ABSTRACT

FRANTZ, NEHA JOSHI. Characterization of Complex Protein Mixtures from Human Plasma Fractionation by Capillary Electrophoresis and Mass Spectrometry. (Under the direction of Dr. Morteza G. Khaledi.)

The purpose of the research has been to develop a simple, quantitative method for characterizing complex protein mixtures using Cohn Fractionation IV-1 Paste as an example. This process step is the starting material for the plasma fractionation process which leads to purified alpha$_1$-antitrypsin, and is an example of a complex protein matrix. Currently, CE analysis of complex plasma samples involves a Borate-NaCl buffer system which does not provide adequate resolution to quantitate protein composition. The goal of this research is to discover optimum CE separation conditions, which would allow rapid, efficient, and inexpensive characterization of these complex samples.

A simple, reproducible system utilizing SDS has been developed which provides better resolution and quantitation of complex protein mixtures. This new method is referred to as "CE-SDS," and although it is an improvement over the current method, orthogonal methods such as mass spectrometry can provide additional information not captured by this method.

CHARACTERIZATION OF COMPLEX PROTEIN MIXTURES FROM HUMAN PLASMA FRACTIONATION BY CAPILLARY ELECTROPHORESIS AND MASS SPECTROMETRY

by

NEHA JOSHI FRANTZ

A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Master of Science

CHEMISTRY

Raleigh

2006

APPROVED BY:

_____	_____
Edmond F. Bowden	Lin He

_____

Morteza G. Khaledi
(Chair of Advisory Committee)

BIOGRAPHY

Neha Joshi Frantz was born in 1974 to Sharad and Meenakshi Joshi in Brooklyn, NY. At age 5, the family relocated and settled in Southern California. In 1992, Neha graduated from Troy High School in Fullerton, CA, and continued on to University of California at Irvine. Undergraduate research on transgenic mosquitoes was performed under the direction of Professor Anthony James and Dr. Kurt Yardley. After graduating from UC Irvine in 1996, Neha moved to Cary, NC with her husband, Dan Frantz. Neha secured a position as an Assistant Research Scientist at Bayer Corporation, Clayton, NC, under the direction of Dr. Lisa Musmanno and Dr. Gerold Mohn. After gaining further experience in clinical research, Neha returned to Bayer Corporation and began graduate studies at North Carolina State University, Raleigh under the direction of Professor Morteza Khaledi. A strong desire for knowledge of protein chemistry and a love of research drove Neha to pursue a graduate degree while remaining employed in industry. The research was funded by Bayer Corporation, now Talecris Biotherapeutics, and the focus of the research was to create high efficiency separations of plasma protein intermediates using capillary electrophoresis. In 2006, Neha completed the Master's Degree in Chemistry and nearly ten years of experience in the biopharmaceutical industry.

ACKNOWLEDGEMENTS

I would like to express my gratitude and admiration for my advisor, Professor Morteza G. Khaledi, for his patience, guidance and expertise in the field of separation science. I am grateful to Bayer Corporation and Talecris Biotherapeutics for funding and encouraging this research.

I would also like to thank my distinguished mentors and colleagues for their support, encouragement and scientific advice in order to help me complete this work: Dr. Lisa Musmanno, Dr. Gerold Mohn, Dr. Hansjeorg Duerr, Dr. Peter Vandeberg, Dr. Barbara Merrill, Dr. Rita Froede, Joseph Buettner, James "Cooper" Cannady, Dr. Jeff Bumgarner, Dr. Todd Willis, Dr. Kevin Wee, Wendy Ryals Sellers, Jennifer White Seymour, Andrea Milbauer Wellhouse, Jeff Warlick, Rebekah Weber, Bill Burgess, Gisela Fleming, Dr. Bassam Nakhle, Dr. Koen van der Drift, Dr. Krisstina Burgess, Dr. Vakhtang Loladze and Dr. Jason Barker. You have been a source of inspiration for me.

I would like to thank my committee members, Dr. Edmond Bowden and Dr. Lin He for their time and flexibility. I would also like to thank the following faculty members: Dr. John Cavannagh for teaching me about protein structure; Dr. Lin He for teaching me about mass spectrometry applications to proteins; Dr. Edmond Bowden for "making" me learn about cutting-edge technology; and Dr. Ken Hanck for teaching me when data is "real."

I would also like to thank my husband, Dan, for "gently applying pressure" without which this work may have been performed, but not published. I could not have done this without you. Thanks to Maximillian for coming along; you have taught me the most important things to treasure in life. Thanks to my parents and brother for providing me a strong foundation upon which to build my life. I could not have predicted that my little brother, Tapan, would be present at my defense, but then again, I've been "at his defense" throughout his life.

Thanks to The Lord who is my strength and ultimate guidance. Thanks for always looking out for me.

Thanks to my former educators who have inspired me to work hard, be creative, and show leadership: Mrs. Karen McClung, Mrs. Michelle Rudd, Mr. Richard Liem, Dr. Chris Heaton, and Mr. William Bledsoe. I would also like to express my admiration for my undergraduate research advisor, Dr. Anthony A. James, who is one of the coolest smart people I have ever met.

A couple of heroes of mine (whether they know it or not) include Dr. P. Michelle Fitzsimmons and Dr. Clara Schreiner.

A note to any potential readers of this thesis: As in capillary electrophoresis, you are "peaking" through a narrow window into my life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. INTRODUCTION

Background – Cohn Fractionation Process

Modified Cohn Fractionation of human plasma involves various steps including ethanol and salt precipitation, centrifugation, polymer suspension/extraction, pH adjustment, ultrafiltration/diafiltration and various modes of chromatography. Originally, Dr. E.J. Cohn[1] invented the process to provide serum albumin as a substitute for whole blood for wounded soldiers in the field during World War II. Purified serum albumin is stable at ambient temperature for several years and can be administered to prevent death due to loss of blood. Based on Dr. Cohn's principles, many other therapeutic proteins of interest can be purified upstream of serum albumin. Newer technology has provided better, more efficient purification techniques so that plasma purification can be performed at large-scale. Figure 1.1 shows a diagram of the fractionation and purification processes used to produce various therapeutic products from human plasma.

Much of the work presented in this research will focus on Fraction IV-1 Paste. This process step serves as an example of separation of a complex protein mixture, comprised of approximately twenty or more proteins. However, the same separation conditions can be applied to subsequent process steps leading up to the final product, and can be a quantitative measure of yield. Ideally, a fast, inexpensive method to obtain a protein profile can easily be introduced in-line during the manufacturing process saving millions of dollars per year for biotech companies.

The benchmark techniques used for separation of protein mixtures are sodium dodecylsulfate polyacrylamide gel electrohporesis (SDS-PAGE) and two-dimensional gel electrophoresis. SDS-PAGE provides separation of proteins based on size in a gel matrix. The protein bands must be stained and detected off-line by band densitometry. Two-dimensional gel electrophoresis combines the separation based on isoelectric point (pI) in one dimension and size in a second dimension. Multiple spots can arise from the same protein. The spots must also be

stained and detected off-line by band densitometry. These techniques are almost unrivaled for their resolution capabilities, however, they are manual and time-consuming. Capillary electrophoresis has great potential to mimic these separations with high efficiency, better reproducibility and higher throughput.

The main issues surrounding CE of complex protein mixtures include protein adsorption to the silica wall, solubility, protein heterogeneity (factors affecting surface charge), pH and ionic strength of the background electrolyte (BGE) which affect mobility and resolution. Reliance on commercially available kits and coated capillaries may lead to problems with reproducibility if these materials become discontinued or modified by the vendor.

Applicable Modes of Capillary Electrophoresis

Various classes of CE conditions appropriate for protein separations were investigated in this research. The traditional method is referred to as the clinical method, and is an example of capillary zone electrophoresis (CZE)[2]. Human serum protein separation is based on cellulose acetate electrophoresis where the separation zones or regions are defined by gamma globulins, beta globulins, alpha globulins and albumin. This type of electrophoresis is used in clinical settings on individual patient serum samples and can diagnose disease states. The separation is based on overall charge-to-mass ratio, and it is a generally low resolution method.

Another mode of separation is Micellar Electrokinetic Chromatography or MEKC. In MEKC, surfactants such as sodium dodecylsulfate (SDS), form micelles in solution, and analytes partition between the micellar phase and the solution phase. The micelles act as a pseudo-stationary phase, and separation is based on differential partitioning. It is important to note that human plasma proteins are generally too large to partition into micelles, but the use of surfactants in CE separations of proteins is widespread.

Alternatively, a polymer, such as dextran or hydroxypropyl methylcellulose (HPMC), can be added to provide a viscous sieving matrix where proteins can ideally be separated by size.

High pressures are required in order to pack the capillary prior to each sample injection. Many of these types of run buffers are now commercially available.

Coated capillaries are able to reduce or eliminate electrosmotic flow and may provide better resolution. Coating also helps to avoid protein adsorption to the capillary walls. Many types of coated capillaries are commercially available, and dynamic coatings using divalent amines are popular as well.

Capillary isoelectric focusing (cIEF) relies on the separation of proteins based on their isoelectric points (pI). Unfortunately, many of the proteins in human plasma have a pI range and not a single point. Often the pI ranges overlap which can lead to co-migration.

All of these methods have the potential to provide an efficient, quantitative profile of complex protein mixtures. However, given the small light path length for absorbance detection, some sensitivity is lost which can adversely affect the detection of low abundance proteins. In order to identify low abundance proteins, orthogonal methods using reverse-phase liquid chromatography coupled to mass spectrometry was applied to IV-1 Paste.

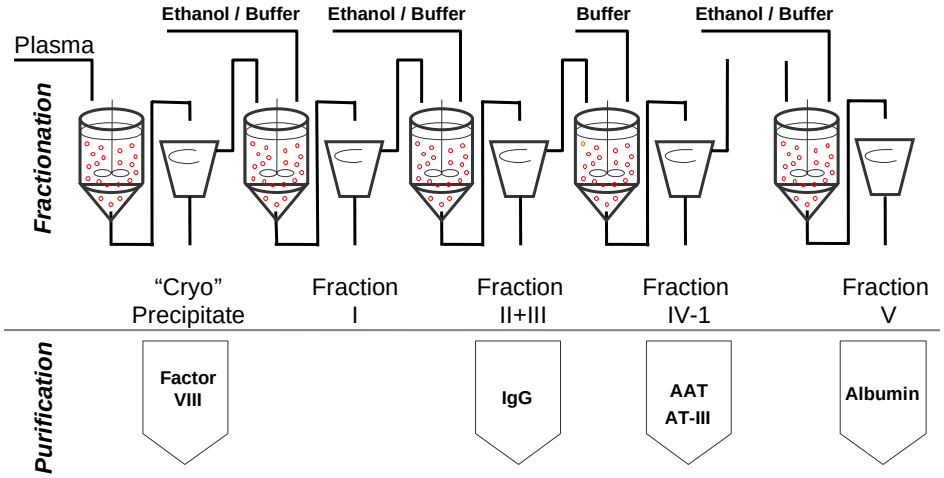


Figure 1.1. General Overview of the Modified Cohn Fractionation Process

References

1. Cohn, E.J.; Strong, L.E.; Hughes, W.L.; Mulford, D.J.; Ashworth, J.N.; Melin, M.; Taylor, H.L. Preparation and Properties of Serum and Plasma Proteins. IV. A System for the Separation into Fractions of the Protein and Lipoprotein Components of Biological Tissues and Fluids *J. Amer. Chem. Soc.* **1946**, *68*, 459-475.
2. Palfrey, S.M. Serum Protein Electrophoresis. *Clinical Applications of Capillary Electrophoresis* Humana Press, Inc.: New Jersey, 1999; pp 11-19.

CHAPTER 2. CAPILLARY ELECTROPHORESIS METHODS DEVELOPMENT

Introduction

Human plasma protein analysis has historically been performed using electrophoresis-based techniques. These techniques include free-solution electrophoresis (Tiselius 1937), high resolution agarose gel electrophoresis (HRAGE), cellulose acetate electrophoresis, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (gel support) [4]. These techniques result in approximately five to six bands which reflect the wide distribution of proteins found in plasma. Capillary electrophoresis has advantages over the historical techniques including the capability of automation and high resolution. This chapter will focus on the application of capillary electrophoresis to the separation and quantitation of human plasma proteins.

The electrophoretic flow produced in capillary electrophoresis involves the interaction of the negatively-charged silanol groups lining the walls of the capillary with positively charged ions provided by the background electrolyte. A double-layer of charge is formed by the attraction of the buffer cations to the silanoate groups, where the first layer of cations are tightly bound and the second cation layer is loosely bound (mobile layer). When voltage is applied across the capillary, the buffer cations in the mobile layer are pulled toward the cathodic end of the capillary dragging the buffer along causing flow through the capillary. This flow is referred to as the electroosmotic flow, or EOF, and it is partly responsible for the migration of analytes through the capillary. Electrophoretic mobility of analytes depends upon the ionic character of the analyte, charge-to-size ratio [1,2].

When the analytes are proteins, inherent challenges are present. Proteins are large molecules with heterogeneous surface charge character (zwitterionic). Capillary electrophoresis of proteins has the potential to capitalize on the differences in charge-to-size ratio in order to achieve separation. The high efficiency of capillary electrophoresis, being an open tubular method, has the potential to provide high resolution with short analysis times. Also, due to the

size of proteins, their diffusion is slower when compared with small molecules, which can lead to sharp peaks [1].

Experimental

Chemicals

Tris hydrochloride, boric acid (sodium salt), sodium phosphate (anhydrous), 1,4-diaminobutane (putrescine), sodium dodecylsulfate (SDS), Triton X-100, cetyl trimethylammonium bromide (CTAB), sodium cholate, and phytic acid (sodium salt) were purchased from Sigma Chemical Company (St. Loius, MO). Sodium chloride and 5N sodium hydroxide were purchased from Fisher Scientific. cIEF kit was purchased from Beckman-Coulter. The Protein 200 kit was purchased from Agilent Technologies, and the Bioanalyzer instrument was borrowed from Agilent Technologies.

Capillary Electrophoresis Conditions

Several different conditions were investigated, and the run conditions are listed in each figure legend. In general, samples were diluted into run buffer for compatibility. Where commercially available kits were used, run conditions were per kit instructions.

Capillary Zone Electrophoresis

Capillary Zone Electrophoresis, or CZE, of human serum proteins has origins in paper electrophoresis, cellulose acetate electrophoresis and agarose gel electrophoresis [3]. Common to these techniques is that human plasma proteins are separated into regions: gamma, beta, alpha-2, alpha-1 and albumin. In this research, the CZE method utilizes a simple borate buffer system. Table 2.1 outlines the CZE regions and which plasma proteins are expected in each region. Figure 2.1 shows a typical electropherogram obtained for IV-1 Paste samples by the CZE method. The CZE method can provide a qualitative profile of IV-1 Paste, however, integration is difficult.

Also, due to co-migration of the many protein components expected in this sample, this method is not considered quantitative. There are variations of CZE method used in clinical settings to analyze patient sera [3].

In this research, electrolytes consisting of borate, phosphate, a combination of borate and phosphate, and tris/HCl were investigated. Alkaline pH (>9) is preferred in order to minimize the interaction of the proteins with the silica wall. Also, the isolectric points of the various proteins in IV-1 Paste requires a pH above 7.5 in order to maximize solubility and mobility by creating a net negative charge for all proteins. Figure 2.2 shows an example of the results obtained using a tris buffer at varying pH. The separation in not a significant improvement over the current separation, and co-migration is evident. Varying the pH does not appear to provide any advantages or disadvantages to the separation. In the case of the tris buffer system (Figure 2.2), it appears that the increase in pH decreased the overall separation time by increasing mobility. The bands became more narrow, but no improvement in resolution was achieved.

In general, run buffers prepared without additives did not produce significant resolution for this complex mixture.

Capillary Isoelectric Focusing, cIEF

Isoelectric focusing can be performed in a capillary, cIEF. An ampholyte mixture of a selected pH range is flushed through the capillary and creates a pH gradient. When a sample plug is introduced and a voltage is applied, the proteins migrate and focus into the zone in the capillary corresponding to the isoelectric point, pI. Adding pressure causes the focused proteins to move past the detector. Most proteins have a pI range, due glycosylation and other post-translational modifications. The pI range of the major protein constituents in IV-1 Paste tended to overlap, which defeated the purpose of the separation. A commercially available kit manufactured by Beckman-Coulter (circa 1999) was tried but resulted in poor reproducibility. In this kit, the ampholyte mixture ranged from pH 3-10. For IV-1 Paste, several sharp peaks were noted,

however, many of the peaks were below 3:1 signal-to-noise. It is difficult to tell which peaks are real as the peak shapes are unusually sharp resembling spikes (Figure 2.3). It should be noted that the matrix of IV-1 Paste may also adversely affect the separation by cIEF. These conditions were not further explored.

"Micellar Electrokinetic Chromatography," MEKC

Micellar Electrokinetic Chromatography, or MEKC, is another widely-used CE technique. In MEKC, addition of surfactants creates micelles in solution and can enhance resolution and provide selectivity. Consider the example of SDS, where the critical micellar concentration, CMC, is 7-10 mM in water[7]. In a CE application, this is not a traditional MEKC application in that plasma proteins are too large to partition into SDS micelles, although interaction of hydrophobic portions of a protein with the micelles is possible[8,9]. More likely, SDS binds to proteins, disrupting the secondary structure due to hydrophobic interactions and resulting in a net negative surface charge. This net negative charge will cause proteins to repel from the capillary wall and from each other. For this reason, the term "MEKC" is used loosely when discussing CE separations of proteins using SDS. For this research, CE applications involving SDS will be referred to as "CE-SDS."

Surfactants are preferred for protein separations by CE. These reagents provide solubility and help keep the proteins from adsorbing to the capillary walls. In this research, the anionic surfactants sodium dodecyl sulfate (SDS) and sodium cholate were investigated. Cetyl trimethylammonium bromide is an example of a cationic surfactant investigated in this research. Triton X-100 is an example of a nonionic surfactants examined in this research. Table 2.2 outlines some properties of these surfactants which are important to their application in MEKC[7]. For this research, the ionic nature of the head group, the length of the alkyl chain, the CMC and the aggregation number of each representative detergent were considered. These properties can influence the selectivity of the separation. For example, the aggregation number, or the number

of detergent molecules in a single micelle, can influence the separation by creating a larger micelle, which can allow smaller proteins to partition.

Consider the use of SDS in the separation of the protein constituents of IV-1 Paste. SDS binds to proteins in a ratio of 1.4 grams of SDS per gram of protein[10]. However, this ratio is not constant when one considers that some proteins have internal infrastructure, such as disulfide bonds, which cannot be disrupted by the detergent alone. These types of bonds provide rigidity to the protein structure and require reducing agents in order to break the disulfide bond. Nevertheless, the binding of SDS to proteins results in conformational change, which is not always a linear or rod-shaped conformation. The differential binding of SDS to protein based on the protein structure provides selectivity to SDS-based CE separations.

Upon addition of SDS in sub-micellar concentrations, a difference in resolution is seen between pH 8.3 and 9.5 (Figures 2.4 and 2.5). A better separation is obtained at pH 9.5 where most of the proteins in IV-1 Paste are soluble. Even though SDS is present in sub-micellar concentration, its presence has affected the separation when compared to run conditions without SDS (Figures 2.9 and 2.10). When SDS is not included in the separation conditions, and when the concentration of SDS is replaced with salt, the separation results in co-migration similar to the current CZE method. The overall effect of adding salt is reduction of electroosmotic flow and subsequent loss of resolution. One can conclude that ionic strength of the run buffer alone is not adequate for resolving the protein mixture.

Where SDS is added to the separation conditions above the critical micellar concentration, the separation is significantly improved. Once again, a better separation is achieved at higher pH (Figures 2.6 and 2.7). If the SDS concentration is increased further, there appears to be no advantage, but the disadvantage of broader peaks and lower detection of the minor constituents (Figure 2.8).

Consider the effect of the background electrolyte, in this case boric acid. When the buffer concentration is reduced in the presence of micellar SDS, the separation is negatively

impacted and resembles the separation obtained with sub-micellar SDS at lower pH (Figures 2.6 and 2.11). One can conclude that the buffer capacity of boric acid was greatly reduced and the pH was affected by the proteins and excipients in the sample. When the buffer concentration is increased in the presence of micellar SDS, the separation is negatively impacted as seen by a loss of resolution (Figure 2.12). Here, the conductivity of the run buffer caused a decrease in resolution by an increase in current. An increase in current can lead to an increase in temperature within the capillary, decreased buffer viscosity and subsequent decrease in electroosmtic flow. In essence, all the proteins migrated rapidly through the capillary (as seen by the short run time) at the speed of the electroosmotic flow. Typically, an increase in ionic strength should result in a decrease in electroosmotic flow. However, in this case, one can speculate that the increase in borate concentration led to an increase in current, capillary heating and a subsequent decrease in viscosity. This may have been caused by an enrichment of the charge double layer with sodium ions which decreases the zeta potential.

These experiments led to the optimized buffer conditions of 65 mM boric acid, 20 mM SDS, pH 9.5 for separation of IV-1 Paste.

Another anionic surfactant, sodium cholate, was investigated to see if a different selectivity could be obtained. Sodium cholate has a CMC of 13-15 mM and an aggregation number of 2-4. Again, proteins do not partition into micelles, but the CMC and aggregation number are provided only to illustrate the difference between the two anionic surfactants. For simplicity, sodium cholate was used above the CMC only at the same concentration as SDS in the previous experiments (20 mM). In essence, sodium cholate was substituted for SDS into the optimized CE-SDS run buffer. Figure 2.13 shows that there was no advantage for using sodium cholate over SDS.

The cationic surfactant, cetyltrimethylammonium bromide (CTAB), is another popular surfactant used in MEKC. In MEKC, a difference in selectivity can be achieved by using a surfactant of an opposite charge as seen with small molecules and peptides. For proteins,

published literature has shown examples of high resolution protein separation using CTAB for basic proteins using covalent and dynamic coated capillaries [12-13]. For the purpose of this research, CTAB was used above the micellar concentration at the pH appropriate for solubility of the acidic proteins in IV-1 Paste. The resulting separation shown in Figure 2.14 shows a lack of resolution, which may be attributed to the lower association constant of CTAB to proteins as compared to that of SDS. There is also very little change to the protein structure upon binding of CTAB to proteins as reported in literature [11]. In this case, it is likely that CTAB does not provide adequate denaturation or charge variation to produce separation.

Triton X-100 is an example of a nonionic surfactant. Triton X-100 has a large aggregation number and a low CMC. In comparison with SDS, Triton X-100 creates a larger micelle at lower concentrations. Literature states that the interaction of Triton X-100 with proteins is dependent on their hydrophobicity or hydrophilicity. TX has much higher association with hydrophobic proteins for example those associated with lipid membranes [8,14]. The association of TX with hydrophilic proteins is less and due in part to dipole interaction with the oxygen in the polar head group of this neutral surfactant [8]. When 1% Triton X-100 was applied to capillary electrophoresis separation of IV-1 Paste, no protein peaks were visible after 45 minutes regardless of polarity. This surfactant was difficult to solubilize, and was higher in viscosity than other additives used. This detergent was not further pursued, however, a lower concentration and a larger inner diameter capillary would be possible avenues.

Capillary Gel Electrophoresis, CGE

Capillary gel electrophoresis (CGE) aims to mimic SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Many kits are now commercially available. In CGE, a coated capillary is used, and the run buffer contains polymer additives, which act as a sieving matrix [15]. For the purpose of this research, these kits were investigated but avoided as the basis of this research in order to develop a novel approach without relying upon commercially available

reagents. These kits may be modified or discontinued in the future, which poses a problem for regulated industries. The separation of IV-1 Paste proteins using the Beckman SDS CGE kit was impressive (Figure 2.15). The proteins are theoretically separated by molecular weight due to the presence of SDS and high concentrations of polymer. The negative points involve the reproducibility of the separation, the requirement for high pressure in order to pack the capillary with the replaceable gel buffer, and the long run times. Also, it is apparent that the later migrating proteins have broader peak shapes. For the purposes of this research, it was not prudent to perform spike studies to identify the peaks in this separation due to the fact that a novel system was desired.

In order to further explore size-based separation by CE without the use of commercial products, the addition of polymers, such as polyethylene glycol (PEG), has been suggested in literature [16]. For this experiment, 0.8% PEG was added to the optimized CE-SDS buffer system to see if any further improvement could be made (Figure 2.16). Although the peak shapes were improved for early migrating peaks, the migration order did not appear to change. A loss of peak shape is noted with the apolipoprotein A-1 peak, and the baseline appears noisy. The separation is also shorter in run time, which was unexpected. One would expect the retardation of the proteins by the polymer would slow down the migration times. One explanation might be an increase in current, which led to faster migration time and some baseline noise.

Capillary electrophoresis is easily adapted with lab-on-a-chip technology. Caliper Technologies and Agilent Technologies have co-developed a new instrument called the Bioanalyzer. The instrument and chemistry involve SDS-CGE on a chip with LIF detection. The separation is theoretically based on molecular size, however, it appears that the two major protein constituents in IV-1 Paste are switched in order with respect to molecular weight. Albumin and $Alpha_1$-Antitrypsin are inverted where AAT migrates as the first peak and ALB is the second peak (Figure 2.17). The apparent molecular weight of other proteins is also inaccurate when compared with the protein standards (Table 2.3). This may be attributed to the presence or

absence of glycosylation. For IV-1 Paste, no new proteins are identified using this separation, and it appears that there may be co-migration with the lower marker.

Dynamic Coated Capillaries

Dynamic coating of the capillary walls can be achieved using polyamines (polycations) such as putrescine (1,4-diaminobutane). This is a simple buffer additive that can have dramatic effects through EOF suppression and reduced protein adsorption [17, 18]. In contrast with covalently coated capillaries, dynamic coatings are replaced with every run and do not require much more than a bare fused silica capillary. Using conditions suggested in literature, putrescine was studied for the separation of IV-1 Paste proteins. In this experiment, albumin was run as a purified standard protein to compare with IV-1 Paste. Figures 2.18 and 2.19 show a complex separation of IV-1 Paste, with a negative impact on the baseline. This suggests that there are secondary interactions or possible matrix effects from non-protein components in IV-1 Paste. Under these conditions, albumin is now a split peak, which shows a variant that was not previously seen. In literature, multiple peaks are obtained for purified glycoproteins through the use of these dynamic coatings and the resulting EOF suppression [17, 18]. For complex protein mixtures, the resolution capability of this dynamic coating may further complicate the protein profile and have a negative impact on the separation.

Conclusion

The challenge with separation of complex protein mixtures, such as human plasma proteins from the Cohn fractionation process, is the heterogeneity of the protein constituents with respect to concentration, size, glycosylation, surface charge and structural properties. Each protein has its own microheterogeneity, which further complicates the profile. Several capillary electrophoresis techniques have been explored in this research in an attempt to capitalize on these differences while balancing the need for resolution of all constituents.

SDS-CGE and cIEF showed excellent promise for resolving the proteins in IV-1 Paste. These applications require some optimization. Overall, the most efficient CE separation was developed and optimized in-house without relying on commercially available products. The method utilizes a bare fused silica capillary and a simple borate, SDS buffer at pH 9.5.

As a side note, the proprietary cIEF and SDS-CGE kits used in this research are older generation kits and have since been improved by the manufacturers. Instrumentation has also been improved in order to achieve higher and more consistent pressures.

Table 2.1. Capillary Zone Electrophoresis Protein Identification

CZE Region	Expected Proteins
Gamma	Immunoglobulins: IgG, IgM, IgA
Beta	Transferrin, Plasminogen, C3 (complement), C-reactive Protein, Free Hemoglobin, Lipoproteins (LDL, VLDL)
Alpha-2	Alpha-2-macroglobulin, Antithrombin III, Ceruloplasmin, Haptoglobin
Alpha-1	Alpha-1-antitrypsin, Alpha-1-acid Glycoprotein, Alpha Fetoprotein, High Density Lipoprotein (HDL)
Albumin	Albumin

Table 2.2 Surfactant Properties[7]

Class	Detergent Name	MW of Monomer	CMC (mM)	Aggregation #	MW of Micelle
Nonionic	Triton X-100	650	0.2-0.9	100-155	80,000
Cationic	CTAB	364.5	1.0	170	62,000
Anionic	SDS	288.5	7-10	62	18,000
Anionic	Sodium Cholate	430.6	9-15	2	900

Table 2.3. Apparent Molecular Weight by Agilent Bioanalyzer

Protein	Expected Molecular Weight (Da)	Molecular Weight by Bioanalyzer (Da)
IgG	150,000	162,000
Alpha1-Antitrypsin	54,000	75,000
FVIII	285,000	210,000
Albumin	67,000	54,000

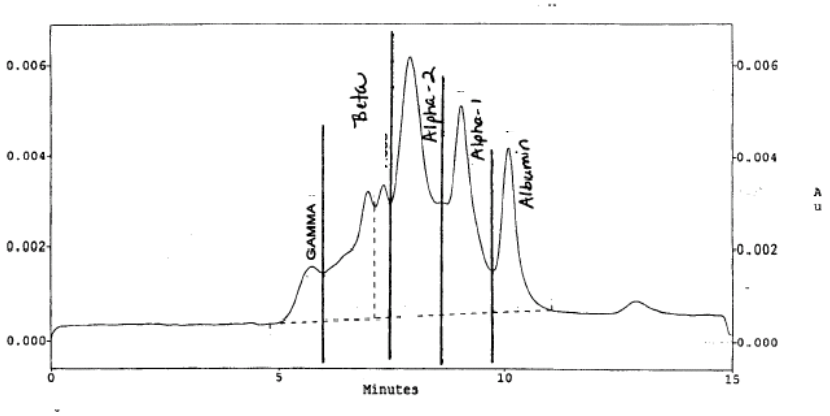
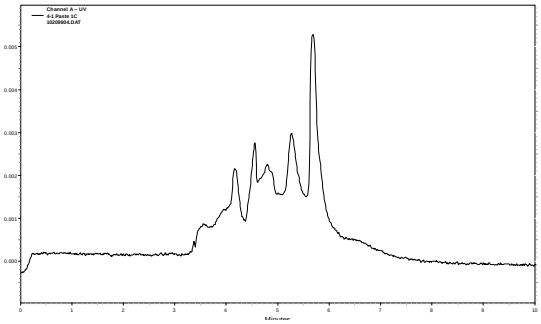
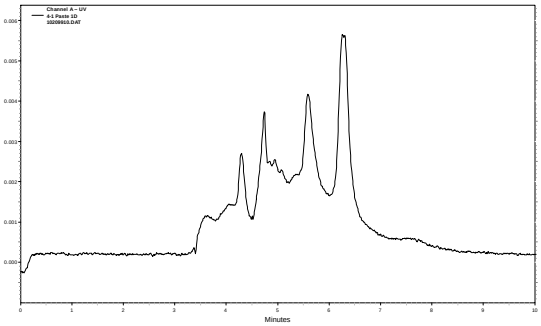


Figure 2.1. IV-1 Paste by Capillary Zone Electrophoresis (CZE) Method: 200mM Borate, 130mM NaCl, pH 9.8; 31.2cm x 20μm bare fused silica capillary; UV detection at 214nm; System: Beckman MDQ.

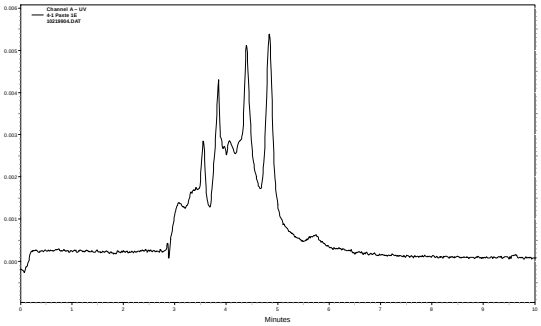
A



B



C



D

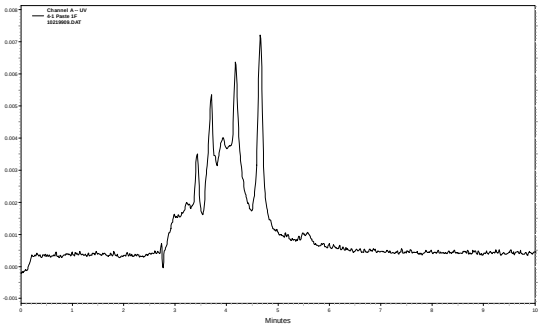


Figure 2.2. IV-1 Paste: 50mM Tris/HCl, 100mM NaCl; 37cm x 20µm bare fused silica capillary; 10kV; UV detection at 214nm; System: Beckman P/ACE 5000.

A. pH 7.5
B. pH 8.0
C. pH 8.5
D. pH 9.0

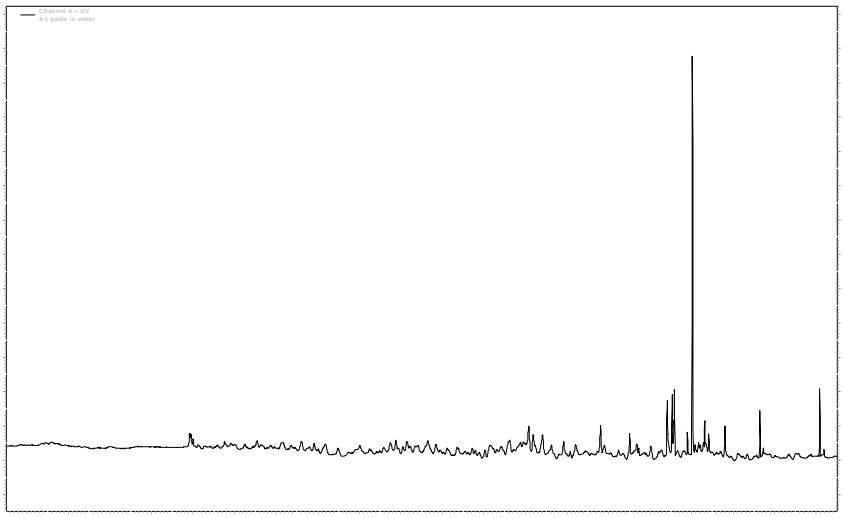


Figure 2.3 Capillary Isoelectric Focusing of IV-1 Paste Using Beckman cIEF kit. Run conditions per kit instructions.

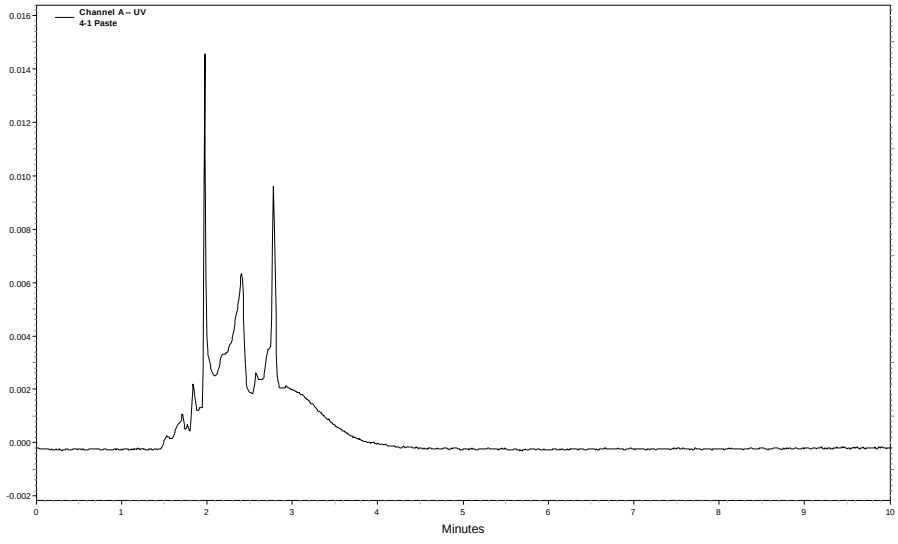


Figure 2.4 IV-1 Paste Using Sub-Micellar SDS, Run Conditions: 65 mM boric acid, 3.5 mM SDS, pH 8.3; 10kV, 37 cm bare fused silica capillary.

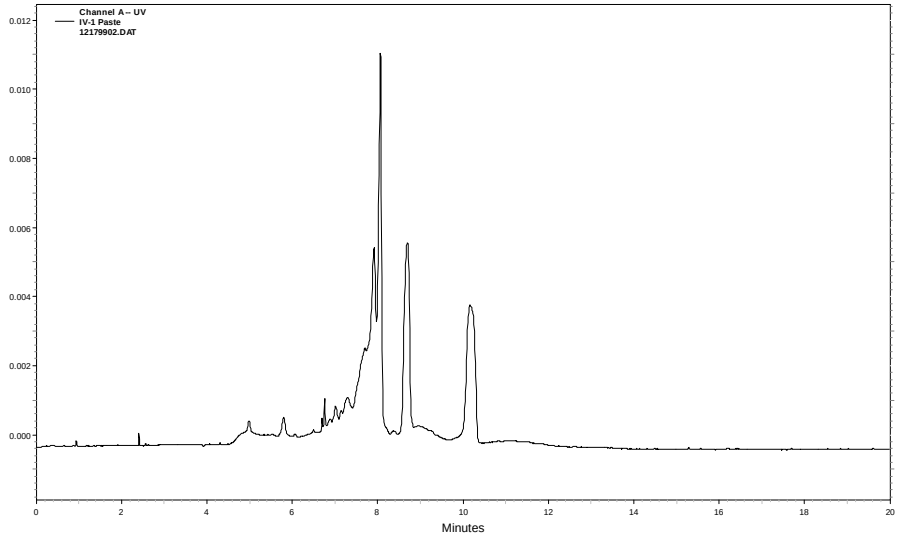


Figure 2.5 IV-1 Paste Using Sub-Micellar SDS, Run Conditions: 65 mM boric acid, 3.5 mM SDS, pH 9.5; 10kV, 37 cm bare fused silica capillary.

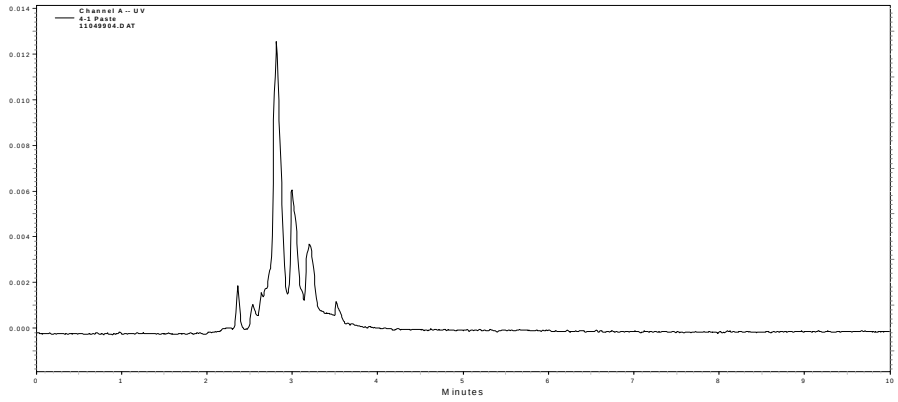


Figure 2.6 IV-1 Paste Using Micellar SDS, Run Conditions: 65 mM boric acid, 20 mM SDS, pH 8.3; 10kV, 37 cm bare fused silica capillary.

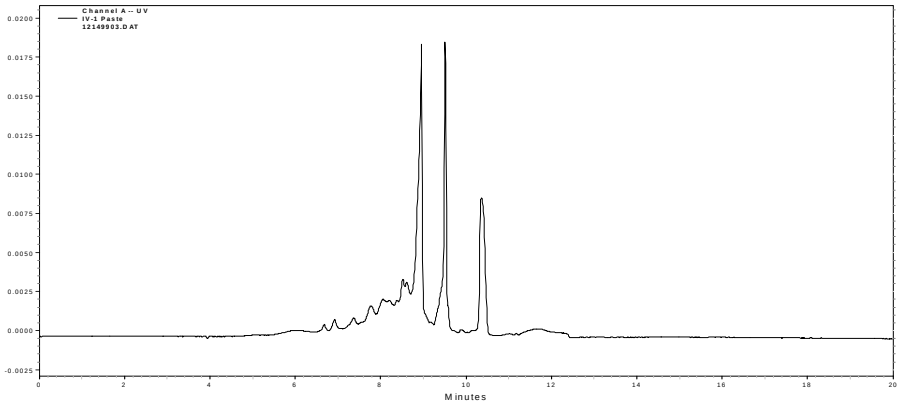


Figure 2.7 IV-1 Paste Using Micellar SDS, Run Conditions: 65 mM boric acid, 20 mM SDS, pH 9.5; 10kV, 37 cm bare fused silica capillary.

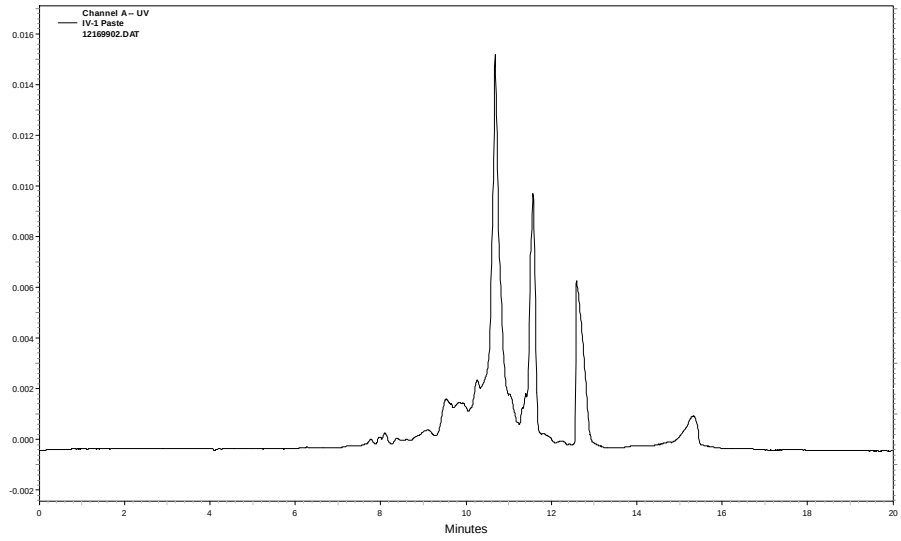


Figure 2.8 IV-1 Paste Using Micellar SDS, Run Conditions: 65 mM boric acid, 50 mM SDS, pH 9.5; 10kV, 37 cm bare fused silica capillary.

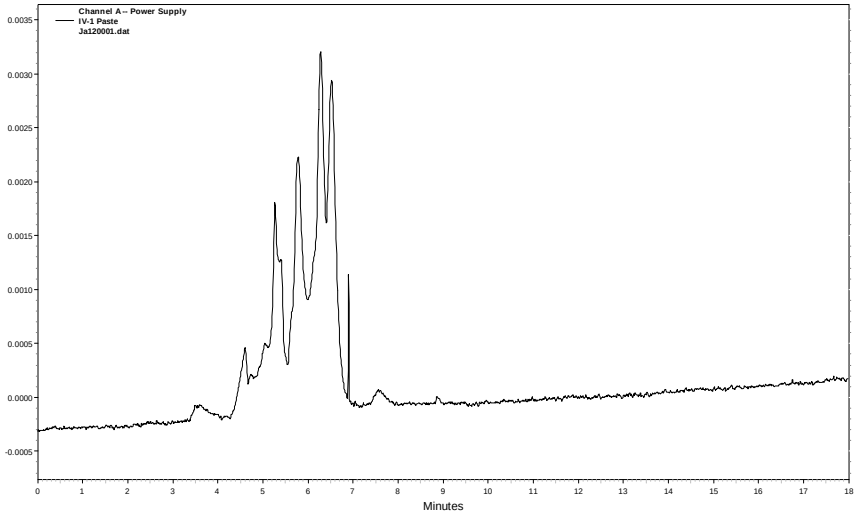


Figure 2.9 IV-1 Paste without SDS, Run Conditions: 65 mM boric acid, pH 9.5; 10kV, 37 cm bare fused silica capillary.

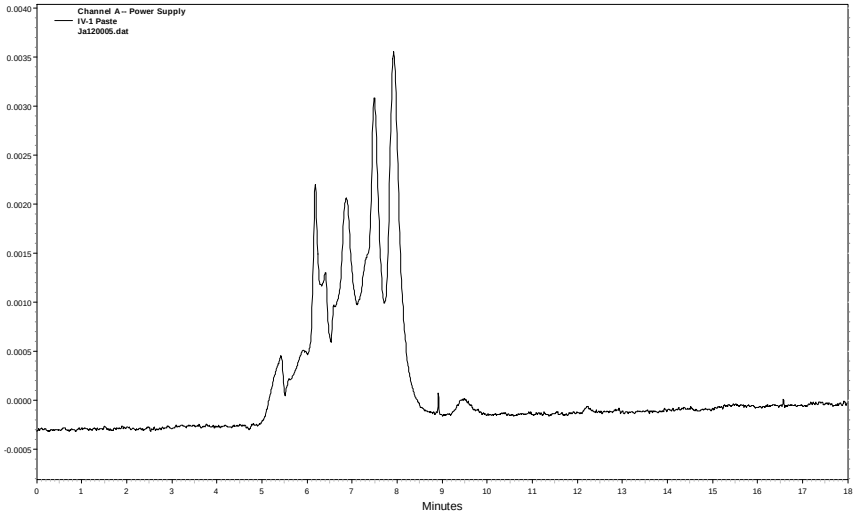


Figure 2.10 IV-1 Paste without SDS adding salt for ionic strength, Run Conditions: 65 mM boric acid, 20 mM NaCl, pH 9.5; 10kV, 37 cm bare fused silica capillary.

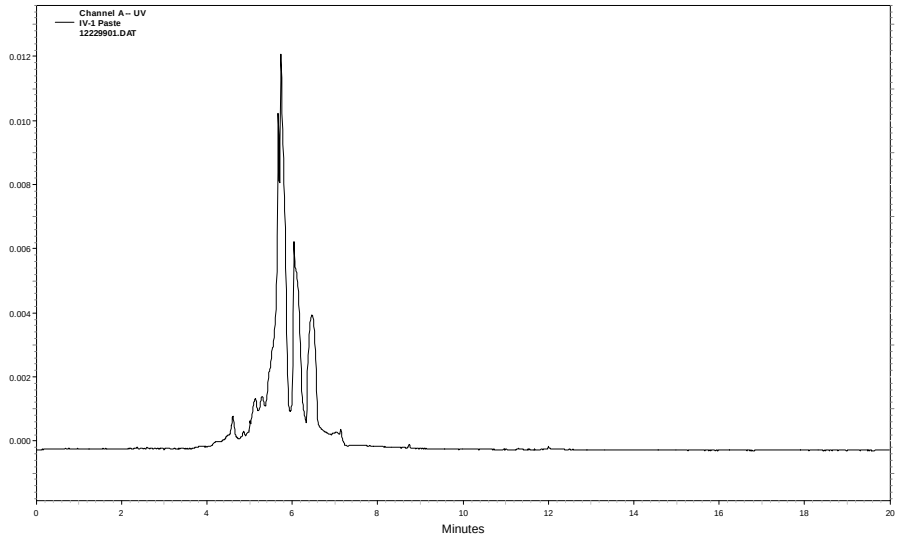


Figure 2.11 IV-1 Paste with Micellar SDS reducing boric acid, Run Conditions: 10 mM boric acid, 20 mM SDS, pH 9.5; 10kV, 37 cm bare fused silica capillary.

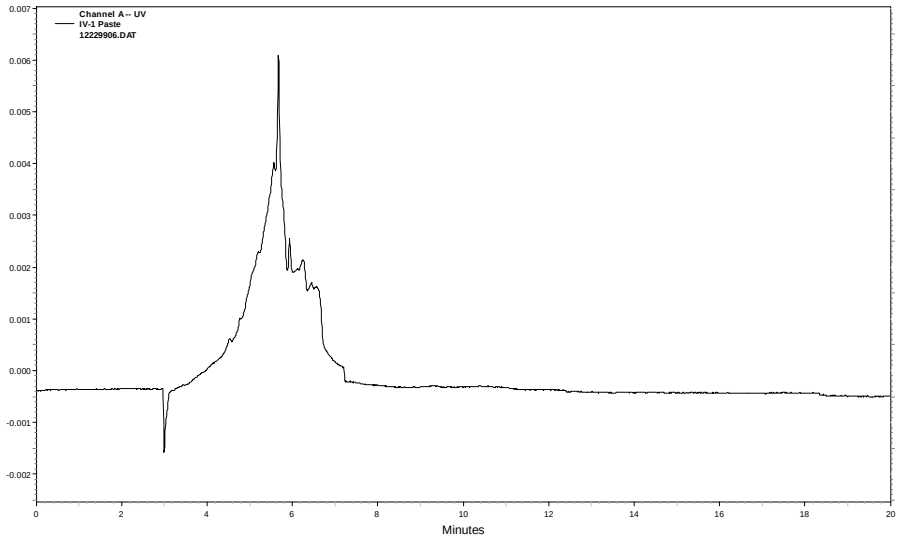


Figure 2.12 IV-1 Paste with Micellar SDS increasing boric acid, Run Conditions: 200 mM boric acid, 20 mM SDS, pH 9.5; 10kV, 37 cm bare fused silica capillary.

