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With its high efficiency and reproducibility, open tubular capillary electrochromatography (OTCEC) is beginning to prove itself as a promising method for the separation of proteins, peptides and pharmaceuticals. OTCEC, when coupled to an absorbance-based detector, suffers from poor sensitivity and high concentration limits of detection. However, laser induced fluorescence coupled to CEC has shown picomolar limits of detections for some compounds.[2] This thesis describes the coupling of OTCEC to a laser induced fluorescence (LIF) detector to optimize both the separation and detection aspects of protein and amino acid analysis. OTCEC capillaries were etched and silanized, and the 4,4' cyanopentoxy biphenyl phase was attached through a radical-initiated process. Such biphenyl phases have shown unique dependence on temperature and mobile phase composition and demonstrated higher selectivity for certain compounds[3] than bare silica capillaries. Proteins and amino acids were modified using pre-column derivatization formats with two different fluorescein-based dyes (fluorescein isothiocyanate, FITC) and Atto-tag FQ (3-(2-furoyl)quinoline-2-carboxaldehyde). These conditions were varied to attempt consistent labeling conditions with varying protein size. Optimizations of the analyses were achieved by modifying conditions such as background electrolyte, pH, voltage, and percent organic modifiers. These modified conditions were used to achieve the highest resolution on protein and amino acid derivatives. Results determined that high sensitivity and efficiency are achieved when

LIF detection is coupled to cyano pentoxy biphenyl coated capillaries using optimal conditions. These conditions include 1) the use of Atto-tag FQ derivatization and 2) an 80:20 acetonitrile: 50 mM phosphate buffer (pH 2.00) background electrolyte with a positive electrical current applied.

Open Tubular Capillary Electrochromatography-Laser Induced Fluorescence for the Separation and Detection of Proteins and Amino Acids

By

Kellie M. Davis

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Approved by

_____
Committee Chair

APPROVAL PAGE

This thesis has been approved by the following committee of the

Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____
Dr. G. Brent Dawson

Committee Members _____
Dr. Alice Haddy

_____
Dr. