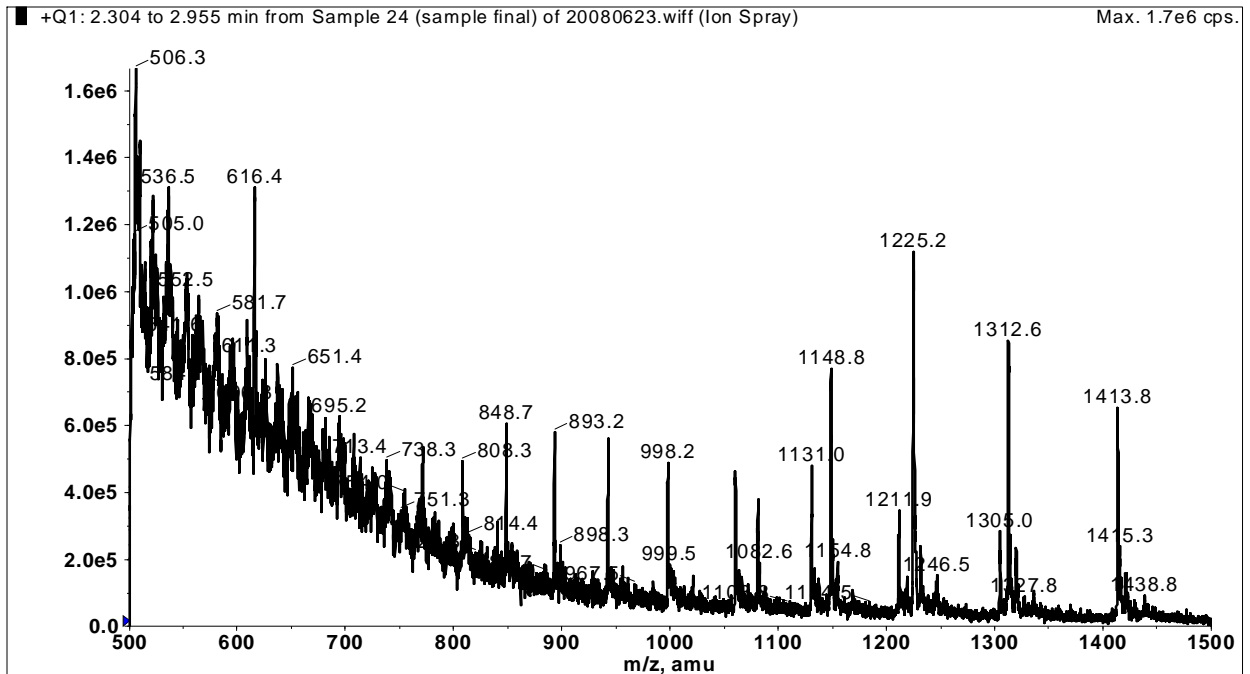


Figure 3.26: Average mass spectrum of time B.

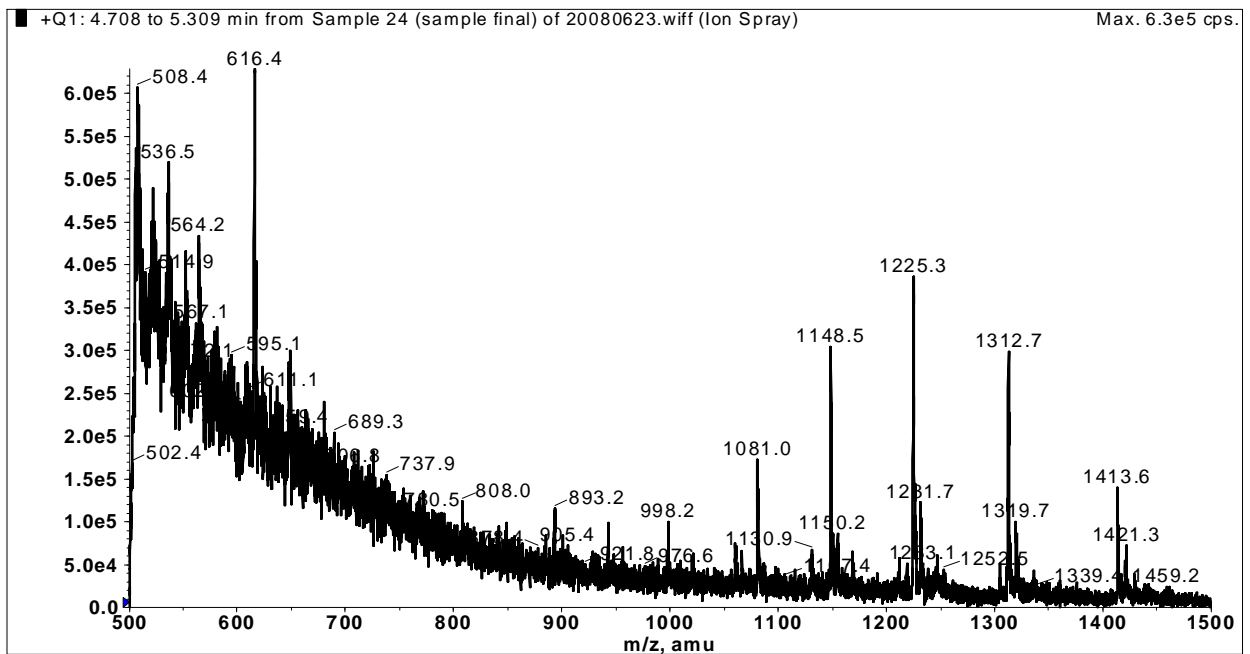


Figure 3.27: Average mass spectrum of time C.

Figure 3.23 indicates the MS TIC of samples after the IEF separation of β -lactoglobulin

and myoglobin, and it was stable. It could be seen from Figure 3.25 that the MS fingerprints of myoglobin emerged slowly, which indicates myoglobin was focused in stage A; similarly, the β -lactoglobulin was focused in stage C (Figure 3.27). However, both the fingerprints of β -lactoglobulin and myoglobin emerged in stage B at the same time as shown in Figure 3.26, which indicates some β -lactoglobulin and myoglobin are still mixed in stage B. It might be due to the dead volume in the connection capillary that destroyed the separation performance, resulting in the focused zone mix a little. It is anticipated that the separation efficiency could be improved if another mobilization method could be applied.

3.4 Conclusions

The microcross design is suitable for coupling CE with MS, especially in coupling CIEF with MS. A low CA concentration demonstrated efficient focusing, and at the same time, does not cause significant signal interference of MS detection. CIEF has been coupled with MS using nanospray.

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Chapter 4 Preparation of capillary monolithic column with immobilized pH gradient for coupling CIEF with mass spectrometry

4.1 Introduction

Capillary isoelectric focusing is a mode of capillary electrophoresis that exhibits powerful separation ability for ampholytic molecules such as proteins. The separation principle is based on the differential isoelectric points in a chemically formed pH gradient under the influence of an applied electric field. Commercial carrier ampholytes (CAs) are commonly used for establishing a pH gradient. After finishing the IEF process, the focused analytes zones are mobilized to pass through the detection point for UV absorbance detection. Due to the high absorbance of CAs at low wavelength, the detection sensitivity is very low.

Much effort has been made to perform CIEF without CAs including autofocusing and temperature- and electrolysis-generated pH gradient.¹⁻⁴ Furthermore, omitting CAs will facilitate coupling of IEF with mass spectrometry (MS), which is a wonderful detection technique for proteomics research. Hochstrasser et al. prepared a CA-immobilized pH gradient (CA-IPG) in a glass tube.⁵ Zhang's group developed an immobilized pH gradient in a monolithic capillary column.⁶ CAs were immobilized at different positions in the column according to their pIs, generating a monolithic immobilized pH gradient (M-IPG). The IEF was implemented in such a column without soluble CAs. In our case, the M-IPG columns were prepared and coupled with MS after IEF of protein separation.

4.2 Experimental Sections

4.2.1 Chemicals and materials

Glycidyl methacrylate (GMA), ethylenedimethacrylate (EDMA), glutaraldehyde (50% in water), dodecanol, cyclohexanol, 1,4-butanediol, *n*-propanol, azobisisobutyronitrile (AIBN), sodium borohydride (NaBH₄), acrylamide, *N,N'*-methylenebisacrylamide, dimethyl sulfoxide (DMSO), 3-methacryloxypropyltrimethoxysilane (γ -MAPS), and 1, 4-butanediol were obtained from Sigma (Canada). Ammonia, methanol, acetic acid, and ethanol were obtained from Fisher Scientific (Canada). Modified upchurch microcross union was obtained from Proxeon Biosystems (USA), spray capillary for modified union (Pico Tip™) was obtained from New Objective (USA). Capillary with 100 μ m i.d. \times 360 μ m o.d. for monolithic column and connection was obtained from Polymicro Technologies (USA). Water bath 150D is purchased from VWR.

4.2.2 Preparation of monolithic capillary column

Fused-silica capillary was successively washed with NaOH (1.0 mol/L), water, HCl (1.0 mol/L), water, and methanol for 30 min, then dried in a gas chromatographic oven with nitrogen flush at 70 °C for 3 hours. The cleaned capillary was filled with a solution of γ -MAPS (30% v/v in methanol) and kept at room temperature with both the ends sealed by silicon septa. About 24 hours later, unreacted γ -MAPS was washed off using 0.5 mL of methanol. The capillary was rinsed by nitrogen at room temperature for 30 min to remove the residual methanol.

Poly-base (GMA-*co*-EDMA) monolithic capillary column was synthesized by filling the capillary with the mixture of 30 μ L GMA, 10 μ L EDMA, 110 μ L cyclohexanol, 10 μ L dodecanol, and 0.3 mg AIBN. The capillary that was about 10 cm long was filled with the solution and then was put in a water bath at 60 °C. The polymerization was stopped after 18 hours, and unreacted

monomers were removed by flushing the capillary with methanol using an HPLC pump.

Similarly, the polyacrylamide-based monolithic capillary column was also synthesized. EDMA was replaced by acrylamide and *N,N'*-methylenebisacrylamide. 1,4-Butanediol and DMSO was used as porogenic solvent. GMA (18.9 mg), acrylamide (16.7 mg), and *N,N'*-methylenebisacrylamide (40.7 mg) were dissolved in the mixture of 333.3 mg of DMSO, 148.2 mg of 1,4-butenediol, and 185.1 mg of dodecanol. After the addition of AIBN (% wt with respect to monomers), the mixture was vortexed for a few minutes, and then degassed with ultrasonic for 2 minutes. Subsequently, the mixture was injected into the pretreated capillary. With both ends sealed, the capillary was put in an oven at 75 °C for 24 hours. The monolith in the capillary was then washed with ethanol for 2 hours, followed by water for another 1 hour using an HPLC pump.

4.2.3 Immobilization of CAs onto the monolithic columns

For preparation of poly-based (GMA-*co*-EDMA)-based M-IPG, ammonia (1 mol/L) was pressed through to react with the epoxy groups for 4 hours. A solution of 5% glutaraldehyde was pumped in and the column was kept at room temperature for 12 hours. Both ends of the capillary without polymer were cut away. The capillary was filled with 10% Pharmalyte. The two ends of capillary were immersed into cathode and anode electrolytes. 100 mmol·L⁻¹ phosphoric acid and sodium hydroxide were used as anolyte and catholyte, respectively. 1500 V was applied on to the column for the first 3 minutes, and then followed by 3000 V 27 minutes. The capillary was kept at room temperature for another 12 hours after the IEF. A solution of NaBH₄ (20 mmol/L in 100 mmol/L tris-HCl, pH 8.1) was pumped through the column for 9 hours. Finally the capillary was washed with methanol for 10 minutes and then with H₂SO₄ (10 mmol/L) for 2 hours followed by

flushing with water for 10 minutes. After these treatments, the capillary could be used in CIEF.

For preparation of polyacrylamide-based M-IPG, the capillary column was directly filled with 10% pharmalyte. A 1500 voltage was applied on to the column for the first 3 minutes, and a 3000 voltage was applied and kept for the rest 27 minutes. The current of the whole process was recorded by the instrument as shown in Figure 4.3.

The capillary was kept at room temperature for another 12 hours after the immobilization. After immobilization, the capillary was washed with water for 10 minutes.

4.2.4 Coupling M-IPG-based CIEF with MS

β -Lactoglobulin and myoglobin were selected as model analytes. β -Lactoglobulin and myoglobin with 40 $\mu\text{g}/\text{mL}$ concentration were mixed in water solution. The mixture was injected into monolithic column by the syringe and the injection lasted for 3 minutes to fill the monolithic column. After sample introduction, the column was inserted into the electrolyte reservoirs, which are similar to the iCE 280 separation cartridge. The end with higher pH immobilized was dipped into the cathode while the end with low pH immobilized was dipped into the anode. 100 $\text{mmol}\cdot\text{L}^{-1}$ phosphoric acid and sodium hydroxide were used as anolyte and catholyte, respectively. The voltage used for focusing was 500 V for the first 2 minutes and 3000 V for the next 8 minutes. The voltage was provided by iCE 280 instrument.

After the focusing in the iCE280 instrument, the electric potential was turned off and the cartridge was taken out. One connecting capillary next to the anode was connected to a syringe pump using capillary adapter and sleeves at a flow rate of 167 nL/min , while the capillary next to the cathode was connected to one end of the microcross union using capillary adapter and sleeves. The makeup solution, consisting of 50% methanol, 49% water, and 1% acetic acid

(v/v/v), was introduced from the other end by capillary at a flow rate of 167 nL/min using a syringe pump. The mixture was pushed to the spray tip for ESI. MS can record the spectra of the whole process as shown in figure 4.5

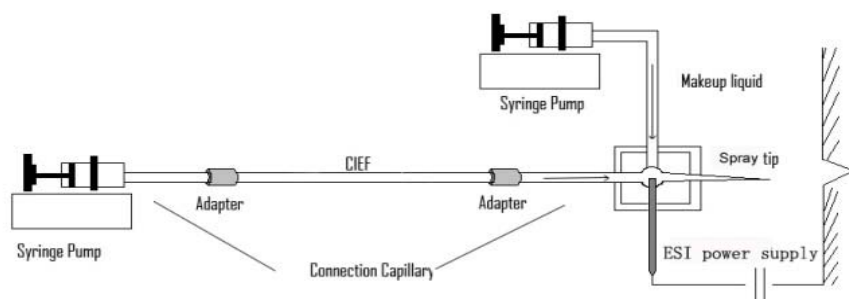


Figure 4.1 Schematic connection of coupling M-IPG with MS.

4.3 Results and discussion

4.3.1 Preparation and characterization of M-IPG

Monolith has been widely used in various scientific and industrial fields such as supporting of catalysts, enzyme immobilization, molecular imprinting, and separation supports.⁷ In this project, two kinds of monolithic columns were prepared to accomplish IPG in the form of monoliths. Figure 4.2 and 4.3 showed the SEM images of the prepared poly-based (GMA-co-EDMA) and polyacrylamide-based monolithic capillary columns. As shown in Figure 4.2A and Figure 4.3A, the organic polymer monolith was well immobilized onto the inner wall of capillary, which is helpful when run several times. The macro pores with diameters of several micrometers

can be clearly observed in Figure 4.2 B and Figure 4.3 B. Additionally; some micropores with diameters of several hundreds of nonometers are seen. The large surface area can be used for further derivatization. Thus, the monolithic columns were used to immobilize the CAs.

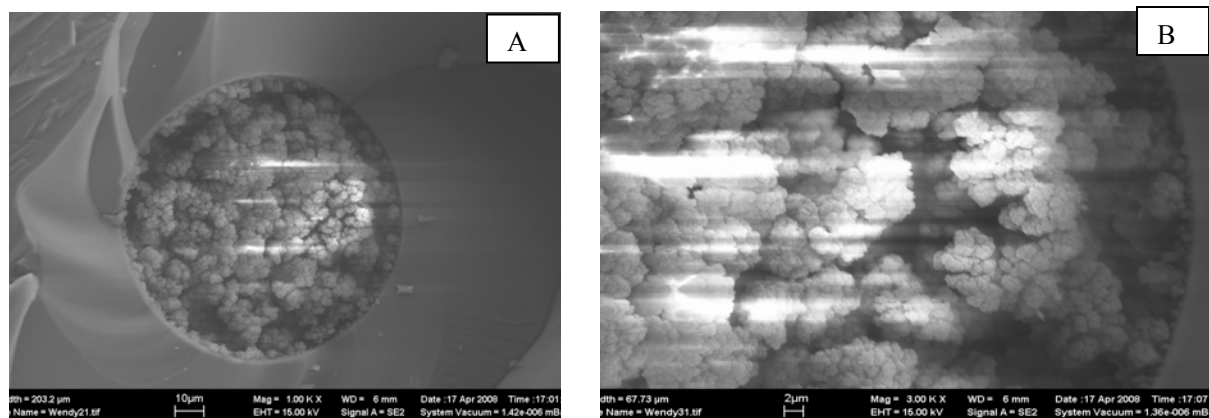


Figure 4.2: SEM imaging of poly (GMA-co-EDMA) based monolithic capillary ($\times 1000$, $\times 3000$)

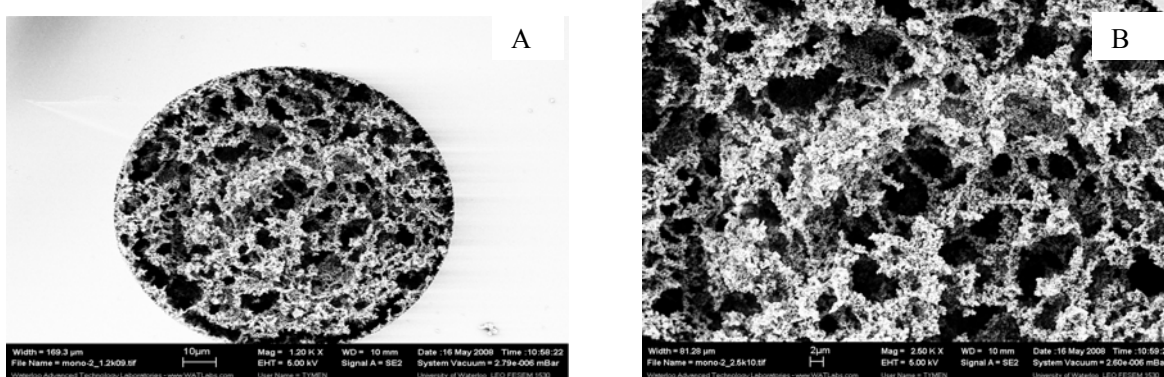


Figure 4.3: SEM imaging of poly-based (GMA-co-acrylamide) monolithic capillary ($\times 1200$, $\times 2500$)

4.3.2 Immobilization of CAs

As mentioned in the method of immobilization of CAs onto the monolithic columns, a 1500 voltage was applied on to the column for the first 3 minutes, a 3000 voltage was applied and kept on for the rest of the 27 minutes. Figure 4.4 recorded the current profile during the

whole immobilization process. The dropping down of current indicated the CAs gradually moved to the points, which equal to their pIs.

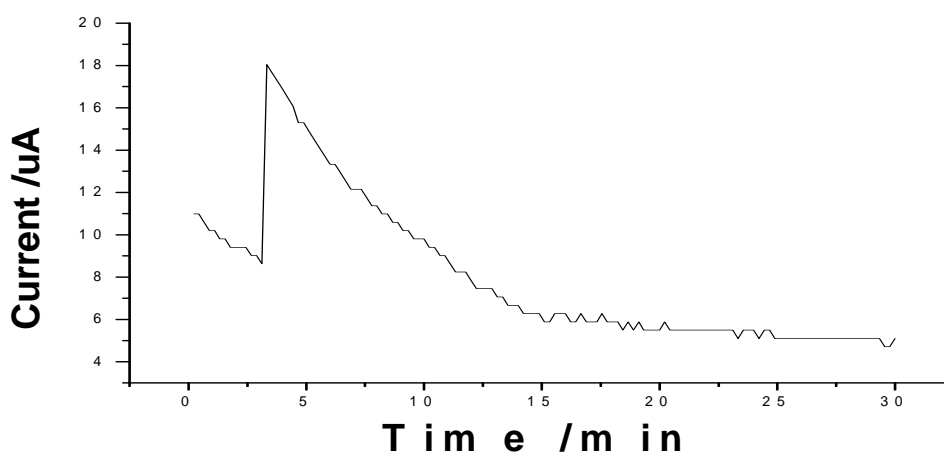


Figure 4.4: Current vs Time during the immobilization procedure. The capillary was filled with 10% pharmalyte. The two ends of capillary were immersed into anode and cathode electrolytes. 100 mmol·L⁻¹ phosphoric acid and sodium hydroxide were used as anolyte and catholyte, respectively. A 1500 V was applied on to the column for the first 3 minutes, followed by 3000 V for 27 minutes. The power supply is provided by iCE280.

4.3.3 Coupling M-IPG with MS

β -Lactoglobulin and myoglobin were selected as model analytes. The monolithic capillary was filled with a mixture of β -lactoglobulin and myoglobin water solution. The concentration of β -lactoglobulin and myoglobin was 40 $\mu\text{g}/\text{mL}$. After filling, the end of low pH was dipped into the anode reservoirs; the other end was dipped into the cathode reservoirs. A 3000 V was applied for 10 minutes. After focusing, the monolithic capillary was taken out. The

anode end of monolithic was connected to syringe pump using capillary adapter and sleeves, the cathode end was connected to one end of the microcross union using capillary adapter and sleeves. The makeup solution (methanol, water, and acetic acid) was introduced from the other end by capillary and syringe pump. The mixture was pushed to the spray tip for ESI. MS can record the spectra of the whole process. From the total ion current spectrum, any time point and any time range can be chosen to check the mass spectrum of analyte at that time point or check the average mass spectrum within that time range.

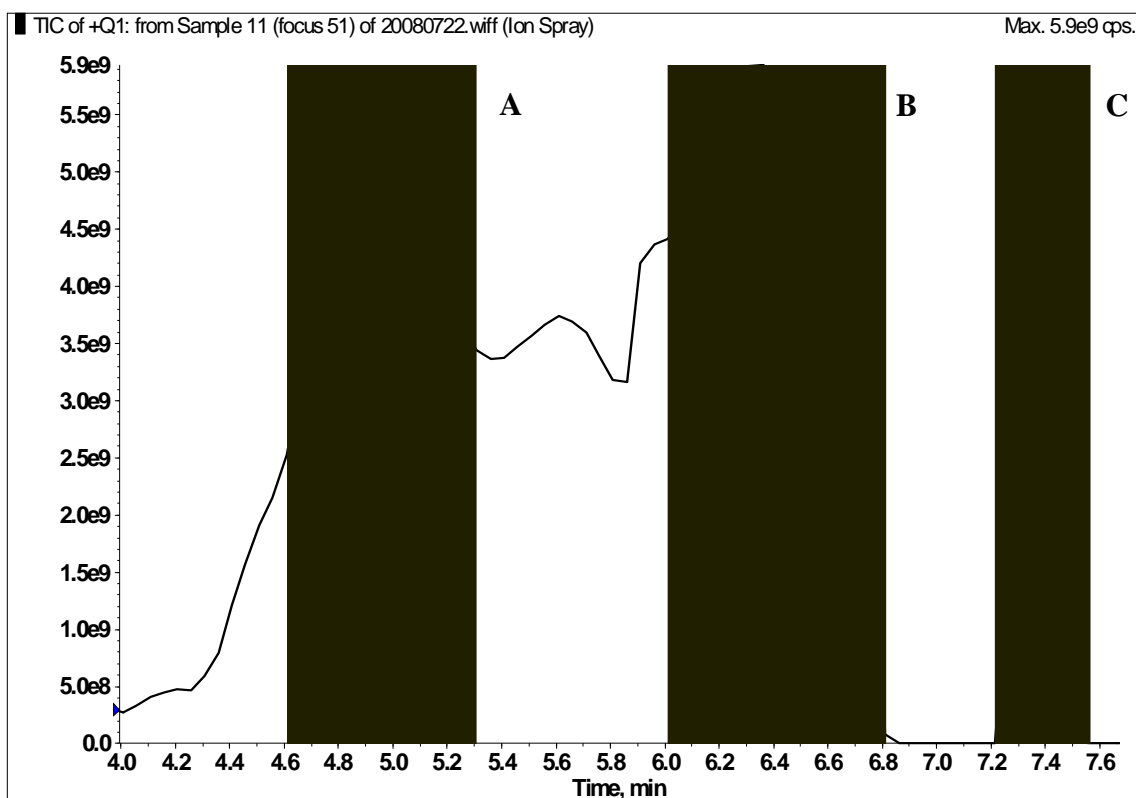


Figure 4.5: MS TIC of samples after the separation in CIEF (M-IPG).

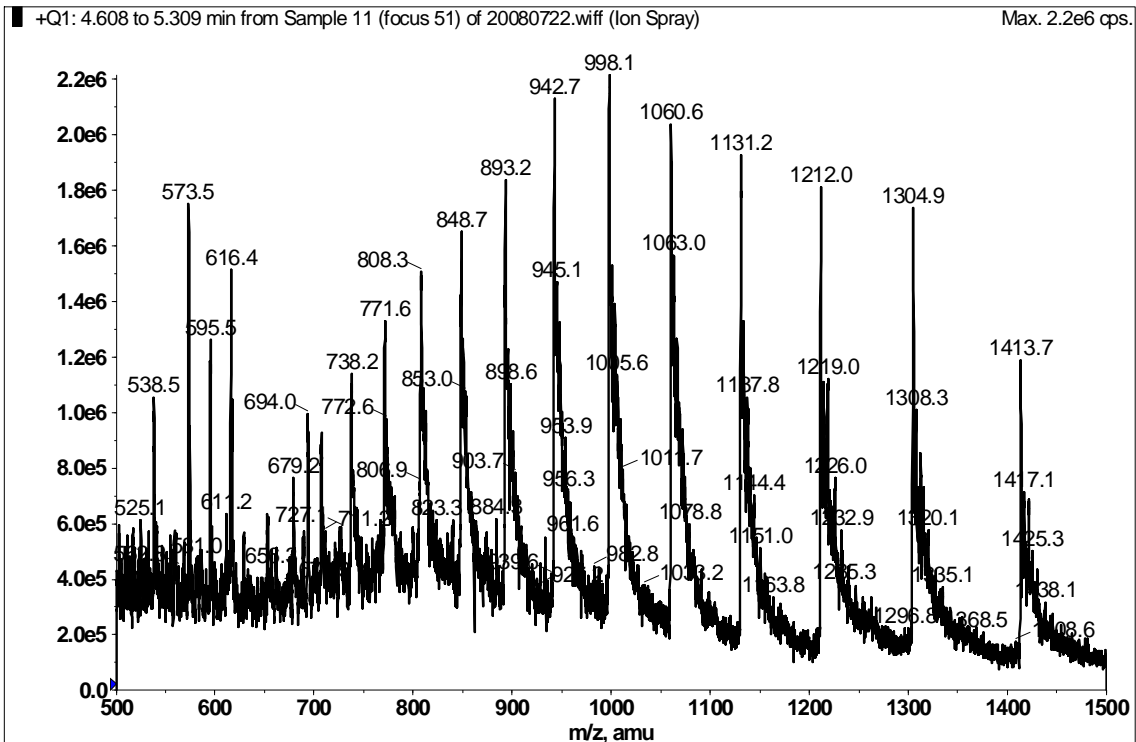


Figure 4.6: Average mass spectrum of time range A.

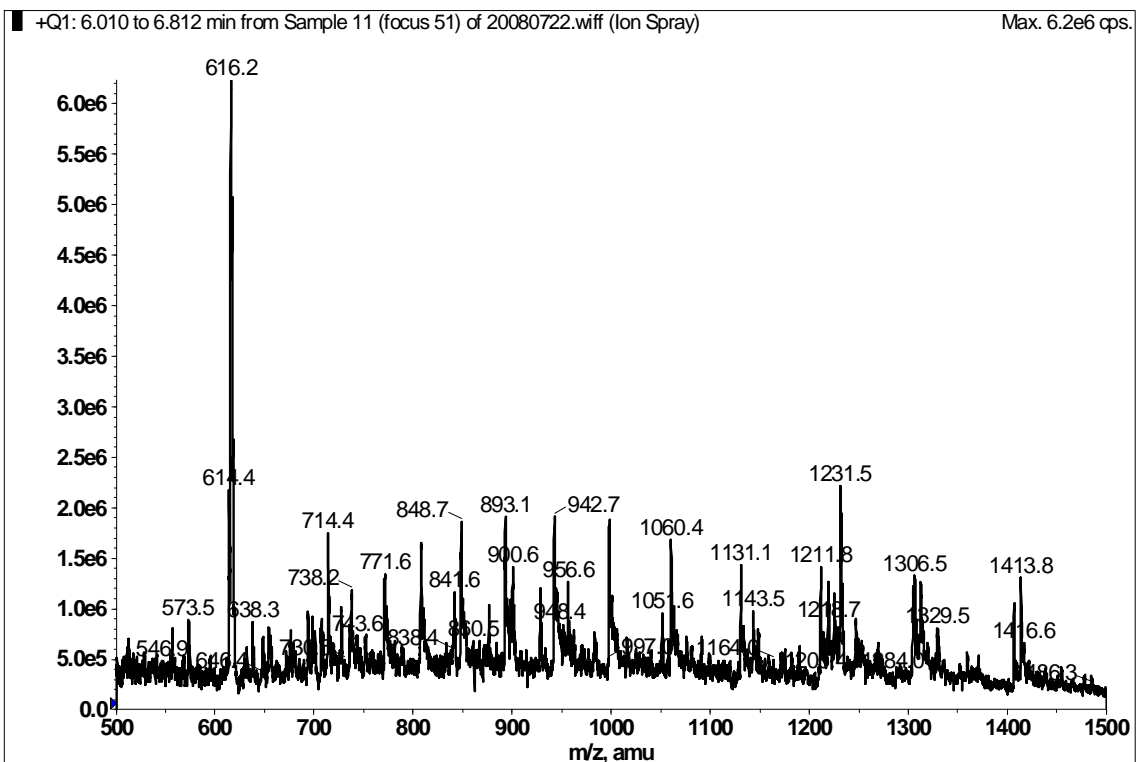


Figure 4.7: Average mass spectrum of time range B.

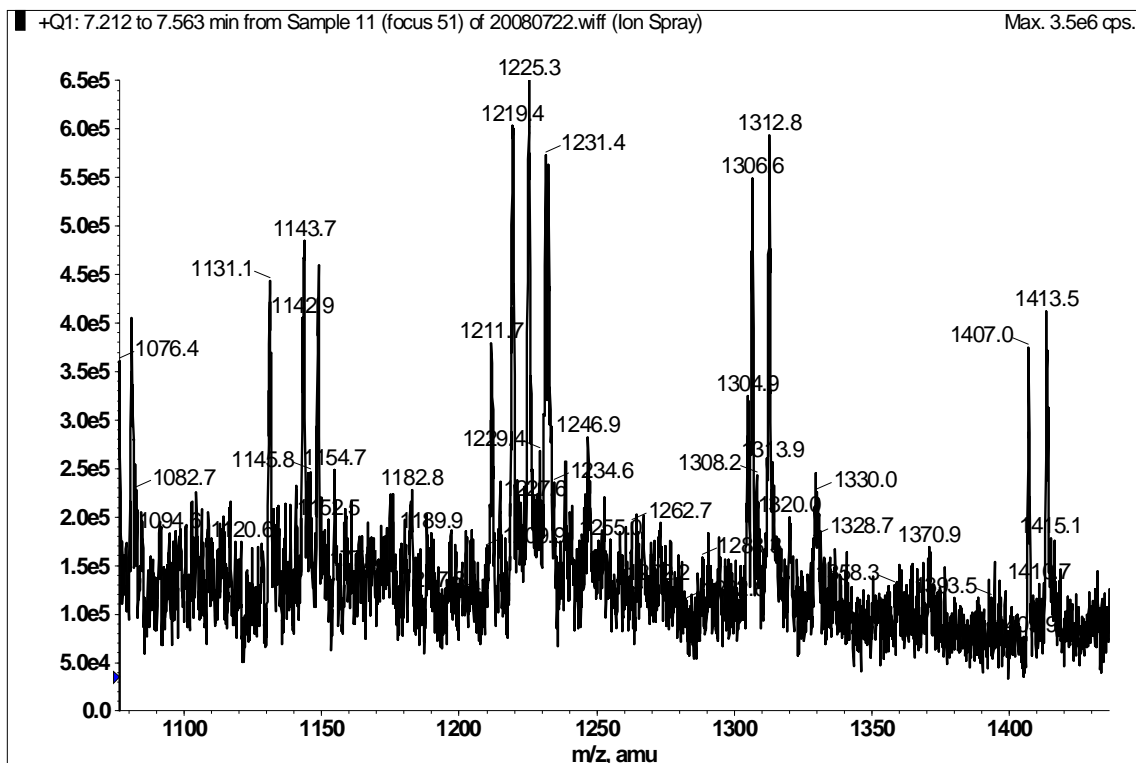


Figure 4.8: Average mass spectrum of time range C.

Figure 4.5 indicated the MS TIC of samples after the IEF separation of β -lactoglobulin and myoglobin. The mass spectrum should be stable, however, in this case, it was not stable. It might be because of the adapters that connected the syringe pump, the separation capillary, and the spray tip. The adapter may not prevent air bubbles from being trapped in the capillary, which may lead to the instability of total ion current. It could be seen from Figure 4.6 that the MS fingerprints of myoglobin were the sole emergents, which indicated that myoglobin was focused in stage A; similarly, the β -lactoglobulin was focused in stage C. These results prove that β -lactoglobulin and myoglobin could be successfully focused under the electrical field in the

prepared monolithic IPG column. However, both the fingerprints of β -lactoglobulin and myoglobin emerged in stage B at the same time as shown in Figure 4.7, which indicated that some β -lactoglobulin and myoglobin are still mixed in stage B. It might be due to air bubbles trapped in the adapter or the dead volume in the connection capillary, which destroyed the separation performance, resulting in the focused zone mixed a little.

It is anticipated that the separation efficiency could be improved if connectors could be improved.

4.4 Conclusions

Two kinds of monolithic columns were prepared and two methods were used to immobilize a pH gradient into two kinds of monolithic capillary column by attaching CAs (Phamalytes) to porous polymer monolith. A poly-based (GMA-co-EDMA) monolithic column did not produce good results. Isoelectric focusing of two proteins was only carried out in polyacrylamide-based M-IPG monolithic capillary column without additional CAs. MS was coupled to the M-IPG as the detector of CIEF. No typical noise of CAs was observed in the MS spectrum.

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