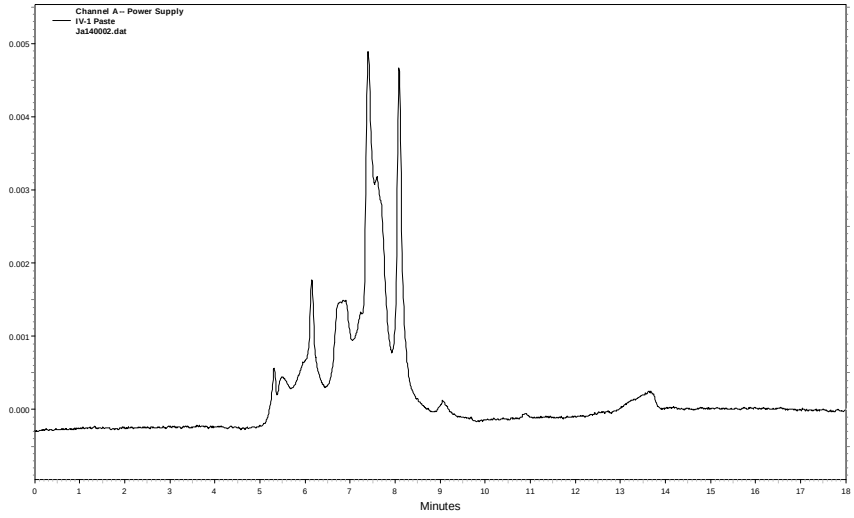


Figure 2.13 IV-1 Paste Using Sodium Cholate, Run Conditions: 65 mM boric acid, 20 mM sodium cholate, pH 9.5; 10kV, 37 cm bare fused silica capillary

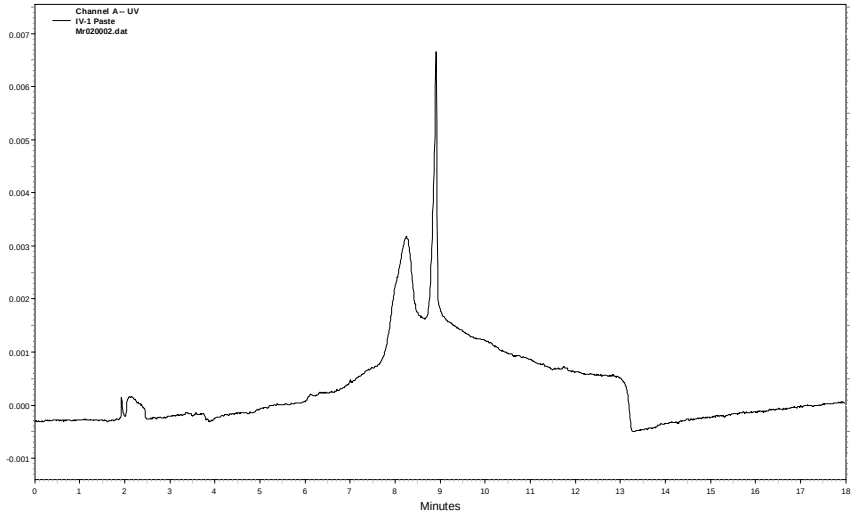


Figure 2.14 IV-1 Paste Using CTAB, Run Conditions: 65 mM boric acid, 3.5 mM CTAB, pH 9.5; -10kV, 37 cm bare fused silica capillary

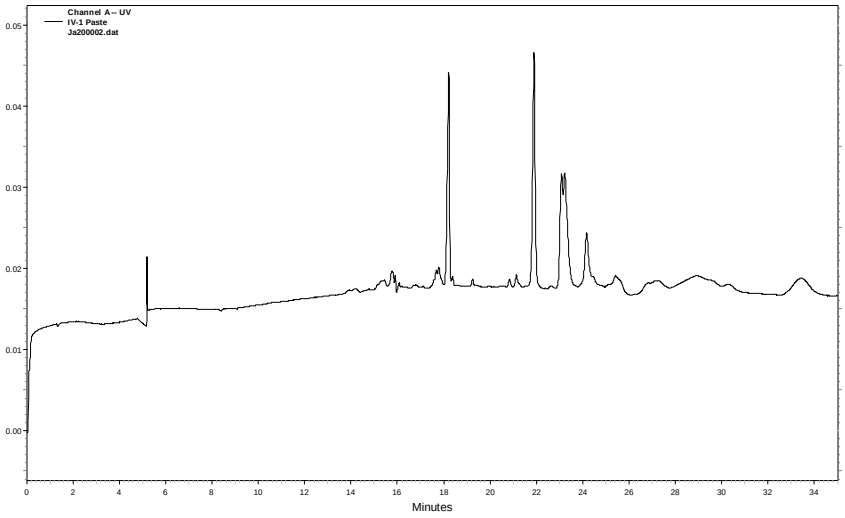


Figure 2.15 IV-1 Paste by Beckman SDS CGE Kit; Run conditions per kit instructions.

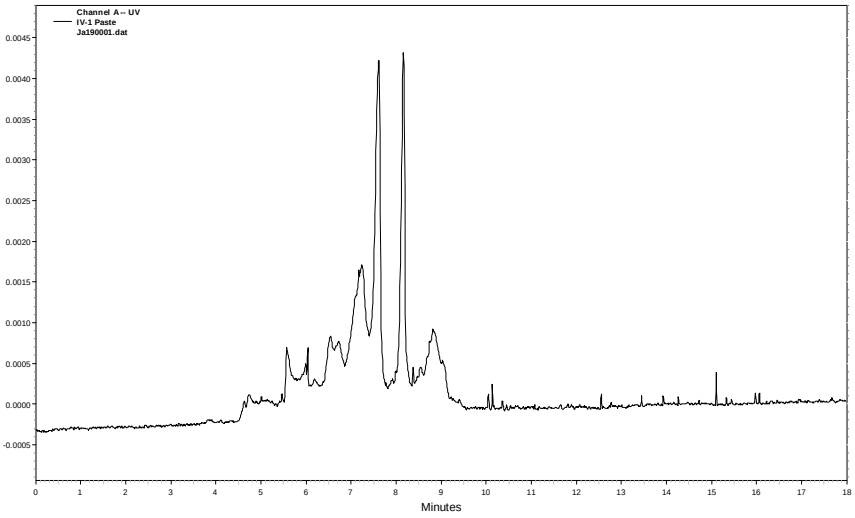


Figure 2.16 IV-1 Paste by CE-SDS with PEG, Run Conditions: 65 mM boric acid, 20 mM SDS, 0.8% PEG, pH 9.5; 10kV, 37 cm bare fused silica capillary.

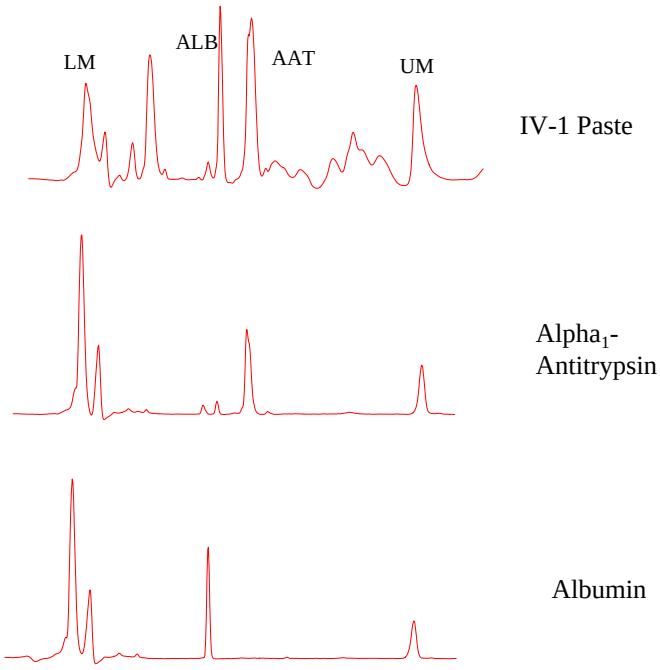


Figure 2.17 IV-1 Paste by Agilent Bioanalyzer (Protein 200 Chip); LM = lower marker, UM = upper marker.

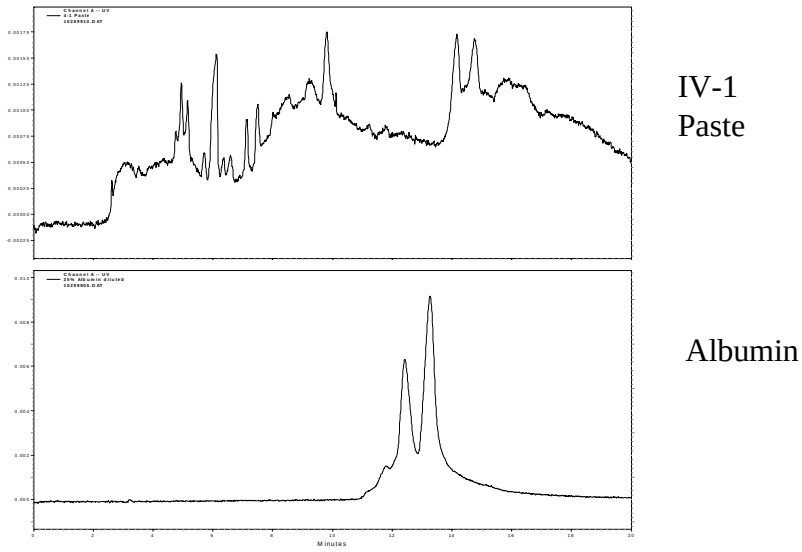
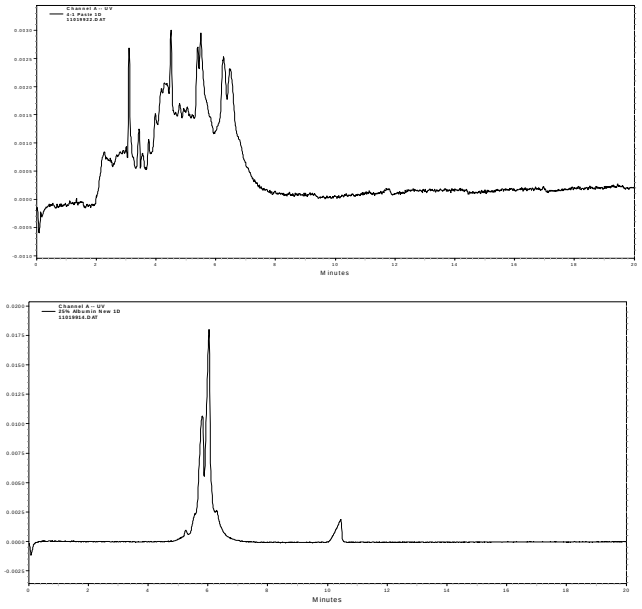


Figure 2.18 IV-1 Paste and Albumin, Run Conditions: 100 mM boric acid, 5 mM diaminobutane, pH 8.5; 20kV, 37 cm bare fused silica capillary.



IV-1
Paste

Albumin

Figure 2.19 IV-1 Paste and Albumin, Run Conditions: 20 mM sodium phosphate, 50 mM boric acid, 5 mM diaminobutane, pH 8.5; 20kV, 37 cm bare fused silica capillary.

References

1. Baker, D.R. *Capillary Electrophoresis*; John Wiley and Sons, Inc.:NewYork, 1995; pp 19-51.
2. Khaledi, M.G. *High Performance Capillary Electrophoresis: Theory, Techniques and Applications*; Wiley and Sons: New York, 1998.
3. Palfrey, S.M. Serum Protein Electrophoresis. *Clinical Applications of Capillary Electrophoresis* Humana Press, Inc.: New Jersey, 1999; pp 11-19.
4. Jenkins, M.A.; Guerin, M.D. Capillary Electrophoresis Procedures for Serum Protein Analysis: Comparison with Established Techniques. *Journal of Chromatography B*. **1997**, *699*, 257-268.
5. Chen, F-T. A. Rapid Protein Analysis by Capillary Electrophoresis. *Journal of Chromatography*. **1991**, *559*, 445-453.
6. Gordon, M.J.; Lee, K-J.; Arias, A. A.; Zare, R.N. Protocol for Resolving Protein Mixtures in Capillary Zone Electrophoresis. *Analytical Chemistry*. **1991**, *63*, 69-72.
7. Bhairi, S.M. A Guide to the properties and Uses of Detergents in Biology and Biochemistry, Calbiochem-Novabiochem Corporation, 2001; pp 35-41.
8. Strege, M.A.; Lagu, A. Micellar Electrokinetic Chromatography of Proteins. *Journal of Chromatography A.* **1997**, *780*, 285-296.
9. Terabe, S. Micellar Electrokinetic Chromatography, Beckman Instruments, Fullerton, CA, 1992.
10. Reynolds, J.A.; Tanford, C. The Gross Conformation of Protein-Sodium Dodecyl Sulfate Complexes. *Journal of Biological Chemistry*. **1970**, *245* (19), 5161-5165.
11. Nozaki, Y.; Reynolds, J.A.; Tanford, C. The Interaction of a Cationic Detergent with Bovine Serum Albumin and Other Proteins. *Journal of Biological Chemistry*. **1974**, *249* (14), 4452-4459.

12. Cifuentes, A.; Rodriguez, M.A.; Garcia-Montelongo, F.J. Separation of Basic Proteins in Free Solution Capillary Electrophoresis: Effect of Additive, Temperature and Voltage. *Journal of Chromatography A.* **1996**, *742*, 257-266.
13. Kleindienst, G.; Huber, C.G.; Gjerde, D.T.; Yengoyan, L.; Bonn, G.K. Capillary Electrophoresis of Peptides and Proteins in Fused-Silica Capillaries coated with Derivatized Polystyrene Nanoparticles. *Electrophoresis*. **1998**, *19*, 262-269.
14. Makino, S.; Reynolds, J.A.; Tanford, C. The Binding of Deoxycholate and Triton X-100 to Proteins. *Journal of Biological Chemistry*. **1973**, *248* (14), 4926-4932.
15. Guttman, A. Capillary Sodium Dodecyl Sulfate-gel Electrophoresis of Proteins. *Electrophoresis*. **1996**, *17*, 1333-1341.
16. Stastna, M.; Radko, S.P.; Chramback, A. Capillary Zone Electrophoresis of Proteins in Semidilute Polymer Solutions: Inter- and Intra-polymer Predictability of Size-dependent Retardation. *Electrophoresis*. **1999**, *20*, 2884-2890.
17. Landers, J.P.; Oda, R.P.; Madden, B.J.; Spelsberg, T.C. High-Performance Capillary Electrophoresis of Glycoproteins: The Use of Modifiers of Electroosmotic Flow for Analysis of Microheterogeneity. *Analytical Biochemistry*. **1992**, *205*, 115-124.
18. Cordova, E.; Gao, J.; Whitesides, G.M. Noncovalent Polycationic Coatings for Capillaries in Capillary Electrophoresis of Proteins. *Analytical Chemistry*. **1997**, *69*, 1370-1379.

CHAPTER 3. CHARACTERISTICS OF THE CE-SDS METHOD

Introduction

In this research, the optimal conditions identified involve a borate-SDS buffer with free-solution separation of proteins in complex matrices. This method was developed in-house, and the performance characteristics of the method will be discussed in this chapter.

Experimental

Chemicals

Boric acid (sodium salt), lauryl sulfate (sodium salt), transferrin (human), Prealbumin (human), apolipoprotein A-I (human), alpha$_2$-macroglobulin (human), haptoglobin (human), IgA (human) and IgM (human) were purchased from Sigma Chemical Company (St. Louis, MO). IV-1 Paste, albumin (human), alpha-1-antitrypsin (human), antithrombin III (human) and IgG (human) were obtained from Bayer Biological Products.

Capillary Electrophoresis Conditions

In general, CE-SDS experiments were performed on Beckman P/ACE 5000 or MDQ CE systems. Later experiments were performed on Agilent G1600A HPC E systems. For experiments performed on the Beckman systems, fused silica capillaries (Beckman) with an inner diameter of 20 μm and a total length of 27 cm were used. The separation voltage was 10 kV (370 V/cm). For experiments performed on the Agilent system, fused silica capillaries (Agilent Technologies) with an inner diameter of 25 μm extended path length and a total length of 47 cm were used. The separation voltage was 17 kV (362 V/cm).

For all experiments, the CE-SDS run buffer consists of 65 mM borate, 20 mM SDS, pH adjusted to 9.5 with 5 N NaOH. All capillaries were first conditioned with 1 N NaOH, followed by deionized water, and then run buffer for approximately ten minutes each. Between sample

injections, capillaries are rinsed with 1 N NaOH followed by water for two minutes each. Prior to sample injection, the capillary is rinsed with run buffer for four minutes. Sample injection was performed by pressure.

Immunonephelometry

Immunonephelometry was performed on a Behring BNII instrument which measures light scattering turbidity formed by antibody recognition complexes.

Sample Preparation

IV-1 Paste was prepared by weighing 0.1 grams of frozen paste and dissolving in five milliliters of CE-SDS run buffer. The solution was vortexed until no pellet was visible. It should be noted that solid IV-1 Paste is considered heterogeneous, and different areas of the solid were sampled in order to be representative.

Other process intermediate samples were diluted a minimum factor of two in order to incorporate SDS and allow to reach equilibrium. If possible, protein concentration was estimated using immunonephelometry of $alpha_1$-antitrypsin, and samples were diluted to fall within the range of the standard curve (0.5 to 5.0 mg/mL).

Results and Discussion

Spike Studies

Spike studies were performed using purified protein standards. In order to identify the migration time of proteins in IV-1 Paste, proteins were injected alone and spiked into IV-1 Paste. The results were varied, where some proteins could be positively identified and others were inconclusive due to multiple peaks or impurities. Also, proteins in run buffer alone did not always have the same migration time as in IV-1 Paste suspension, which made identification by migration time unreliable in some cases. This is attributed to the fact that IV-1 Paste suspension

has a complex matrix and higher ionic strength than purified protein matrices. Most of the early migrating proteins do not resolve completely and exist in low concentrations in IV-1 Paste. The Igs (IgG, IgA and IgM) appear broad and may migrate under several peaks in the distribution. Although the Igs exist in low abundance in IV-1 Paste, they potentially contribute to the broadness of the peaks in the regions preceding the AAT peak. The spike studies using the CE-SDS conditions are summarized in Figures 3.1 to 3.11.

Mechanism of Separation

Figure 3.12 shows the separation and peak identification obtained by the CE-SDS method for IV-1 Paste. It is believed that the mechanism of separation is based on the differential binding and denaturation of the proteins by SDS resulting in elongated structure and overall negative charge. SDS binding to the protein surfaces has a positive effect on the CE separation in that the net negative charge will cause proteins to repel from the capillary wall and from each other. In this method, the separation relies on structural differences of the proteins and how the degree of SDS binding differs between the various structures. Certain protein characteristics are listed in Table 3.1, and proteins are listed in migration order by the CE-SDS method. The selected proteins discussed are not necessarily baseline resolved from each other. However, this chapter aims to characterize the method and its capabilities and limitations.

Consider the example of prealbumin (transthyretin), haptoglobin, tranferrin, alpha$_1$-antitrypsin, albumin and apoliprotein A-I (Figure 3.12). The mechanism of separation of these proteins can be explained by considering the structural characteristics such as beta-sheet or alpha-helical motifs, the presence or absence of disulfide bonds and the degree of glycosylation. One can conclude that the order of migration is dependent on the protein structure primarily, and the presence or absence of disulfide bonds plays a secondary role. The migration order of these selected proteins begins with the early migrating proteins that exist as tetramers (e.g. prealbumin, haptoglobin). The monomeric proteins rich in beta-sheet structure are next in the migration order

(e.g. alpha-2-macroglobulin, transferrin, alpha$_1$-antitrypsin). Finally, the proteins which have mostly alpha-helical structure migrate last (e.g. albumin, apolipoprotein A-I). However, one cannot ignore the fact that albumin contains 17 disulfide bonds, yet is almost last in migration order and tends to separate well from the other proteins. One explanation may be that the disulfide bonds in albumin create nine loops and can be grouped into three homologous domains. Each domain consists of a repeating triplet pattern of long-short-long loops. In solution with SDS, the structure of albumin can be perturbed to form an almost cylinder shape due to the simple adjacent loop structure of the protein [1]. In contrast, prealbumin, or transthyretin, is made up of two homologous dimers through the interactions of the beta-sheet structures [6]. These hydrophobic beta-sheet structures are easily perturbed by SDS, and most likely result in the denaturation of prealbumin into its monomeric form. In other words, the affects of SDS binding are maximized on proteins with mostly hydrophobic structure.

Glycosylation, or the post-translational addition of sugar subunits to proteins, aids in the stability, solubility, folding, biological activity and circulation half-life in the body. For the purpose of this discussion, glycosylation results in additional negative surface charge arising from terminal sialic acid residues. Glycosylation occurs at asparagine residues (N-linked) or threonine residues (O-linked). Not all possible sites of glycosylation for a given protein are occupied by glycans; this results in heterogeneity of the protein population. The presence or absence of glycans affects the binding of SDS, molecular weight and isoelectric point of a protein as well. The example of the model proteins does not allow much correlation between the amount of glycosylation and the migration order other than the fact that the last two proteins (albumin and apolipoprotein A-1) are not typically glycosylated. However, it is important to emphasize the heterogeneity introduced by glycosylation as one explanation for the lack of baseline separation of all of the proteins in the complex mixture.

Quantitation of Alpha$_1$-Antitrypsin by CE-SDS

As mentioned previously, IV-1 Paste is the starting material for the purification of Alpha-1-Antitrypsin by modified Cohn Fractionation. Protein composition of IV-1 Paste was determined by immunonephelometry, which involves immunoprecipitation (monoclonal and polyclonal antibodies) reactions detected by light scattering turbidity, LST (Table 3.2). This technique is used in hospital laboratory settings for quantitation of major plasma proteins of interest. It should be emphasized that this panel of proteins does not represent all of the possible proteins in human plasma. However, it provides a comprehensive profile of the clinically-significant proteins for which quantitation by immunoreaction and LST is accurate within 15% CV.

The three major components in IV-1 Paste include Alpha$_1$-Antitrypsin, Albumin, and Apolipoprotein A-1, where the protein of interest is Alpha$_1$-Antitrypsin (AAT). From Table 3.2, it appears that %Area by CE-SDS has a linear correlation with the fraction of the individual protein of the total protein content, where the protein is identified by this method. Minor components will not likely be identified in part due to the lack of sensitivity from the short path length in CE.

By creating an external standard curve of known AAT concentration versus response factor (peak area), AAT can be accurately quantitated in samples ranging from IV-1 Paste to pre-formulated product. Accurate quantitation is dependent upon a correlation coefficient (R^2) greater than or equal to 0.99 for a standard curve determination (Figure 3.13). Values obtained by CE-SDS for the different process intermediates show reasonable correlation with the values obtained by immunonephelometry (Table 3.3).

Figures 3.14 to 3.18 show the electropherograms of the different process steps during the purification of alpha$_1$-antitrypsin. From the purification process published by Chen, et al in 1998[2], the first step is to suspend the IV-1 Paste in a Tris buffer pH 9.25 to 9.5 and adjust the conductivity by the addition of sodium chloride (Figure 3.14). The second step in the manufacturing process involves the addition of 11.5% PEG for precipitation of viruses and

contaminant proteins, which are removed by centrifugation (Figure 3.15). The level of PEG is then increased to 28.5% in order to precipitate alpha$_1$-antitrypsin. The 28.5% PEG sample is centrifuged to collect alpha$_1$-antitrypsin as a paste and then frozen for further processing (Figure 3.16). When the 28.5% PEG Paste is thawed, it is resuspended in a Tris buffer pH 8.0 and loaded onto a DEAE column for anion chromatography (Figure 3.17). Alpha$_1$-antitrypsin binds to the column resin while many of the remaining contaminant proteins are removed in the flowthrough fraction. Then, the bound alpha$_1$-antitrypsin is eluted and concentrated by ultrafiltration/diafiltration (Figure 3.18).

The CE-SDS method works well for characterizing the process for the purification of alpha$_1$-antitrypsin. One exception is the 11.5% PEG Filtrate sample where the protein profile looks significantly impacted by the sample matrix. (In a previous chapter, the addition of PEG was discussed as a possible sieving polymer for the purpose of size-based separation by CE.) Overall, the purification of alpha$_1$-antitrypsin can be tracked through the process by the CE-SDS method (some level of albumin is present in the final product). The protein profile can be a valuable tool in the manufacturing process to calculate the appropriate column loading based on the amount of alpha$_1$-antitrypsin and contaminant proteins present. The high efficiency and simplicity of this method makes it ideal for the production floor.

Intermediate Precision of the CE-SDS Method

The repeatability (intermediate precision) of the CE-SDS method is outlined in Table 3.4, where the %RSD of the migration time is less than 3% and relative area percent is less than 8% for the three major proteins in IV-1 Paste (n=22 injections). The variability in area percent values can be attributed to the heterogeneity of IV-1 Paste as the same preparation could not be used for these studies (same starting paste). The robustness of this method is proven by the application of this method in different laboratories on different equipment. Early experiments were performed on Beckman P/ACE 5000 and Beckman P/ACE MDQ capillary electrophoresis instruments.

Later experiments were performed on Agilent G1600A HPCE instruments. Minor adjustments were made to the CE conditions in order to compensate for longer and slightly wider capillaries used in the Agilent systems. The only notable difference is seen towards the end of the CE run, where the baseline is affected in samples run on the Agilent system (Figure 3.19). This may beattributed to the temperature thermostatting of the capillary, which is air-cooled in the Agilent system versus liquid-cooled in the Beckman systems. Overall, the profiles are highly conserved.

Limitations

The separation window of the CE-SDS method defined by the migration time of the electroosmostic flow marker, mesityl oxide, and the micelle marker, Sudan III (Figure 3.19). The migration time window is less than eight minutes for which to separate the many protein constituents in IV-1 Paste. Although this separation is not due to differential partitioning, the separation window can still be defined and poses a limitation to the separation capability. Attempts to widen the window of separation by increasing the ionic strength or increasing the SDS concentration did not improve resolution, but only broadened the peaks and reduced detection sensitivity of low abundance proteins.

IV-1 Paste is a downstream sample form the modified Cohn fractionation process, where several proteins of interest have been removed in previous steps. For example, Igs (immunoglobulins) are very minor components in IV-1 Paste (precipitated upstream in II+III Paste). Given the heterogeneity of the Igs, the depletion of Igs from this sample has been advantageous to this separation. It is not possible to separate such a heterogeneous class of proteins by this method even though they are significantly different in size. The CE-SDS method did not result in separation of IgG, IgA and IgM, and the baseline in the early migrating peaks may have been negatively impacted by the presence of the Igs (Figures 3.8, 3.9 and 3.10).

Applicability

The CE-SDS method as developed in this research has the capability for on-line process monitoring during plasma fractionation. Recently, the Food and Drug Administration has introduced the Process Analytical Technology Initiative (PAT), which defines the need for characterization of the manufacturing process through implementation of analytical techniques at various points in the manufacturing process of a biopharmaceutical. Through multivariate analysis of characterization data, critical process parameters which may correlate to yield can be identified. The goal is to analyze samples at multiple checkpoints throughout the process in order to monitor aberrances prior to reaching final product. By improving process control through analytical characterization testing, pharmaceutical and biopharmaceutical companies can avoid discarding final product and save millions of dollars per year.

Biopharmaceutical companies also have to balance the implementation of technological advancements with adherence to the licensed manufacturing process. Theoretically, the PAT Initiative allows for technological advancements in equipment and processes to be implemented without having to reapeat licensure. Comparability protocols have to be performed, however, this is yet another important source of cost savings.

Conclusions

Overall, the CE-SDS method as developed in this research shows excellent precision, accuracy, linearity, and robustness for the detection of proteins of interest in IV-1 Paste. The resolution of the method is a significant improvement over the current serum protein methods. The mechanism of separation involves differential binding of SDS to the proteins due to structural properties, and the solubility and charge repulsion from the capillary wall provided by SDS. The limitations of the method as discussed include the resolution of early migrating proteins, low detection sensitivity to low abundance proteins and the lack of resolution for immunoglobulins.

Table 3.1 Protein Characteristics of Selected Proteins in IV-1 Paste

Migration Order ↓

Protein	Molecular Weight (Da)	pI	Structural Motifs	No. of Disulfide Bonds	Glycosylation	References
Prealbumin (Transthyretin)	65,000	4.7	Homotetramer beta sheet rich	0 1 free Cys	None	SwissProt P02766, Ref 3, 6
Haptoglobin	200,000	4.1	Tetramer of 2 alpha and 2 beta chains Combination of alpha-helix and beta-sheet	7, of which 3 are interchain	4 possible sites (all N-linked)	SwissProt P00738, Ref 4, 7, 8, 9
$Alpha_2$-Macroglobulin	163,000	5.4	beta-sheet rich, low alpha-helix	13, 3 possible internal cross-links	8 possible sites (all N-linked)	SwissProt P01023, Ref 4, 10, 11
Transferrin	85,000	5.5-5.9	Combination of alpha-helix and beta-sheet	1	4 possible sites (3 N-linked, 1 O-linked)	SwissProt P02786, Ref 4, 12
$Alpha_1$-Antitrypsin	51,000	4.2-4.9	Combination of alpha-helix and beta-sheet	0 1 free Cys	3 possible sites (N-linked only)	SwissProt P01009, Ref 3, 5, 13
Albumin	66,500	4.0-5.8	All alpha-helix	17 + 1 free Cys	None	SwissProt P02768, Ref 1, 3, 14, 15, 16
Apolipoprotein A-1	28,000	5.1	All alpha-helix	0	None	SwissProt P02647, Ref 17

Table 3.2 CE-SDS Relative %Composition Compared with Immunonephelometry

Component	% Protein by Immunonephelometry	Rel. %Area by CE-SDS
Alpha-1 Antitrypsin	19.9%	23.83%
IgG	8.3%	12.07%
IgA	8.8%	
IgE	Not significant	unidentified
Antithrombin III	2.7%	0.54%
Albumin	22.3%	17.53%
Transferrin	7.3%	14.29%
Alpha-2-Macroglobulin	7.6%	
Haptoglobin	2.5%	2.86%
Alpha-1-acid Glycoprotein	0.6%	unidentified
Ceruloplasmin	3.3%	unidentified
Apolipoprotein-A1	13.7%	18.17%
Fibronectin	0.5%	Not significant
Plasminogen	0.2%	Not significant
Apolipoprotein-B	0.2%	Not significant
Prealbumin	1.2%	0.84%

Table 3.3 Quantitation of $Alpha_1$-Antitrypsin by CE-SDS in Process Intermediates

Sample	AAT (mg/mL) by Immunoneph.	AAT (mg/mL) by CE-SDS	% Recovery between CE-SDS and Immunoneph*
IV-1 Paste Suspension	2.2	1.9	87%
11.5% PEG Filtrate	0.9	1.1	117%
28.5% PEG Paste Prelim	10.7	11.4	107%
28.5% PEG Suspension, Column Load	16.3	17.0	105%
Concentrated Column Eluate	125	121	98%

Table 3.4 Intermediate Precision of IV-1 Paste (day-to-day, capillary-to-capillary)

N = 22 injections	Migration Time (min)			Area%		
	Alpha-1	Albumin	Apo-A1	Alpha-1	Albumin	Apo-A1
Average	9.2	9.8	10.7	31.3	23.1	13.5
Standard Deviation	0.24	0.27	0.32	1.87	1.76	0.52
%RSD	2.6%	2.8%	3.0%	6.0%	7.6%	3.8%

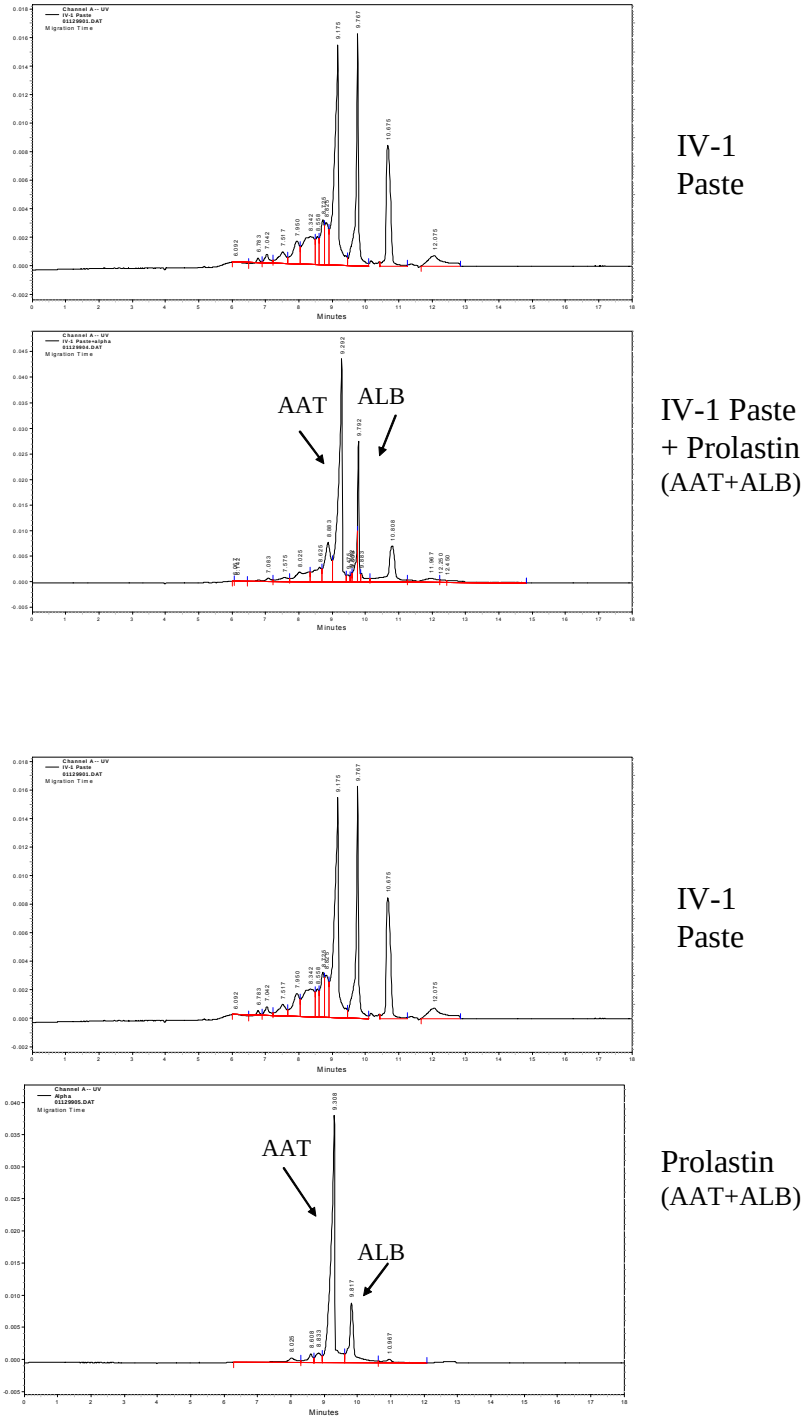
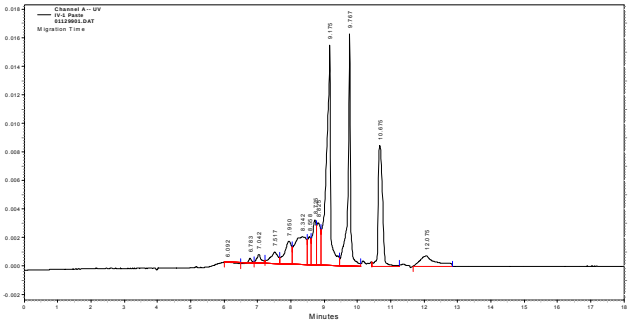
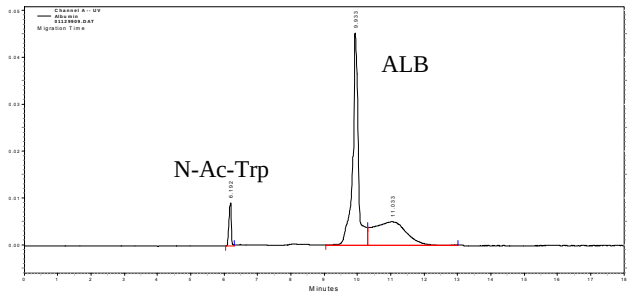


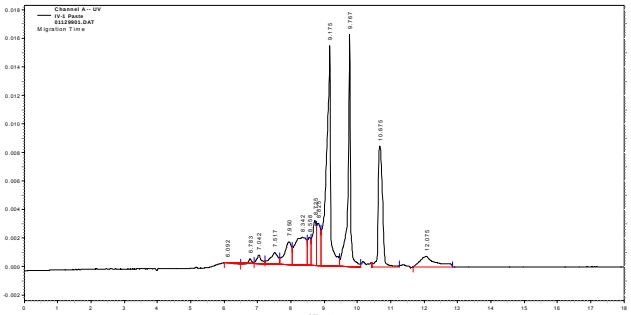
Figure 3.1 Spike Study of Alpha$_1$-Antitrypsin (AAT) and Albumin (ALB) in IV-1 Paste by CE-SDS.



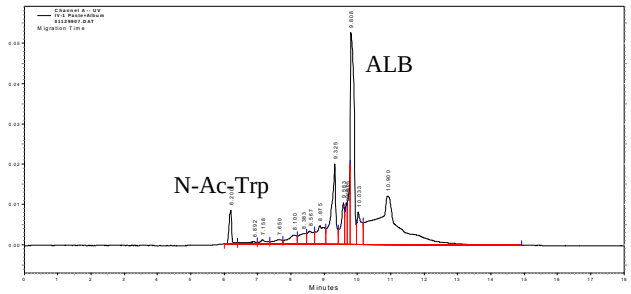
IV-1
Paste



Albumin

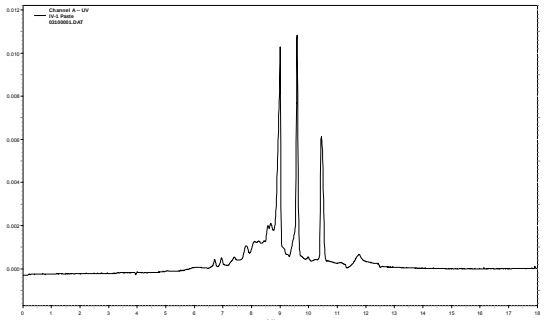


IV-1
Paste

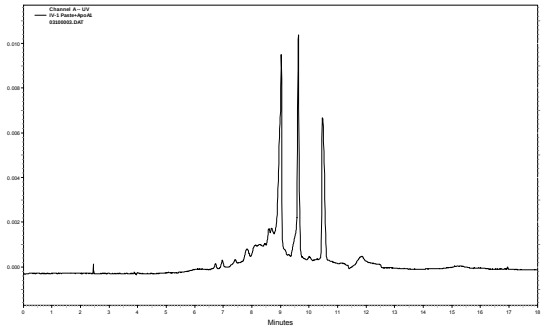


IV-1
Paste +
Albumin

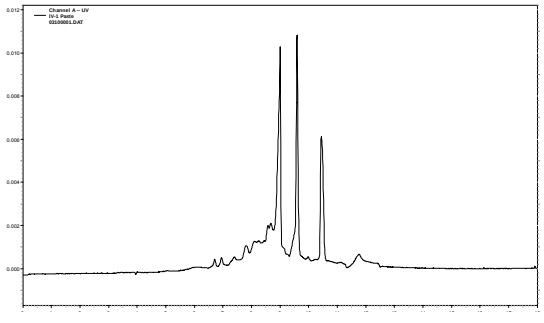
Figure 3.2 Spike Study of Albumin in IV-1 Paste by CE-SDS.



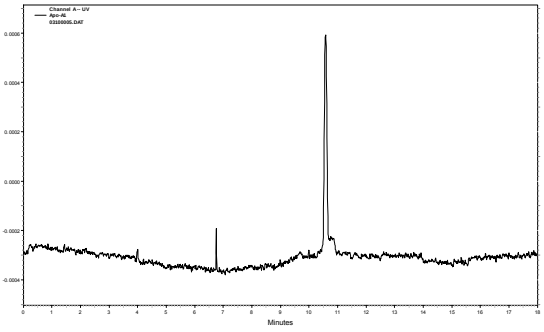
IV-1 Paste



IV-1 Paste + ApoA

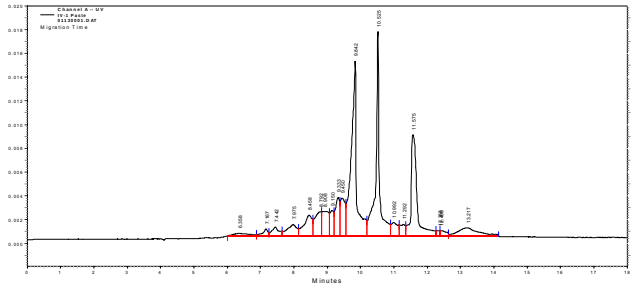


IV-1 Paste

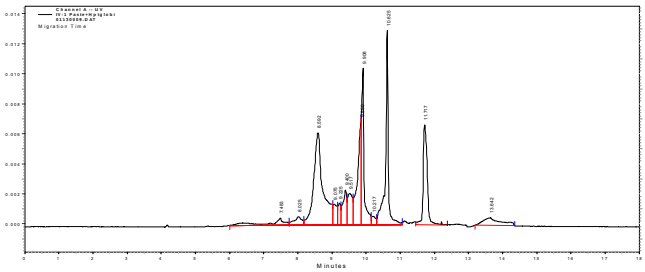


ApoA

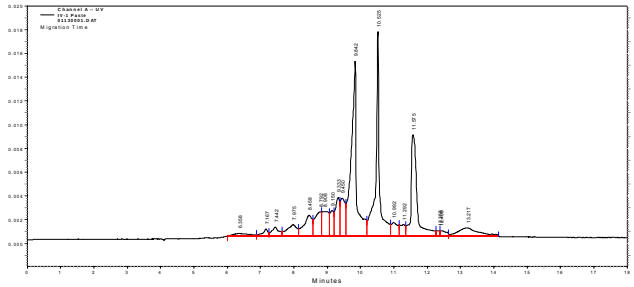
Figure 3.3 Spike Study of Apolipoprotein A-1 in IV-1 Paste by CE-SDS.



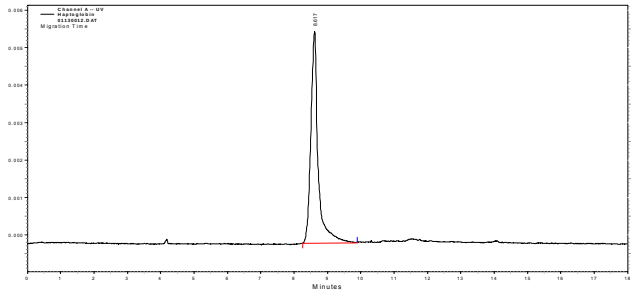
IV-1
Paste



IV-1 Paste +
Haptoglobin

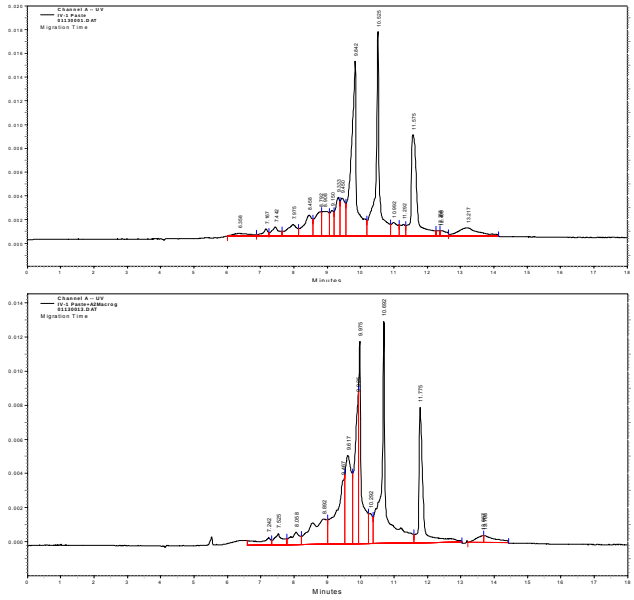


IV-1
Paste



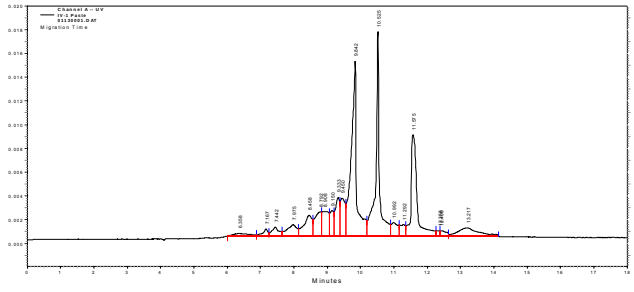
Haptoglobin

Figure 3.4 Spike Study of Haptoglobin in IV-1 Paste by CE-SDS.

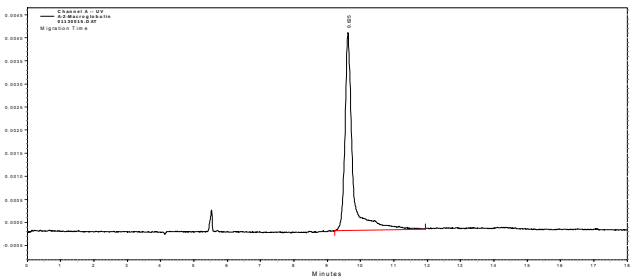


IV-1 Paste

IV-1 Paste + $Alpha_2$-Macroglogulin

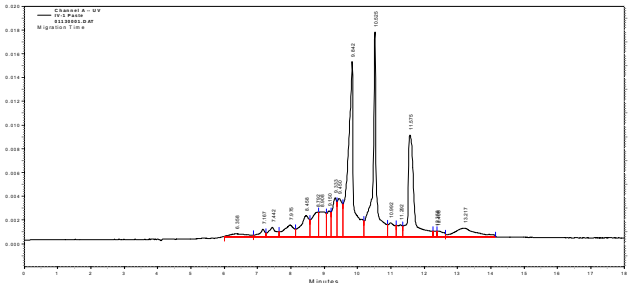


IV-1 Paste

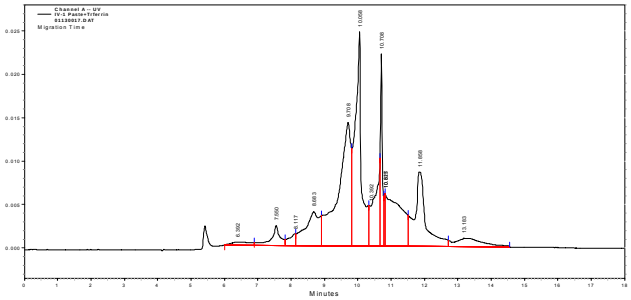


$Alpha_2$-Macroglogulin

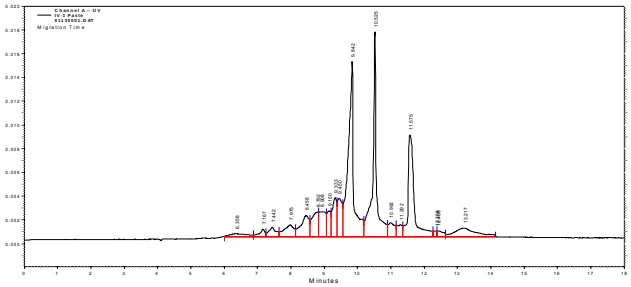
Figure 3.5 Spike Study of $Alpha_2$-Macroglobulin in IV-1 Paste by CE-SDS.



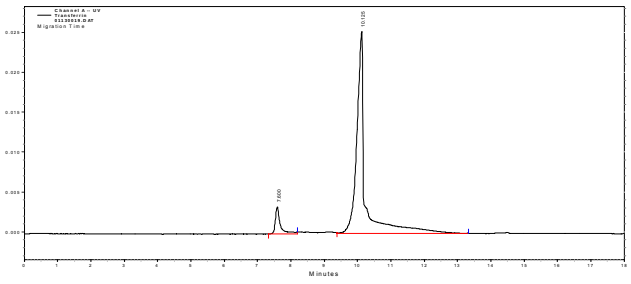
IV-1
Paste



IV-1 Paste
+
Transferrin

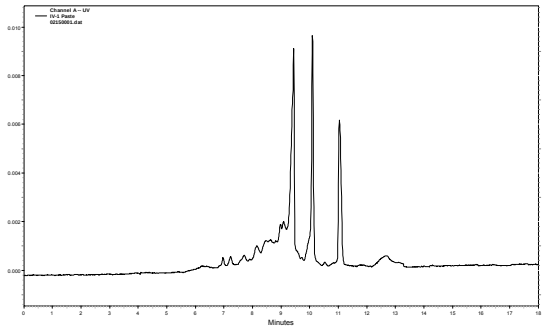


IV-1
Paste

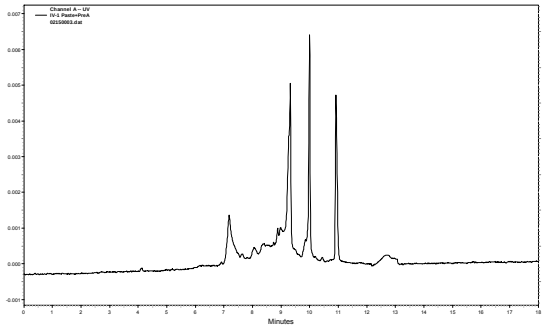


Transferrin

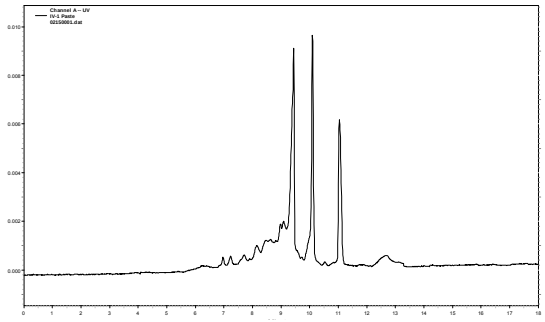
Figure 3.6 Spike Study of Transferrin in IV-1 Paste by CE-SDS.



IV-1 Paste



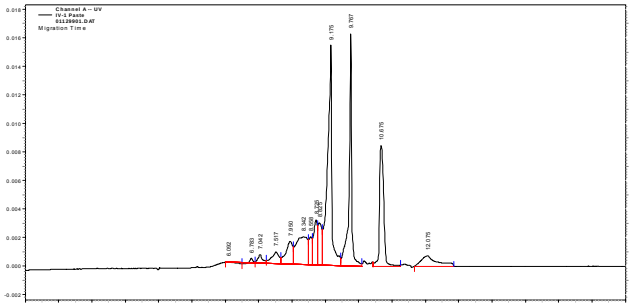
IV-1
Paste+Prealbumin



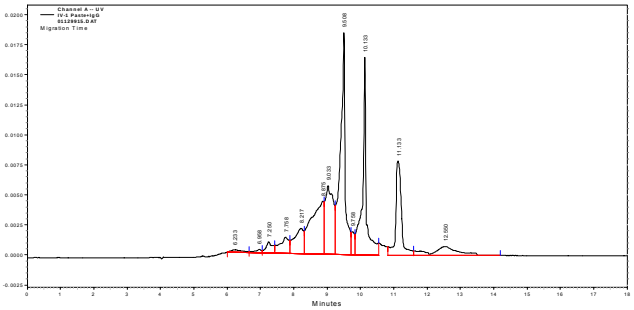
IV-1 Paste

Prealbumin

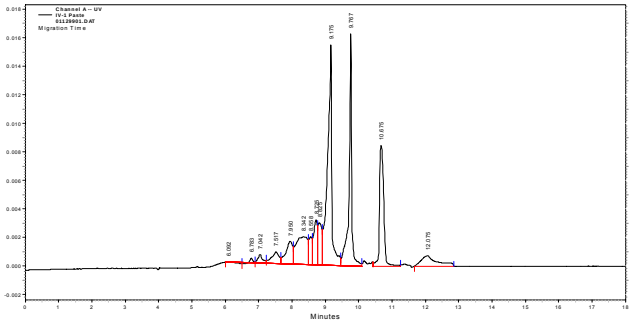
Figure 3.7 Spike Study of Prealbumin in IV-1 Paste by CE-SDS.



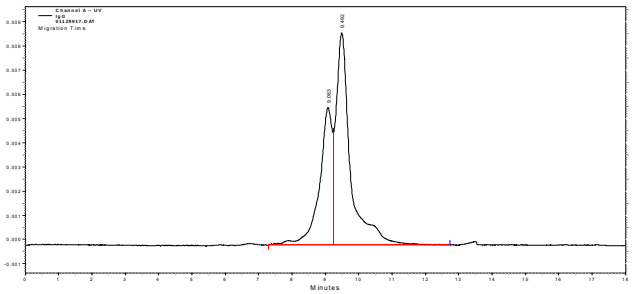
IV-1 Paste



IV-1 Paste + IgG



IV-1 Paste



IgG

Figure 3.8 Spike Study of IgG in IV-1 Paste by CE-SDS.

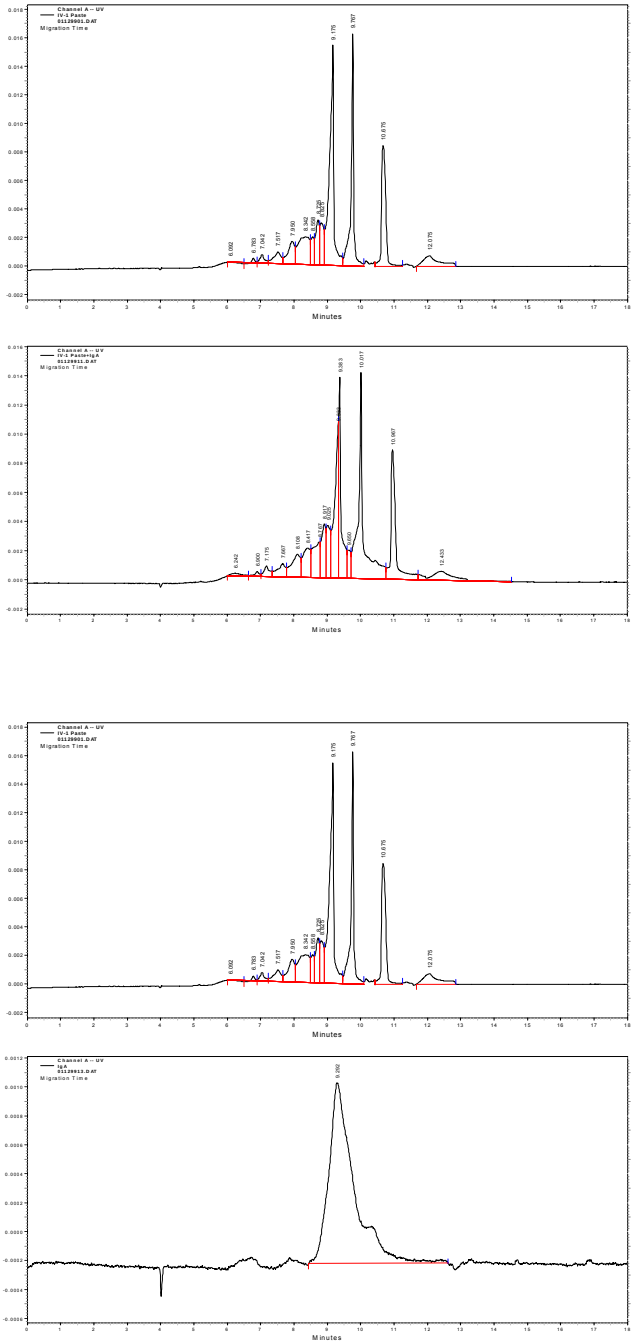


Figure 3.9 Spike Study of IgA in IV-1 Paste by CE-SDS.

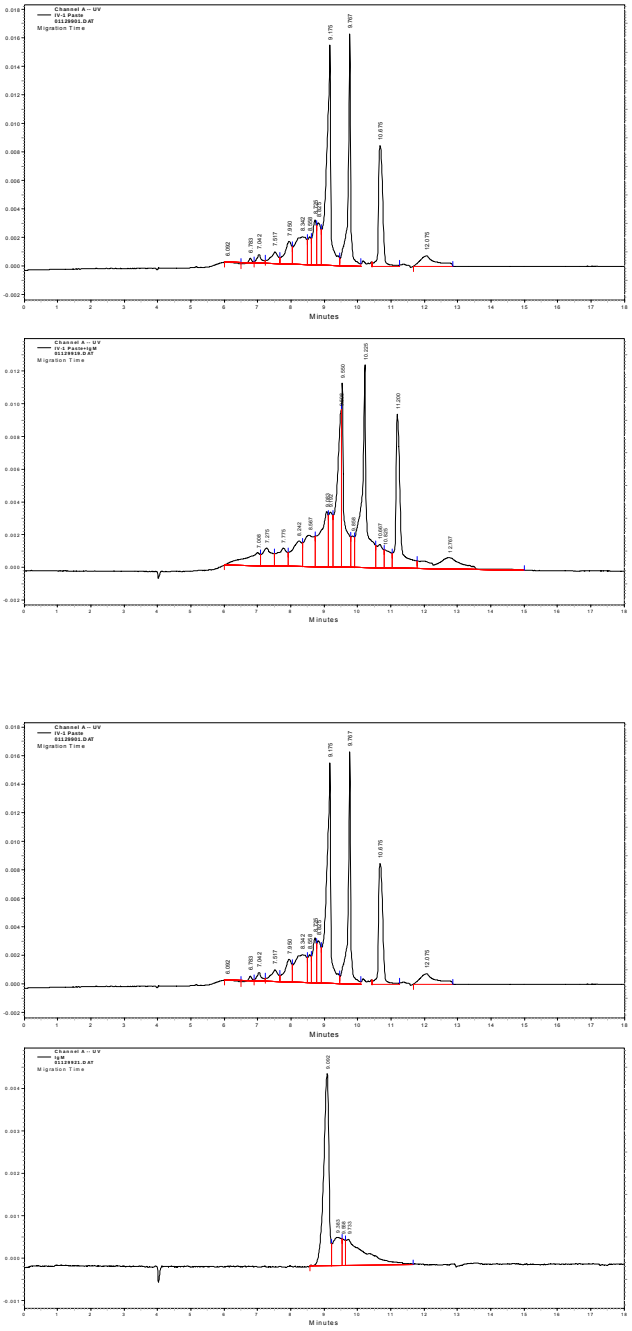
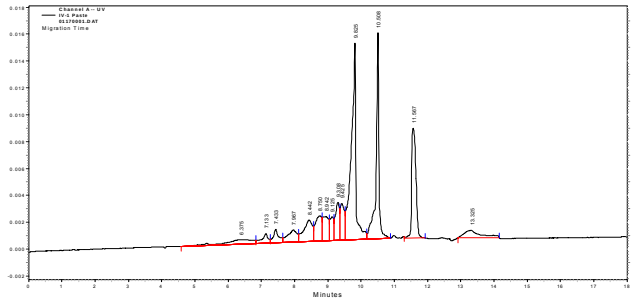
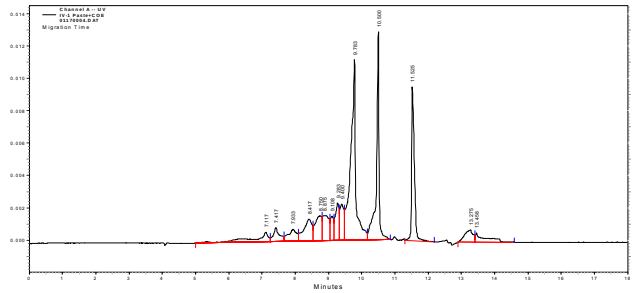


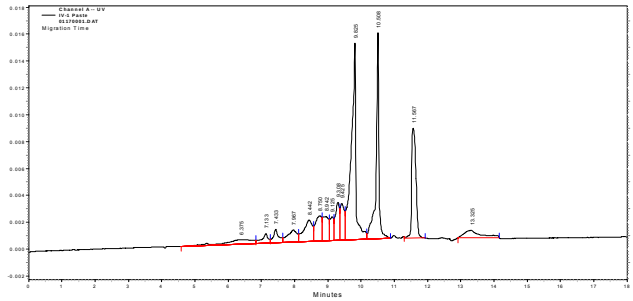
Figure 3.10 Spike Study of IgM in IV-1 Paste by CE-SDS.



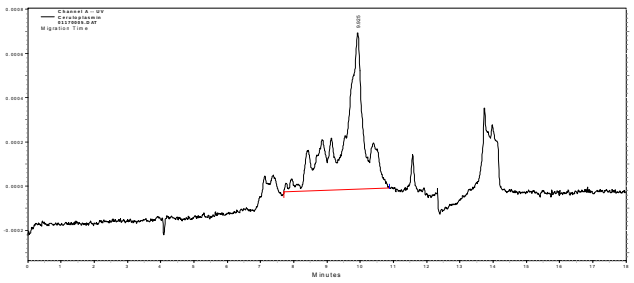
IV-1 Paste



IV-1 Paste +
Ceruloplasmin



IV-1 Paste



Ceruloplasmin

Figure 3.11 Spike Study of Ceruloplasmin by CE-SDS.

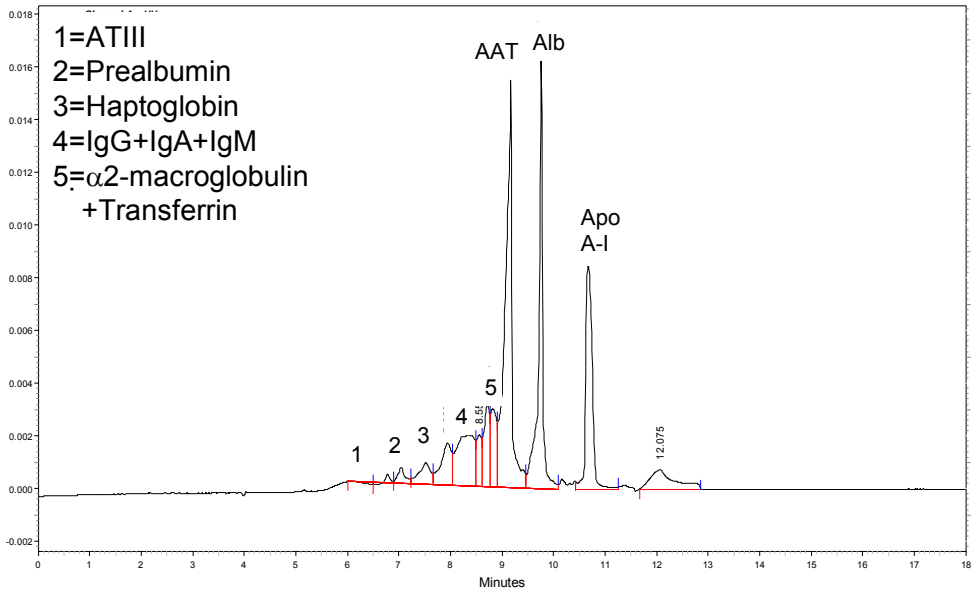


Figure 3.12 Electropherogram of IV-1 Paste by CE-SDS Method.

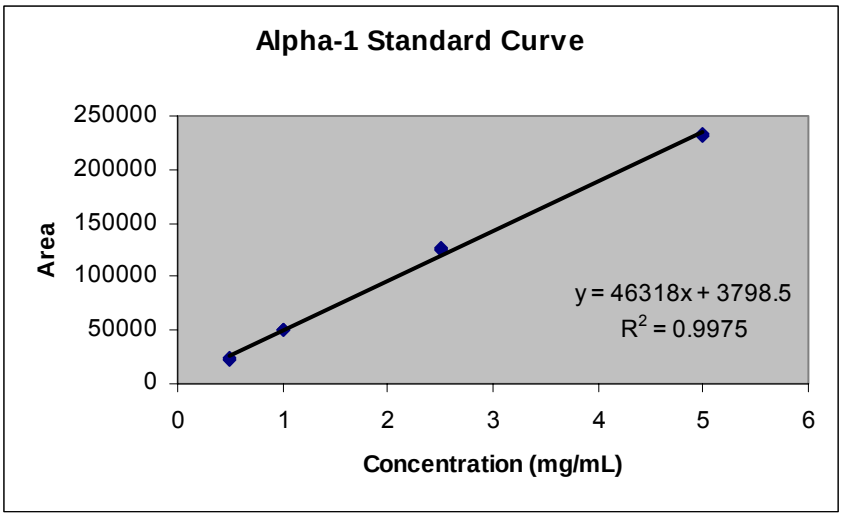


Figure 3.13. Standard Curve of Alpha$_1$-Antitrypsin by CE-SDS Using an In-house Standard.

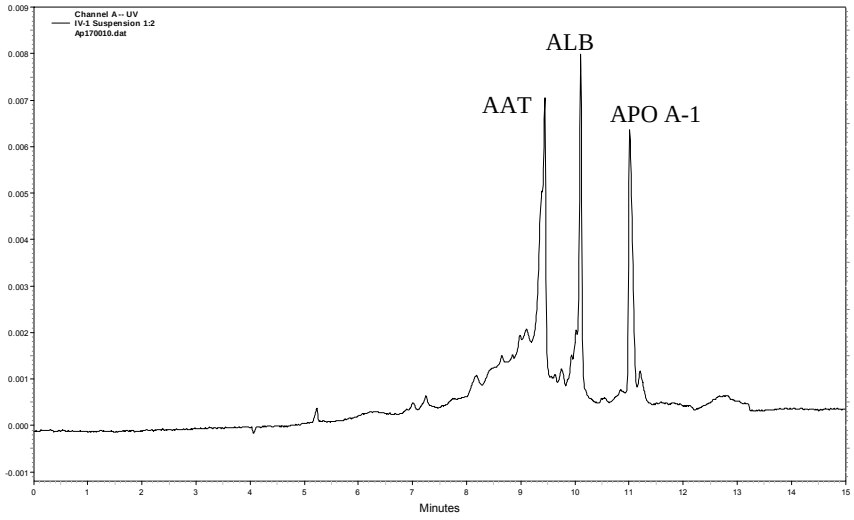


Figure 3.14 IV-1 Paste Suspension Post-NaCl Addition by CE-SDS.

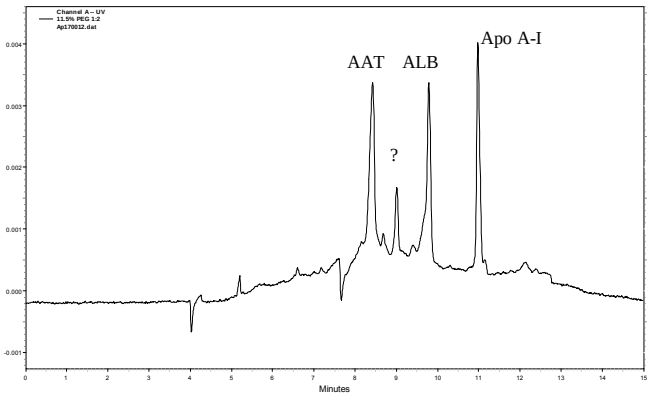


Figure 3.15 11.5% PEG Filtrate Step by CE-SDS.

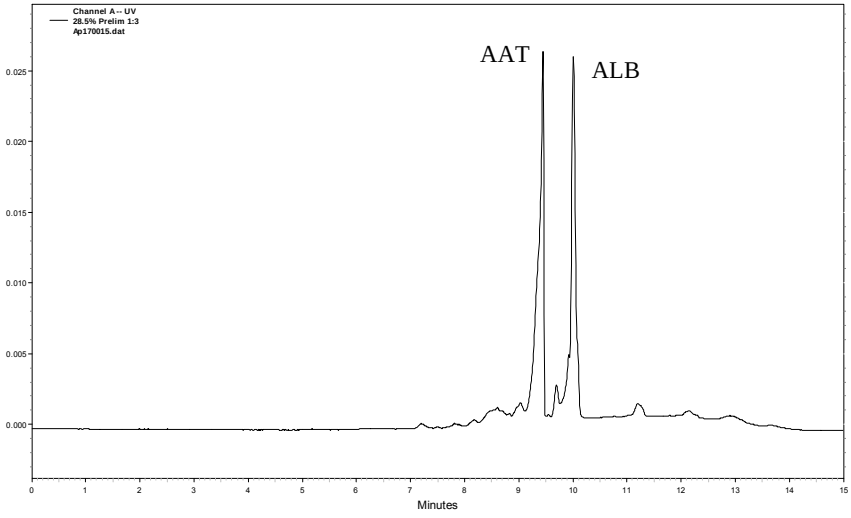


Figure 3.16 28.5% PEG Paste Step by CE-SDS.

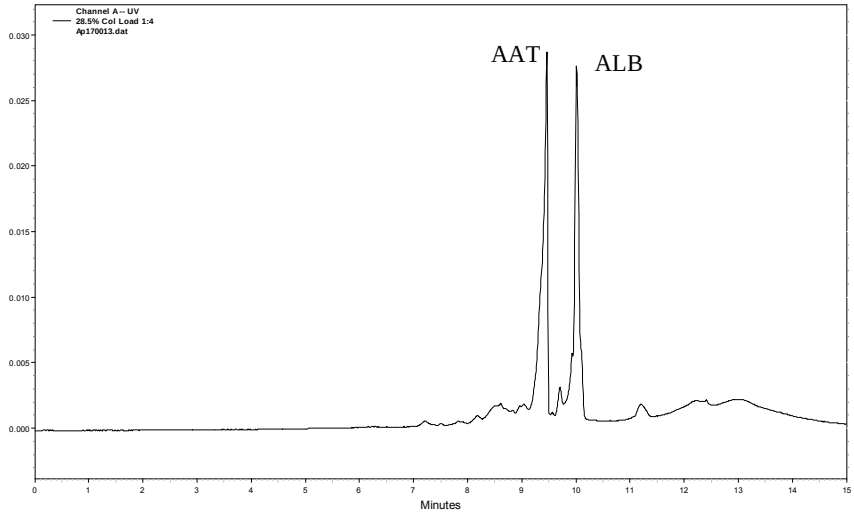


Figure 3.17 28.5% PEG Suspension, Column Load Step by CE-SDS.

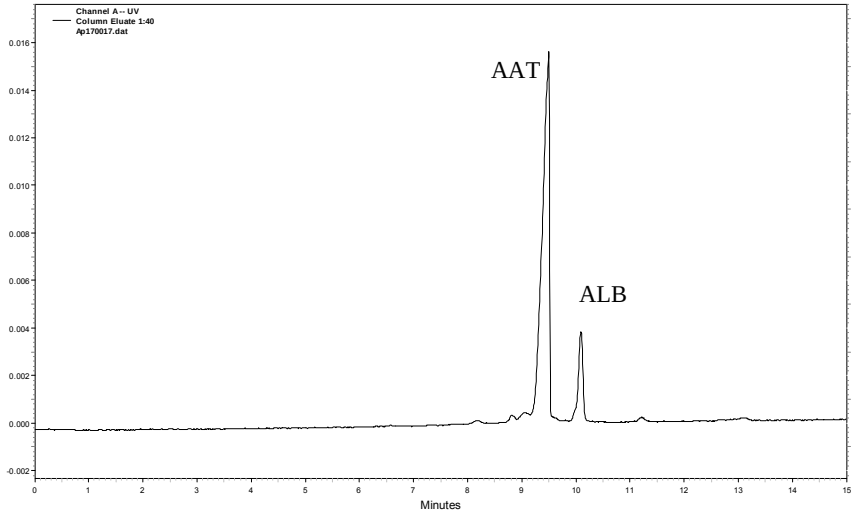


Figure 3.18 Concentrated Column Eluate Step by CE-SDS.

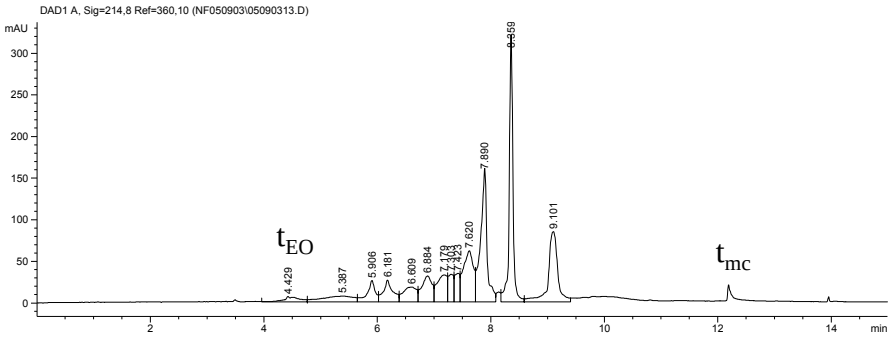


Figure 3.19 IV-1 Paste by CE-SDS on Agilent HPCE System

References

1. Peters, Theodore Jr. All About Albumin, Biochemistry, Genetics and Medical Applications; Academic Press: San Diego, 1996; pp 9-54.
2. Chen, S.A.; Hammond, D.J.; Klos, A.M.; Wood, W.D.; Wydick, J.E.; Lebing, W.R. Chromatographic Purification of Human α_1 Proteinase Inhibitor From Dissovled Cohn Fraction IV-1 Paste. *Journal of Chromatography A.* **1998**, *800*, 207-218.
3. Putnam, F.W. The Plasma Proteins, 2nd ed.,Vol.1, Academic Press,Inc.: New York, 1977,p 422
4. Tietz, NW, Textbook of Clinical Chemistry, 1st edition; Philadelphia: WB Saunders, 1986
5. Colman,R.W., Hematosis and Thrombosis, Basic Principles and Clinical practice 3rd Ed., J.B. Lippincott Co.: Philadelphia, 1994
6. Blake, C.C.F.; Geisow, M.J.; Swan, D.A.; Rerat, C.; Rerat, B. Structure of Human Plasma Prealbumin at 2.5 A Resolution, A preliminary Report on the Polypeptide Chain Conformation, Quaternary Structure and Thyroxine Binding. *Journal of Molecular Biology* **1974**, *88*, 1–12.
7. Budayova-Spano, M., Grabarse, W., Thielens, N.M., Hillen, H., Lacroix, M., Schmidt, M., Fontecilla-Camps, J., Arlaud, G.J., Gaboriaud, C. Monomeric structures of the zymogen and active catalytic domain of complement protease c1r: further insights into the c1 activation mechanism *Structure* **2002**, *10*, 1509-1519.
8. Kurosky A., Barnett D.R., Lee T.-H., Touchstone B., Hay R.E., Arnott M.S., Bowman B.H., Fitch W.M. Covalent structure of human haptoglobin: a serine protease homolog. *Proc. Natl. Acad. Sci.* **1980**, *77*, 3388-3392.
9. Malchy B., Dixon G.H. Studies on the interchain disulfides of human haptoglobins. *Canadian Journal of Biochemistry* **1973**, *51*, 249-264.

10. Sottrup-Jensen, L. α-Macroglobulins: Structure, Shape, and Mechanism of Proteinase Complex Formation. *The Journal of Biological Chemistry* **1989**, *264*, 11539-11542.
11. Qazi, U., Gettins, P.G.W., Stoops, J.K.. On the Sturctural Changes of Native α_2-Macroglobulins upon Proteinase Entrapment. *The Journal of Biological Chemistry* **1998**, *273*, 8987-8993.
12. Cheng, Y., Zak, O., Aisen, P., Harrison, S.C., Walz, T. Structure of the Human Transferrin Receptor-Transferrin Complex. *Cell* **2004**, *116*, 565-576.
13. Loebermann H., Tokuoka R., Deisenhofer J., Huber R. Human alpha 1-proteinase inhibitor. Crystal structure analysis of two crystal modifications, molecular model and preliminary analysis of the implications for function. *Journal of Molecular Biology* **1984**, *177*, 531-556.
14. Carter D.C., He X.-M., Munson S.H., Twigg P.D., Gernert K.M., Broom M.B., Miller T.Y.; Three-dimensional structure of human serum albumin. *Science* **1989**, *244*, 1195-1198.
15. Carter D.C., He X.-M. Structure of human serum albumin. Science 249:302-303(1990).
16. Shaklai, N. Garlick, R.L., Bunn, H.F. Nonenzymatic Glycosylation of Human Serum Albumin Alters Its Conformation and Function *The Journal of Biological Chemistry* **1984**, *259*, 3812-3817.
17. Borhani D.W., Rogers D.P., Engler J.A., Brouillette C.G. Crystal structure of truncated human apolipoprotein A-I suggests a lipid-bound conformation. *Proc. Natl. Acad. Sci.* **1997**, *94*, 12291-12296.

CHAPTER 4. CHARACTERIZATION OF IV-1 PASTE BY MASS SPECTROMETRY

Introduction

The complicated task of human plasma protein analysis can be addressed by multi-dimensional techniques in order to obtain a more complete protein profile. Typical plasma profiling methods include capillary electrophoresis, two-dimensional gel electrophoresis, and SDS-PAGE. Separation techniques can provide semi-quantitative distribution of proteins in a plasma sample. However, a high concentration of albumin in human plasma amounts to approximately 50-60% of the total protein and often creates a problem with detection of low abundance proteins [2]. Mass spectrometry can provide detection sensitivity not afforded by absorbance detection with additional specificity.

One disadvantage of mass spectrometry is that it cannot be considered quantitative due to differences in ionization properties of different proteins and peptides. However, mass spectrometry can be used as a second dimension to identify some of the protein constituents not well separated or detected due to low abundance. Recently the flourishing proteomics field has resulted in a wealth of research in analytical approaches, such as the "top down" or "bottom up" approaches [7].

The "top down" approach to analyzing complex biological samples, such as human serum, cell lysates, and protein complexes, involves coupling of chromatographic separation with mass spectrometry. The "bottom-up" approach involves the enzymatic digestion (e.g. trypsin) of the entire mixture of proteins followed by cation exchange chromatography with fraction collection. Each fraction containing multiple peptides is subsequently injected onto a reverse-phase separation column with direct infusion into a mass spectrometer (Figure 4.1). Tandem mass spectrometry provides a higher level of peptide sequencing information as compared to parent mass only techniques. Peptide sequences lend to protein identification, which is often accomplished through database searching [7,8,9].

Alternatively, differential protein expression is studied by two-dimensional gel electrophoresis, where proteins are separated orthogonally by isoelectric point (pI) and molecular weight. Spots which increase in signal intensity indicate elevated expression of a particular protein. These spots are excised and subsequently subjected to enzymatic digestion followed by the procedures mentioned. Although these methods have great potential to identify unknown or low abundance proteins, they are quite labor intensive and time-consuming. High-throughput analysis using these methods has been achieved, but requires expensive instrumental setups involving multiple capillary LC with high resolution mass spectrometry (e.g. FT-ICR) and robotics for sample preparation. Also, this type of approach makes an inherently complex sample several-fold more complex. For these reasons, this approach is not desirable for the current analytical problem.

In this research, two capillary electrophoresis methods, a clinical method and a newly-developed method (CE-SDS), is compared with a proteomics approach to examine the protein profile of a sample from human plasma fractionation, IV-1 Paste. To illustrate the top-down approach, a reverse-phase LC/MS experiment was performed. The mass spectrometer in this case is a quadrupole ion trap with an electrospray source. As an example of the bottom-up approach, the entire protein mixture was digested with trypsin and analyzed by LC/MS/MS using the same mass spectrometer (Figure 4.2). The protein profile obtained by MALDI-TOF mass spectrometry will provide orthogonal protein profile, since the ionization off proteins by MALDI is different than by electrospray. Also, an albumin-depletion method was used to investigate whether removal of albumin will increase the detection of low abundance proteins in IV-1 Paste.

Experimental

Chemicals

Urea and acetonitrile (JT Baker) were purchased from Fisher Scientific. Dithiothreitol (DTT) was obtained from Invitrogen (10X sample reducing agent). Sequencing grade TPCK-treated bovine

trypsin was purchased from Promega. Trifluoroacetic acid, ammonium bicarbonate, sinnapinic acid, tris, and calcium chloride were purchased from Sigma Chemical Co., St. Louis, MO. Montage albumin depletion kit was purchased from Millipore Corporation, Bedford, MA.

CE "CLINICAL METHOD"

The "Clincal Method" was adapted from M.A Jenkins, et al [1]. The experiments were performed on Agilent G1600A HPCE system. The buffer conditions were 75 mM boric acid, 0.2 mM calcium lactate, pH 10.3. Bare fused silica capillaries (Agilent Technologies) with an inner diameter of 50 μm and a total length of 70 cm were used. The separation voltage was 20 kV (positive polarity). Sample injection was performed by pressure (15 seconds with 50 mbar). Diode array detector was used to obtain absorbance at 214 nm. Data were collected and analyzed using ChemStation software.

CE-SDS METHOD

CE-SDS experiments were performed on Agilent G1600A HPCE system. Bare fused silica capillaries (Agilent Technologies) with an inner diameter of 25 μm extended path length and a total length of 47 cm were used. The separation voltage was 17 kV (positive polarity). The CE-SDS run buffer consists of 65 mM borate, 20 mM SDS, pH 9.5. Sample injection was performed by pressure (15 seconds with 50 mbar). Diode array detector was used to obtain absorbance at 214 nm. Data were collected and analyzed using ChemStation software.

MALDI-TOF

Analysis was performed using a Voyager MALDI-TOF in linear, positive ion mode. Sinnapinic acid matrix was dissolved at 20 mg/mL in 50% acetonitrile/50% water/0.1% TFA. IV-1 Paste suspension was diluted 1:50 in sinnapinic acid matrix and spotted onto a MALDI plate.

LC/MS

Reverse phase LC/MS was performed on an Agilent 1100 HPLC system coupled with a Thermo Finnigan LCQ Classic ESI-Ion Trap mass spectrometer. The column was manufactured by Microhm BioResources PLRP-S 4000Å, 2.0mm x 150mm, and column temperature was maintained at 40°C. Mobile phase A was 0.1% TFA in water and mobile phase B was 0.1% TFA in acetonitrile. Gradient elution 30-40% mobile phase B over 20 minutes, 0.4 mL/min. UV detection was at 210nm. Mass spectral data was collected and analyzed by Xcalibur software.

LC/MS/MS

Reverse phase LC/MS/MS was performed on an Agilent 1100 HPLC system coupled with a Thermo Finnigan LCQ Classic ESI-Ion Trap mass spectrometer. The column was manufactured by Vydac C18 (218MS52) with dimensions 4.1mm x 150 mm, and column temperature was maintained at 40°C. Mobile phase A was 0.02% TFA in water and mobile phase B was 0.02% TFA in acetonitrile. Gradient elution of 2-60% B over 180 minutes at 0.1 mL/min. UV detection was at 210nm. MS parameters involved data-dependent scanning and three scan events: full spectrum, MS/MS of the most abundant ion from full scan, and MS/MS of the second most abundant ion from full scan. Mass spectral data was collected and analyzed by Xcalibur software and SEQUEST.

Tryptic Digest

Tryptic digest protocol began with denaturation of the proteins in IV-1 paste suspension by dilution in 12M urea. Samples are heated at 37°C for 15 minutes to promote denaturation. Samples are cooled to room temperature prior to addition of reducing agent. Disulfide bonds are reduced by adding 0.5M dithiothreitol (DTT). Addition of molar excess of iodoacetamide provides stabilization of free sulfhydryls by alkylation. During the alkylation step, samples are

incubated in the dark at ambient temperature. Samples are diluted to 2M Urea using 400mM ammonium bicarbonate in 5% acetonitrile. Trypsin activation buffer (1X TAB) is comprised of 50mM Tris, 8mM calcium chloride, 5% (v/v) acetonitrile, pH 7.8 The pH of samples should be checked at this step to ensure the ideal pH 8. Trypsin is added (bovine, TPCK-treated, Promega) 1 part trypsin to 50 parts protein, and samples are incubated overnight at 37°C (approximately 16 hours). Samples are frozen to stop enzymatic reaction.

Albumin Depletion

The sample preparation for the albumin depletion experiment was performed per kit instructions. In summary, the column is equilibrated with equilibration buffer prior to the addition of diluted sample (also in equilibration buffer). The column is centrifuged, then washed and the eluate is collected which is the depleted sample. Albumin and other proteins are retained or bound to the affinity resin.

Results and Discussion

Results of Capillary Electrophoresis Methods

Capillary electrophoresis of proteins is quite challenging but can lead to a rewarding separation which can be high-throughput, high resolution and quantitative. Protein adsorption to the capillary wall and protein heterogeneity contribute to the level of difficulty in achieving such a separation.

IV-1 Paste and albumin-depleted IV-1 Paste samples were analyzed by two capillary electrophoresis methods, a generally accepted clinical method and a newly-developed CE-SDS method. The results of the original sample analyzed by the clinical method were inconclusive due to the low resolution and shift in migration time of the expected proteins (Figure 4.3). Only albumin alpha$_1$-antitrypsin and transferrin could be identified qualitatively. Clearly, protein loss

(non-specific removal proteins other than albumin) from the albumin depletion sample preparation is evident. The results from the CE-SDS method also indicate protein loss, but also show an enhanced detection of antithrombin III, AT-III (Figure 4.4). One advantage of the clinical CE method over the CE-SDS method is the ability to resolve transferrin.

Results of MALDI-TOF Analysis of IV-1 Paste

Matrix-assisted laser desorption/ionization, time-of-flight (MALDI-TOF) mass spectrometry allows protein-profiling by mass-to-charge ratio and requires minimal preparation and analysis time. There is no pre-separation technique, and this method is considered qualitative versus quantitative. MALDI-TOF has the advantage of minimal sample preparation, salt tolerance, and a wide mass range. MALDI-TOF of proteins does not yield high resolution mass spectra when compared with other mass spectrometry techniques such as electrospray ionization mass spectrometry (ESI-MS). Rather, the protein spectra resemble chromatographic peaks with broad distributions. Lower molecular weight proteins have sharper peaks, whereas higher molecular weight proteins have broader peaks. This is due mainly to the ionization of proteins by MALDI and the distribution of time for each ionized protein species as it reaches the detector.

The results of the original IV-1 Paste sample and the albumin-depleted sample are shown in Figures 4.5 and 4.6 respectively. No proteins were detected >100,000 m/z, where Igs, alpha-2-macroglobulin, and haptoglobin (tetrameric) may be found. However, the MALDI-TOF profile is quite comprehensive although it is not quantitative. The speed of analysis is a key advantage.

Results of LC/MS Analysis of IV-1 Paste

Reverse-phase liquid chromatography was utilized for separation of IV-1 Paste proteins based on the theory that proteins would separate based on hydrophobicity, where hydrophilic

proteins would elute earlier than hydrophobic, less polar proteins. Reverse-phase methods utilizing volatile solvents are typically interfaced with mass spectrometers. One drawback of reverse-phase methods involves the fact that ion-pairing agents, such as trifluoroacetic acid (TFA) are often added to the mobile phase to improve the chromatographic peak shape and resolution. However, ion-pairing agents can suppress ionization and therefore reduce the resolution capability of mass spectrometers. For these reasons, the TFA concentration had to be reduced, which compromised the chromatographic resolution.

The quadrupole ion trap (QIT) mass spectrometer is ideal for analyzing peptides but has only adequate resolution for obtaining molecular weight of intact proteins. The spectral quality is heavily dependent on the ionization source, which in this case is electrospray ionization. Intact proteins must acquire multiple charges to fall within the mass-to-charge range (m/z) of the mass spectrometer, in this case 300-2000 m/z. Nevertheless, this type of mass spectrometer is ideally coupled with LC.

The chromatographic separation is not optimal; however, the mass spectrometer was able to discern some of the proteins even with the low chromatographic resolution (Figure 4.7). By LC/MS, antithrombin III, $alpha_1$-acid glycoprotein, $alpha_1$-antitrypsin, apolipoprotein A-I, and albumin were conclusively detected (Figures 4.7 and 4.8). Apolipoprotein A-I, a major constituent of IV-1 Paste, was not well resolved by the chromatography but was detected by mass spectrometry due to the spectral quality of the charge envelope (Figure 4.9). $Alpha_1$-antitrypsin, the main component of interest in IV-1 Paste, is positively identified, and the major glycoforms are resolved by mass spectrometry (Figure 4.10). The charge envelopes obtained for antithrombin III are shown in Figures 4.11 and 4.12 respectively. The idenitification of AT-III from the charge envelope and subsequent deconvoluted mass was slightly enhanced by the albumin depletion step. The charge envelope of albumin was complicated, however, upon deconvolution, it is apparent that an isoform of albumin is being resolved, which was also indicated in the chromatography. The mass of the major peak (66,559 Da) is consistent with the

blocked thiol form of albumin (expected 66,556 Da) [4]. The second major peak (66,440 Da) was identified as the free thiol form of albumin (expected 66,438 Da) [4].

Despite the fact that the quadrupole ion trap is considered a low resolution mass spectrometer compared with the Q-TOF and FT-ICR, it is very versatile and the mass accuracy is sufficient for protein identification.

Results of LC/MS/MS Analysis of Tryptic Digest of IV-1 Paste

Reverse-phase LC/MS/MS was performed to identify unresolved proteins and minor components in IV-1 Paste. This experiment is an example of the "bottom-up" approach, where the protein mixture is digested with trypsin, then subjected to reverse-phase LC followed by MS/MS for peptide identification. Figure 4.13 and 4.14 show the reverse-phase LC separation of the peptide mixture (UV traces) as well as the total ion chromatograms (TIC).

By LC/MS/MS, alpha$_1$-antitrypsin was the first protein identified in the original sample. Roughly equal concentrations of albumin and alpha$_1$-antitrypsin is expected, however albumin was the fourth match. In the albumin-depleted sample, albumin peptides were no longer detected, even though albumin is still present. Apolipoproteins made up the majority of the protein matches. AT-III was not detected, however, transferrin was detected. The results are summarized in Table 4.3.

Conclusion

The resolution and/or detection of the protein constituents of IV-1 Paste by the various methods are summarized in Table 4.1. Although the removal of albumin was successful, in most cases, albumin depletion did not improve the detection or identification of low abundance proteins. This may be attributed to the protein loss due to non-specific binding and dilution of the sample by the preparation.

The CE-SDS method provides a protein profile that correlates well with the immunonephelometry profile (Table 4.2) and MALDI-TOF profile. CE analyses provided protein composition information which was supported by mass spectrometric results. For example, the CE-SDS method exhibited an increase in the detection of AT-III upon albumin removal, and this was supported by LC/MS results. Also, the presence of apolipoprotein A-I is detected by the CE-SDS method and supported by the MALDI and LC/MS/MS results.

LC/MS/MS provides high quality data for peptide matching and subsequent protein identification. This is particularly useful for the identification of low abundance proteins.

In general, no single technique was able to capture all of the proteins regardless of abundance. Single-dimension separation such as capillary electrophoresis and liquid chromatography are capable of producing a quantitative profile but with limited dynamic range. Mass spectrometry adds a second dimension of "separation" where the co-eluting protein constituents can be identified. The collection of techniques in this research together provide a comprehensive profile of the protein constituents in IV-1 Paste.

Table 4.1 Resolution/Detection of Expected Proteins[a] in IV-1 Paste by Technique

Method	Protein[b]								
	AT-III	PreA	HPT	AAG	A2M	TRF	AAT	ALB	Apo AI
Clinical CE	No	No	No	No	No	Yes	Yes	Yes	No
CE-SDS	Yes	Yes	Yes	No	co-migration[c]		Yes	Yes	Yes
MALDI-TOF	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes
LC/MS	Yes	No	No	Yes	No	No	Yes	Yes	Yes
LC/MS/MS	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes

[a]Expected proteins are based on immunonephelometry panel results (see table 4.2).

[b]Abbreviations are as follows: AT-III = antithrombin III, PreA = prealbumin (transthyretin), HPT = haptoglobin, AAG = alpha$_1$-acid glycoprotein, alpha-2-macroglobulin, TRF = transferrin, AAT = alpha$_1$-antitrypsin, ALB = albumin, Apo AI = apolipoprotein A-I

[c]Co-migration of transferrin and AAT also exists.

Table 4.2 Example of Immunonephelometry Results (Expected Protein Profile) of IV-1 Paste (n=30 lots); Comparison with CE-SDS Method

Component	% Protein by Immunonephelometry	Rel. %Area by CE-SDS
Alpha-1 Antitrypsin	19.9%	23.83%
IgG	8.3%	12.07%
IgA	8.8%	
IgE	Not significant	unidentified
Antithrombin III	2.7%	0.54%
Albumin	22.3%	17.53%
Transferrin	7.3%	14.29%
Alpha-2-Macroglobulin	7.6%	
Haptoglobin	2.5%	2.86%
Alpha-1-acid Glycoprotein	0.6%	unidentified
Ceruloplasmin	3.3%	unidentified
Apolipoprotein-A1	13.7%	18.17%
Fibronectin	0.5%	Not significant
Plasminogen	0.2%	Not significant
Apolipoprotein-B	0.2%	Not significant
Prealbumin	1.2%	0.84%

Table 4.3 LC/MS/MS Protein Identification in Order of Peptide Matches

Order	Original Sample	Albumin-depleted Sample
1	$Alpha_1$-antitrypsin	Apolipoprotein A-I
2	Apolipoprotein A-I	$Alpha_1$-antitrypsin
3	Albumin	Apolipoprotein A-II
4	$Alpha_2$-macroglobulin	$Alpha_2$-macroglobulin
5	Haptoglobin	Transferrin

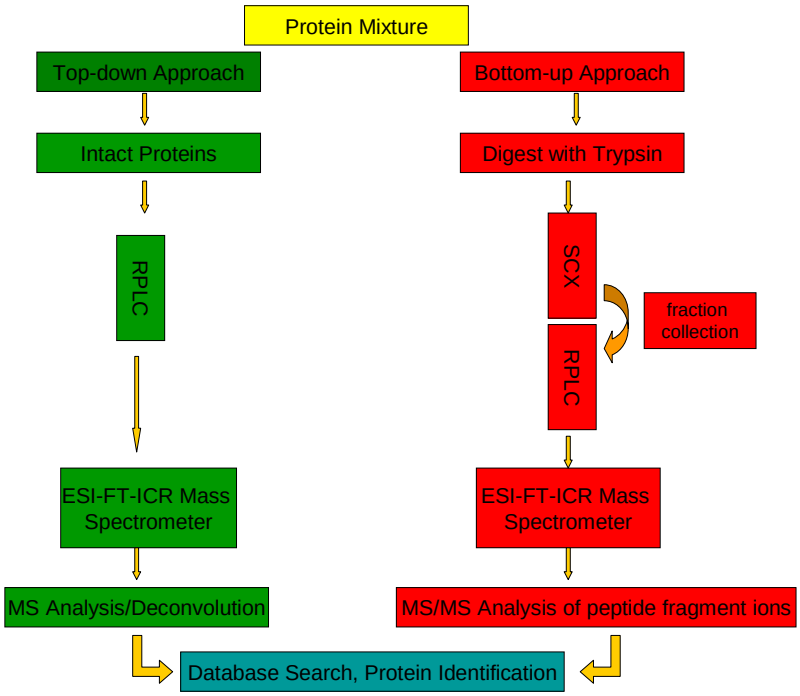


Figure 4.1 Ideal Proteomics Approach

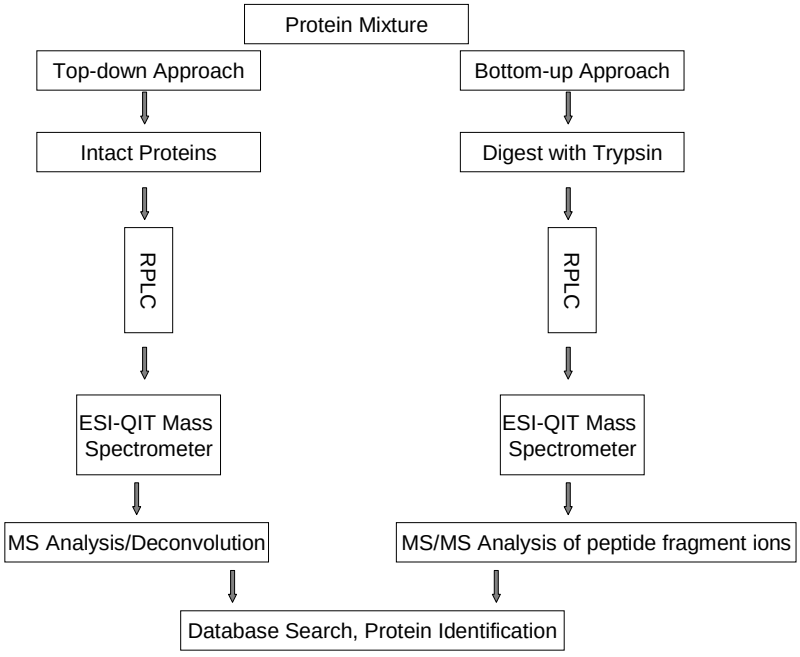
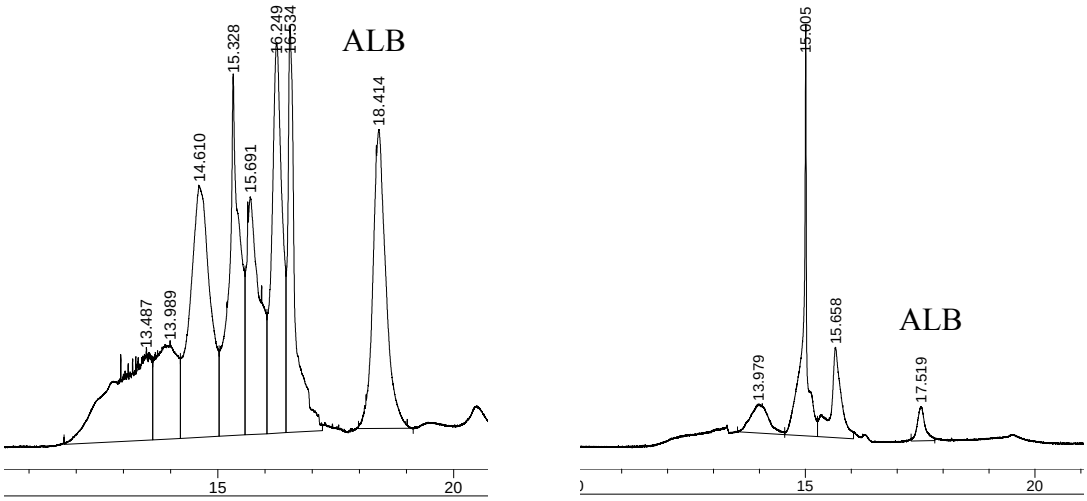


Figure 4.2 Proteomics Approach for Current Research



A. Original Sample B. Albumin-depleted sample

Figure 4.3 IV-1 Paste by the Clinical Method[1]. Electropherogram of original sample was not identical to literature reference, possible due to matrix effects of IV-1 Paste.

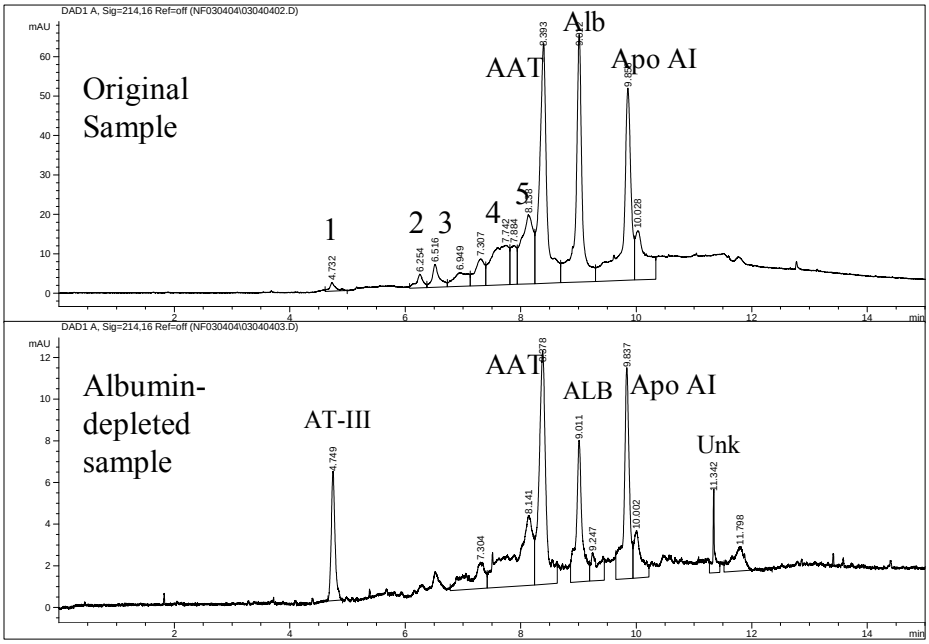


Figure 4.4 IV-1 Paste by CE-SDS Method. Comparison of original and albumin-depleted sample. Region 1=ATIII, Region 2=Prealbumin, Region 3=Haptoglobin, Region 4=IgG+IgA+IgM, Region 5=α-2-macroglobulin+Transferrin. Unk = unknown peak.

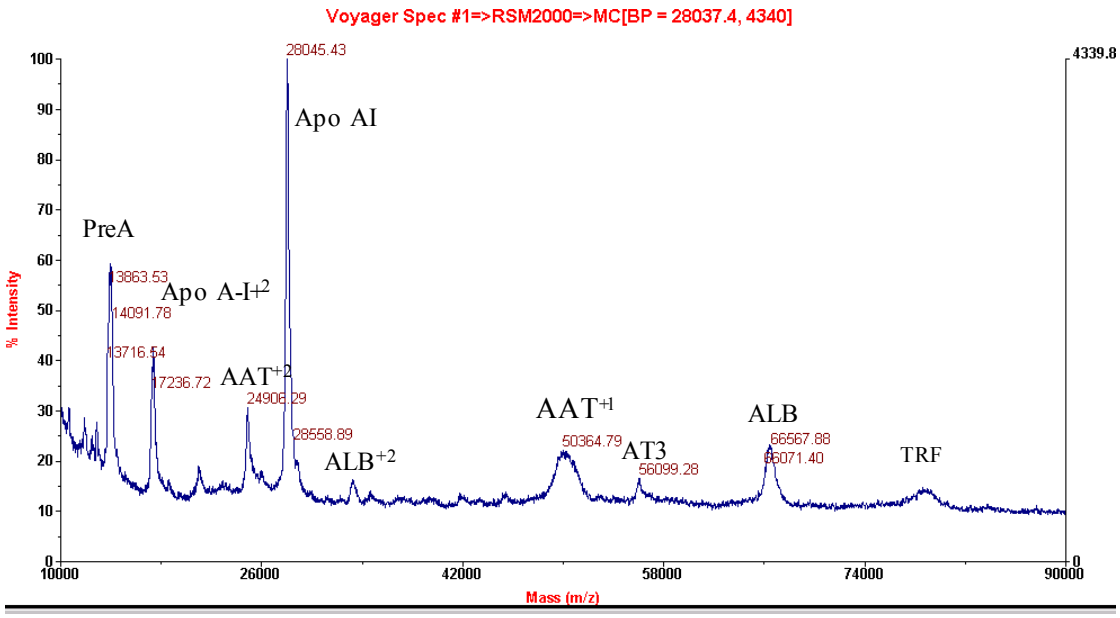


Figure 4.5 MALDI-TOF Analysis of Original IV-1 Paste Sample. PreA = prealbumin (transthyretin), AAG = alpha$_1$-acid glycoprotein, AT3 = antithrombin III, AAT = alpha$_1$-antitrypsin, Apo AI = apolipoprotein A-I, ALB = albumin, TRF = transferrin

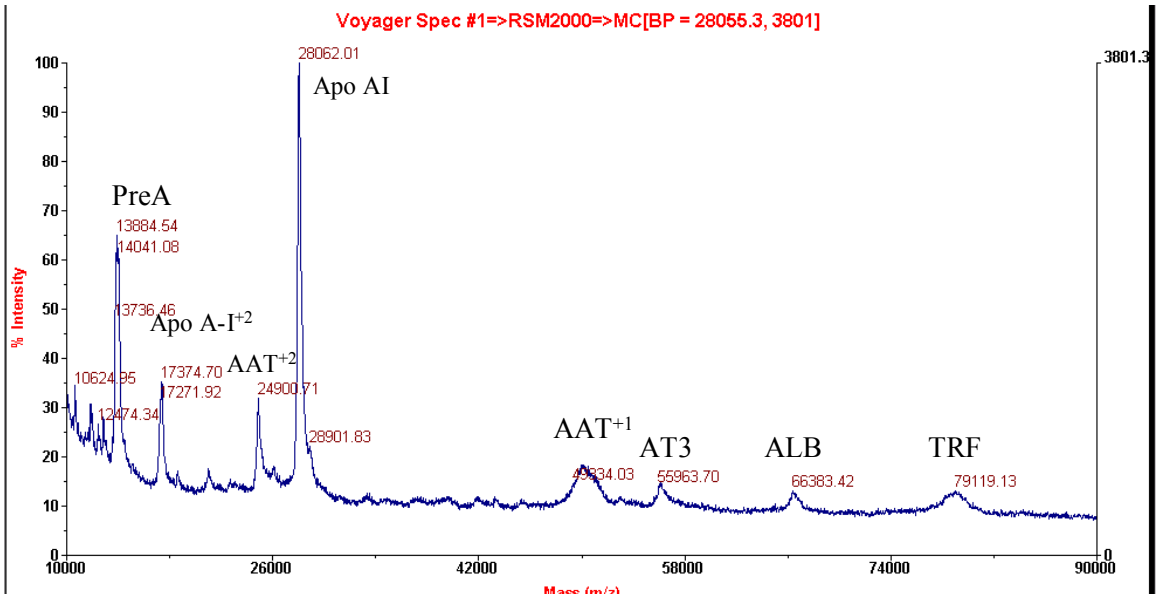


Figure 4.6 MALDI-TOF Analysis of Albumin-depleted IV-1 Paste Sample. PreA = prealbumin (transthyretin), AAG = alpha$_1$-acid glycoprotein, AT3 = antithrombin III, AAT = alpha$_1$-antitrypsin, Apo AI = apolipoprotein A-I, ALB = albumin, TRF = transferrin

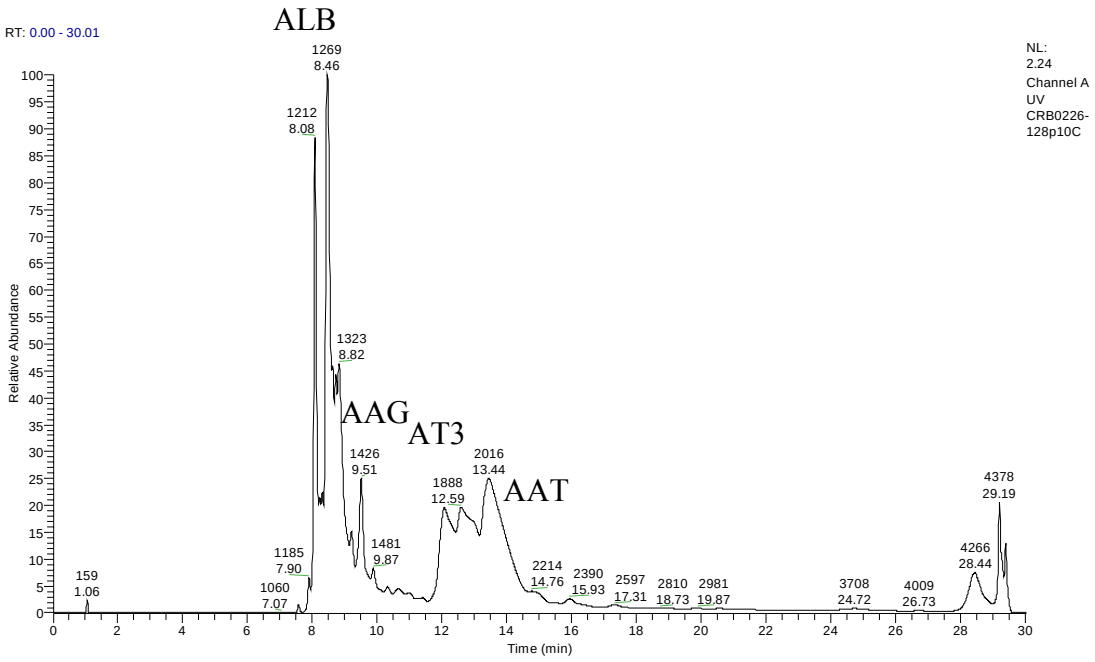


Figure 4.7 Reverse-phase LC-MS UV Chromatogram of Original Sample. ALB = albumin, AAG = alpha$_1$-acid glycoprotein, AT3 = antithrombin III, AAT = alpha$_1$-antitrypsin.

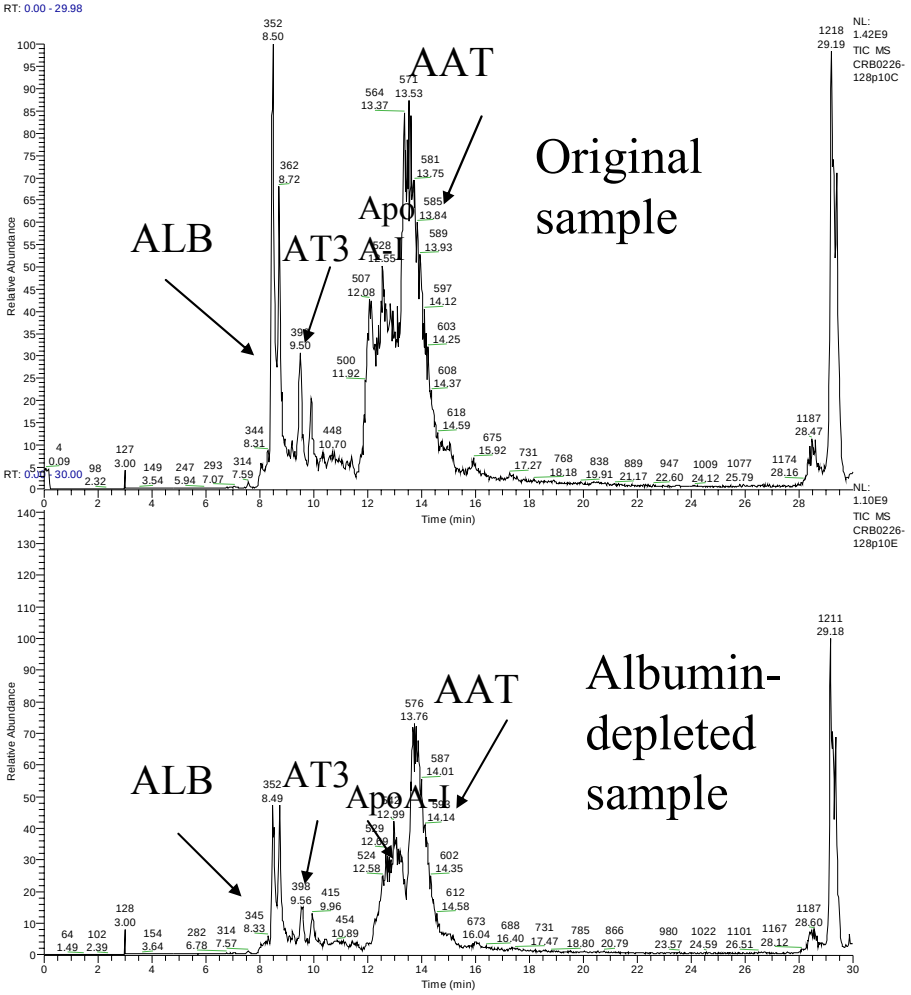


Figure 4.8 Reverse-phase LC-MS Total Ion Chromatogram (TIC). ALB = albumin, AT3 = antithrombin III, AAT = alpha$_1$-antitrypsin.

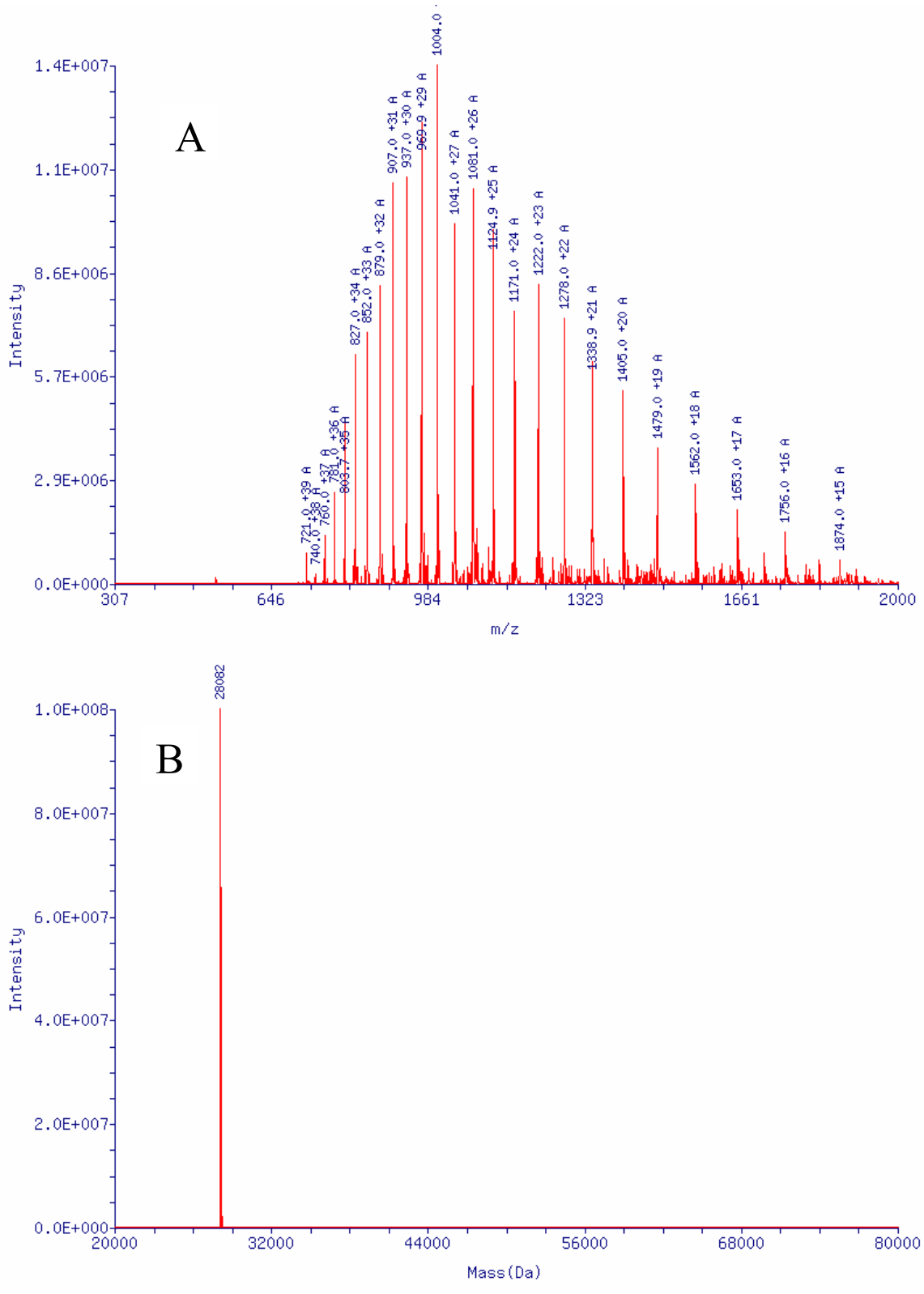


Figure 4.9 A. Charge envelope of Apo A-I. B. Spectral deconvolution of Apo A-I.

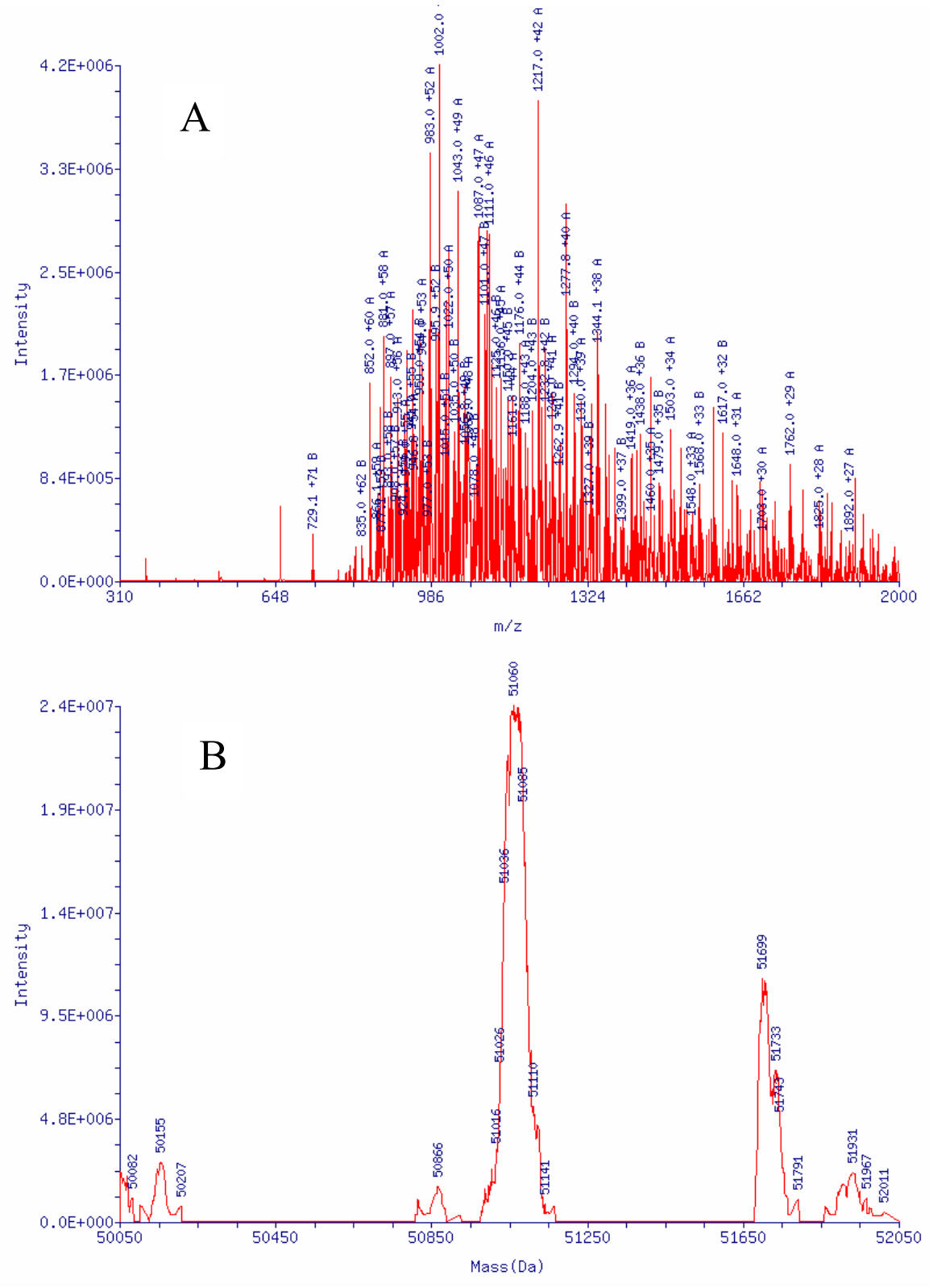


Figure 4.10 A. Charge envelope of Alpha$_1$-Antitrypsin (AAT). B. Spectral deconvolution of AAT.

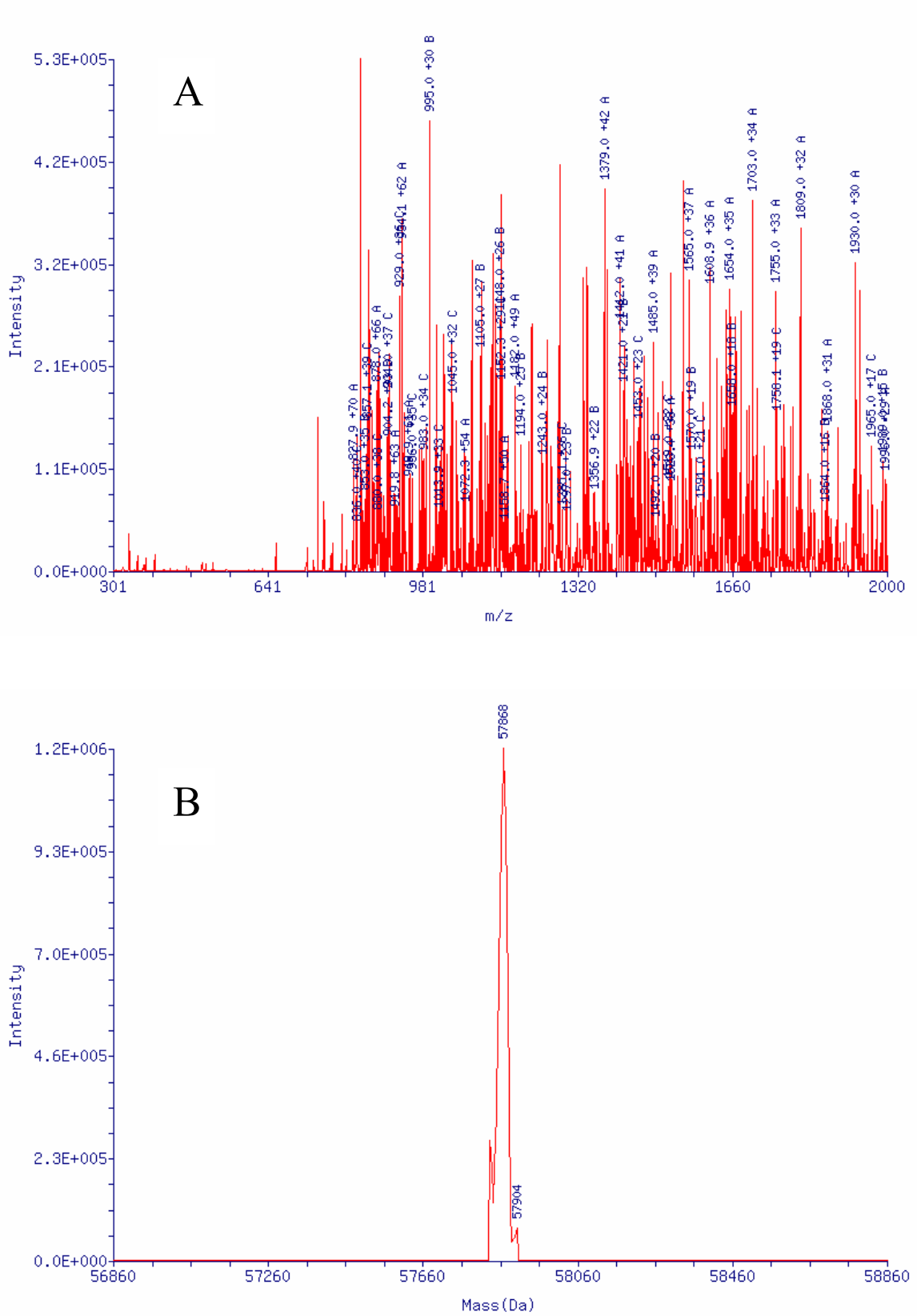


Figure 4.11 A. Charge envelope of AT-III. B. Spectral deconvolution of AT-III.

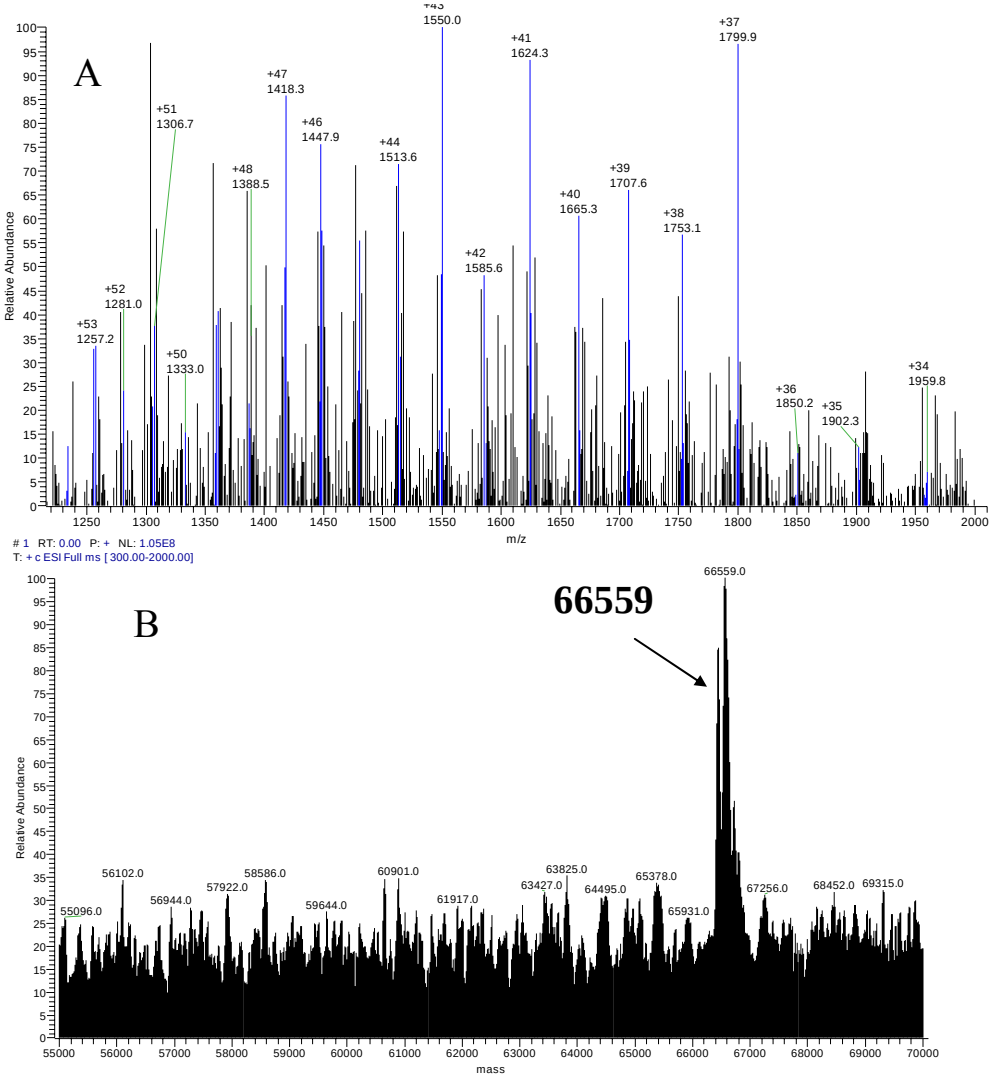


Figure 4.12 A. Charge envelope of Albumin. B. Spectral deconvolution of Albumin.

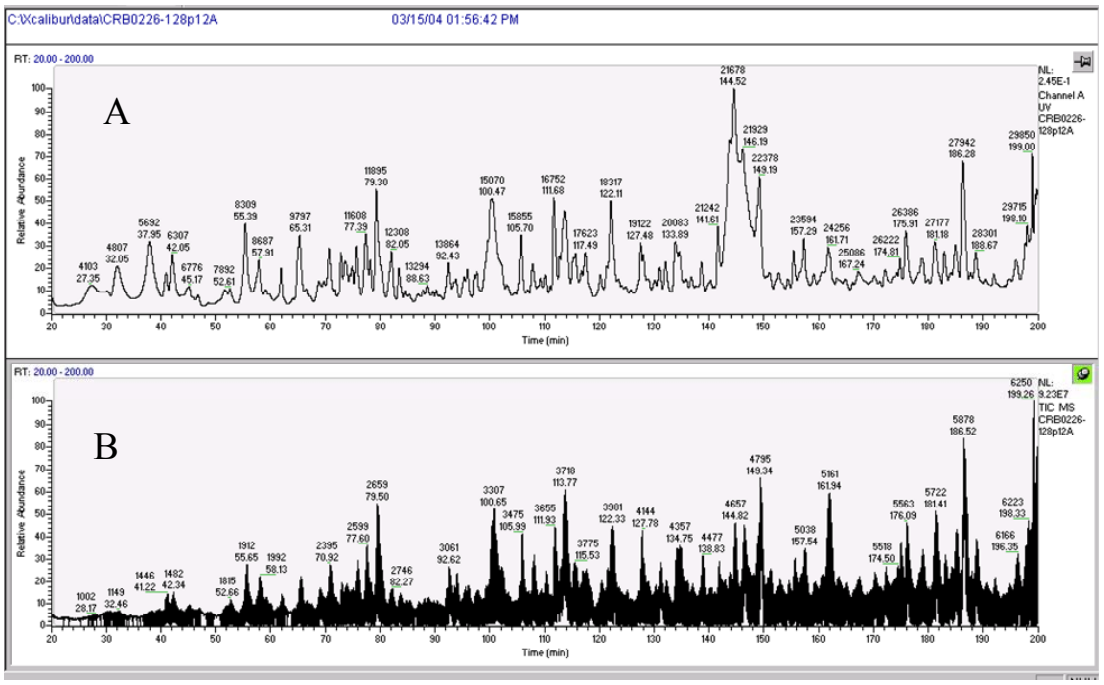


Figure 4.13 LC-MS-MS Tryptic Digest of Original IV-1 Paste Sample. A UV Chromatogram. B. Total Ion Chromatogram.

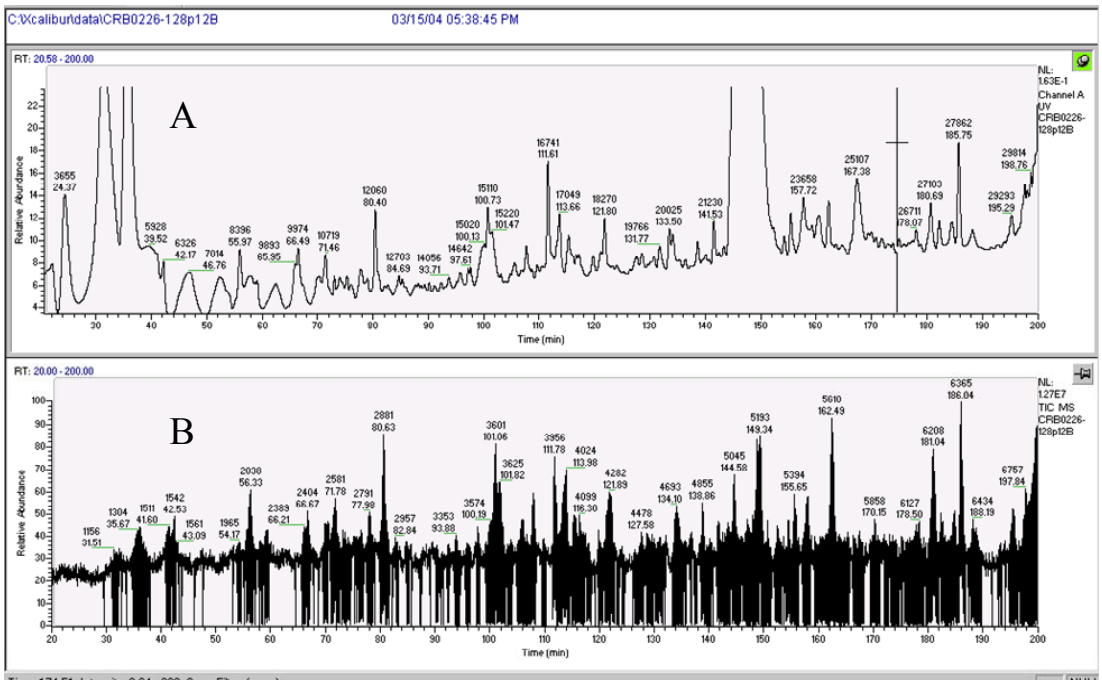


Figure 4.14 LC-MS-MS Tryptic Digest of Albumin-depleted IV-1 Paste Sample. A UV Chromatogram. B. Total Ion Chromatogram.

References

1. Jenkins, M.A. Methods in Molecular Medicine, Vol 27: Clinical Applications of Capillary Electrophoresis. Humana Press, Inc., Totowa, NJ 1999
2. Reif, O.W., Lausch, R., Feitag, R. High Performance Capillary Electrophoresis of Human Serum and Plasma Proteins, Advances in Chromatography, Vol. 34 1994
3. Mass Spectrometry of Biological Materials, Second Edition, Larsen, B.S., McEwan, C.N., Marcel Dekker, Inc. New York, NY 1998
4. Chan, B.; Dodsworth, N.; Woodrow, J.; Tucker, A.; Harris, R. Site-specific N-terminal auto-degradation of human serum albumin. *European Journal of Biochemistry*. **1995**, *227*, 524-528.
5. http://prospector.ucsf.edu/
6. http://us.expasy.org/
7. Kelleher, N.L., Lin, H.Y., Valaskovic, G.A., Aaserud, D.J., Fridriksson, E.K., McLafferty, F.W., Top Down versus Bottom Up Protein Characterization by Tandem High-Resolution Mass Spectrometry. *Journal of the American Chemical Society* **1999**, *121*, 806-812.
8. McLafferty, F.W., Tandem mass spectrometric analysis of complex biological mixtures. *International Journal of Mass Spectrometry* **2001**, *212*, 81-87.
9. Shen, Y., Jacobs, J.M., Camp II, D.G., Fang, R., Moore, R.J., Smith, R.D., Ultra-High-Efficiency Strong Cation Exchange LC/RPLC/MS/MS for High Dynamic Range Characterization of the Human Plasma Proteome. *Analytical Chemistry* **2004**, *76*, 1134-1144.

CHAPTER 5. CONCLUSIONS AND FUTURE RECOMMENDATIONS

Abstract

Protein profile analysis of complex process intermediates from Cohn plasma fractionation was investigated. In this research, protein profiling of Fraction IV-1 Paste was analyzed by comparison of separation techniques with mass spectrometry techniques. Capillary electrophoresis methods are simple, reproducible, provide acceptable resolution and detection. An example of CE-SDS of proteins was applied to IV-1 Paste and resulted in a significant improvement in resolution over current analytical CE applications. However, the use of SDS in CE and the interaction of SDS with proteins both in solution and in the micellar phase could be further explored. Reverse phase HPLC was similarly capable of resolving and detecting the major protein constituents, but low abundance proteins were still not well detected. Mass spectrometry coupled with bioaffinty interactions, such as antibody-protein recognition, has the potential to add another dimension of separation and identification of proteins in such a complex mixture. Specifically, SELDI and SPR coupled with MALDI-TOF may be a perfect alternative to quantitate and identify low abundance proteins in complex protein matrices.

Introduction

Modified Cohn Fractionation processing of pooled human plasma is the underlying scheme of many biological therapeutics. In sequential processes, human plasma proteins are separated through alcohol precipitation and chromatography into several fractions [1]. Protein profile analysis of Cohn fractionation intermediates has many advantages, for example, identification of new protein targets, abundance of those proteins, analysis of waste fractions, and impurity analysis. As the raw material for plasma processing is inherently heterogeneous, it is

understood that the immunoglobulin population would cause great interference with most analytical techniques. IV-1 Paste represents an intermediate step where Igs are previously removed and will be used as an example to illustrate the approach.

Single dimension separation techniques, such as capillary electrophoresis and liquid chromatography, can be limited in resolution and detection of the many types of proteins present in human plasma. No single technique can separate such a complex mixture of proteins widely-ranging in properties of pI, hydrophobicity, hydrophilicity and size. Also, the overwhelming concentration of albumin often suppresses the detection of low abundance proteins.

Application of bioaffinity interactions can provide front end specificity to simplify the identification of low abundance proteins in human plasma. Published analytical approaches which combine bioaffinity capture techniques with mass spectrometry include surface-enhanced laser desorption ionization (SELDI) and surface plasmon resonance (SPR). Both SELDI and SPR can be combined with the ease and elegance of MALDI-TOF analysis with the specificity of biomolecular interactions. In effect, bioaffinity capture concentrates a protein of interest.

Future Directions in Capillary Electrophoresis

In this research, a simple CE-SDS method was developed capable of separating the major protein constituents of IV-1 Paste. The addition of SDS to the run buffer significantly improved resolution and peak shape over current CE methods. The mechanism of separation seemed to involve protein structural attributes such as size, structural rigidity, structural motifs and glycosylation, for example. In order to explore the structural basis of the separation by CE-SDS, an experiment similar to Gudiksen, et al. could be performed [1]. Gudiksen et al. examined the unfolding/denaturation of representative proteins by various levels of SDS by calculating the mobility shifts by CE. The representative proteins all underwent different stages of unfolding at various levels of SDS, which could lead to the identification of the optimal SDS concentration at which each protein may be resolved.

This type of experiment could be performed for the major protein constituents found in IV-1 Paste. By varying the levels of SDS at smaller increments, one can study the nature of the SDS-protein binding and denaturation using CE as a tool for studying protein structure. Also, the differential binding of SDS to the various proteins in a mixture is one way to optimize the separation.

Surface-Enhanced Laser Desorption-Ionization Mass Spectrometry (SELDI)

In SELDI experiments performed by Weinberger et al., cell extracts and cell culture supernatants were exposed to several different types of bound stationary phases in an array format[2]. Protein arrays were chosen containing five different stationary phases: strong anion-exchange, weak cation-exchange, hydrophobic, and immobilized metal affinity capture ProteinChip® arrays (Ciphergen Biosystems). This technology attempts protein separation from crude mixtures using principles of liquid chromatography miniaturized to the surface of a microchip. The crude samples are loaded onto the array surface at the appropriate pH and ionic strength for the capture of the protein of interest. A wash step removes unbound species as well as impurities by increasing the ionic strength or varying the pH. Finally, sinnapinic acid matrix is added to desorb bound proteins and crystallize. Laser beam desorption followed by TOF-MS analysis generates a mass spectrum. This technique allows the rapid investigation and identification of the appropriate chromatographic conditions to capture the protein of interest. However, the specificity of the capture is not comparable with that of antibody-antigen recognition.

Biomolecular Interaction Mass Spectrometry (BIA-MS)

Nedelkov et al. have coined the term "biomolecular interaction analysis mass spectrometry" (BIA/MS). Their research focuses on combining the high sensitivity and specificity of SPR and mass spectrometry in a two-dimensional approach to study biomolecular

interactions of proteins of interest [3,4]. Here, antibodies against the protein of interest are used as affinity ligands for capture. SPR serves as the first dimension of capture of the specific proteins. The sensor chip is then washed to elute/release the bound proteins, and these samples are subsequently analyzed by MALDI-TOF (Figure 5.2). The SPR experimental setup is highly specific and nondestructive, which allows for further structural analysis of the proteins of interest by mass spectrometry. In fact, the authors indicate that MALDI-TOF analysis provides validation of the SPR data.

The main advantages of this approach include reduced sample manipulation/preparation, reduced instrumental analysis time and reduced data analysis time. This technique can be used to study many classes of proteins, such as hydrophobic proteins associated with lipid bilayers.

Potential disadvantages include the expense of the instrumental setup as well as expense of individual antibodies. This approach does not identify unknown proteins which may also be of low abundance. In the case of IV-1 Paste, the sample must be depleted of albumin and Igs.

Conclusions

Bioaffinity mass spectrometry through the use of surface plasmon resonance and mass spectrometric analysis may indeed solve the problem of detection of low abundance proteins in human plasma. This approach combines front end specificity, quantitative ability, and validation of results through mass spectrometry.

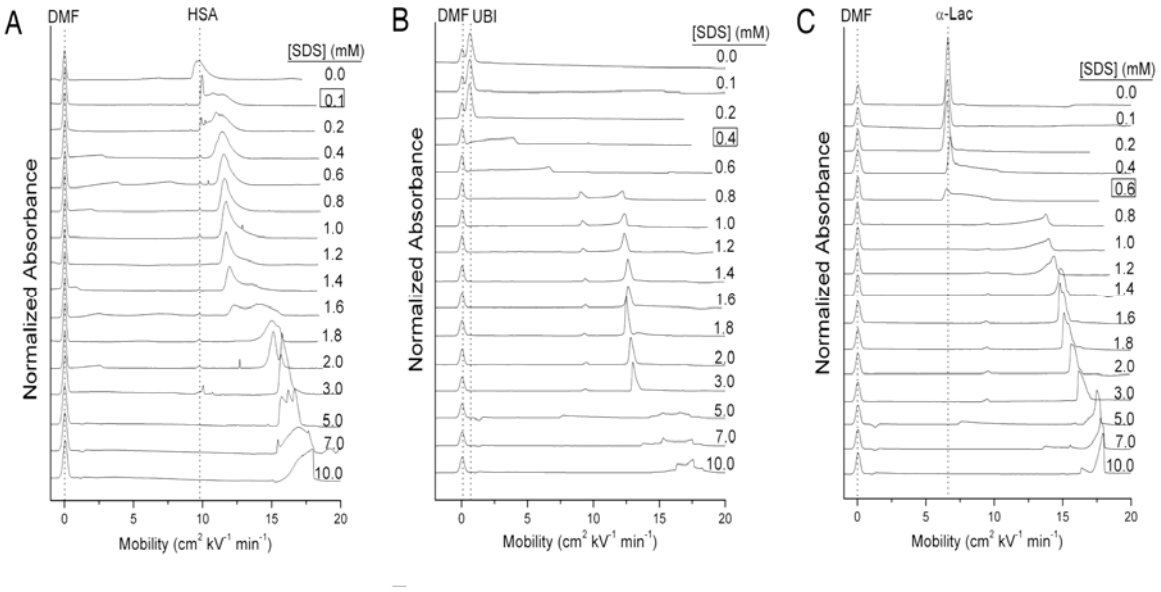


Figure 5.1 Example of electropherograms showing the denaturation of representative proteins by SDS and the subsequent shifts in mobility (Gudiksen et al.)[1]

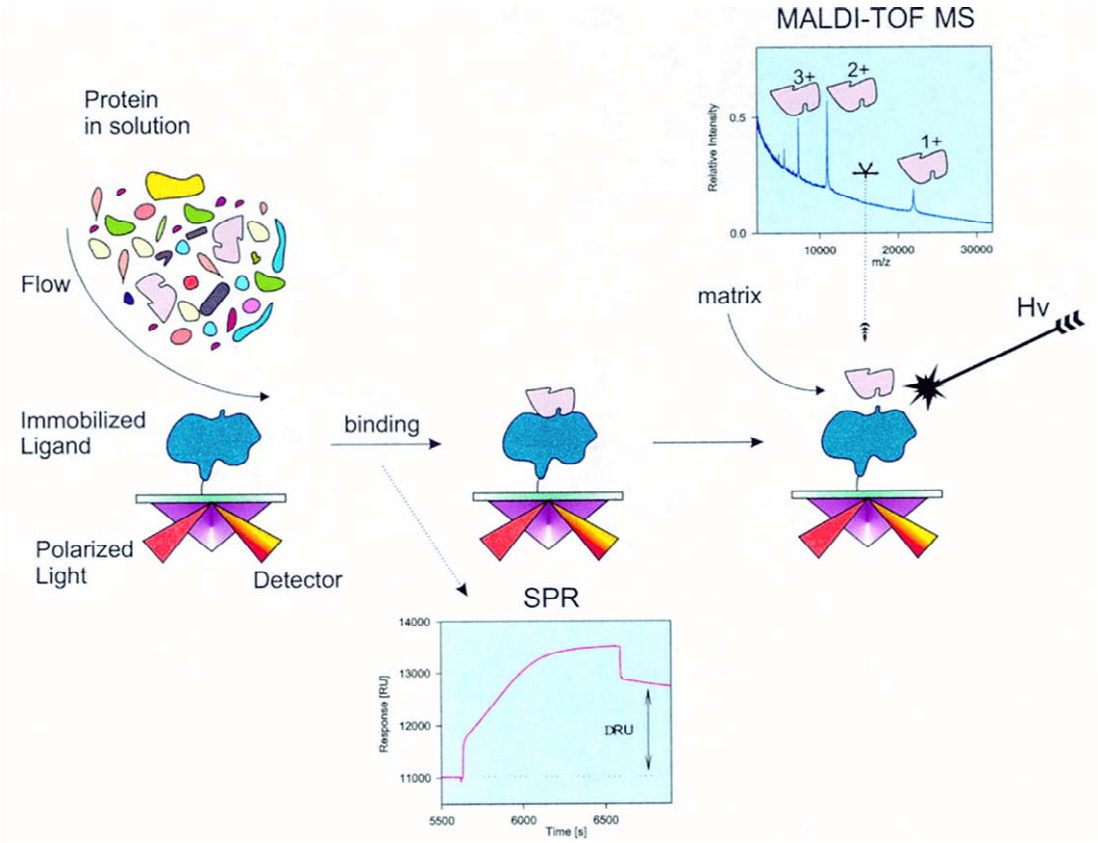


Figure 5.2 BIA-MS Experimental Setup (Nedelkov et al.)[4]

References

1. Gudiksen, K.L.; Gitlin, I.; Whitesides, G.M. Differentiation of proteins based on characteristic patterns of association and denaturation in solution of SDS. *Proceedings of the National Academy of Sciences* **2006**, *103* (21), 7968-7972.
2. Weinberger, S.R.; Boschetti, E.; Santambien, P.; Brenac, V. Surface-enhanced laser desorption-ionization retentate chromatography™ mass spectrometry (SELDI-RC-MS): a new method for rapid development of process chromatography conditions. *Journal of Chromatography B* **2002**, *782*, 307-316.
3. Nedelkov, D., Nelson, R.W.; Kiernan, U.A.; Niederkofler, E.E.; Tubbs, K.A. Detection of bound and free IGF-1 and IGF-2 in human plasma via biomolecular interaction analysis mass spectrometry. *FEBS Letters* **2003**, *536*, 130-134.
4. Nedelkov, D.; Nelson, R.W. Analysis of native proteins from biological fluids by biomolecular interaction analysis mass spectrometry (BIA/MS): exploring the limit of detection, identification of non-specific binding and detection of multi-protein complexes. *Biosensors & Bioelectronics* **2001**, *16*, 1071-1078.
5. Rüdiger, A.H.; Rüdiger, M.; Carl, U.D.; Chakraborty, T.; Roepstorff, P.; Wehland, J. Affinity Mass Spectrometry-Based Approaches for the Analysis of Protein-Protein Interaction and Complex Mixtures of Peptide-Ligands. *Analytical Biochemistry* **1999**, *275*, 162-170.
6. Lee, W.-C.; Lee, K.H. Applications of affinity chromatography in proteomics. *Analytical Biochemistry* **2004**, *324*, 1-10.
7. Florens, L.; Liu, X; Wang, Y.; Yang, S.; Schwartz, O.; Peglar, M.; Carucci, D.J.; Yates III, J.R.; Wu, Y. Proteomics approach reveals novel proteins on the surface of malaria-infected erythrocytes. *Molecular & Biochemical Parisitology* **2004**, *135*, 1-11.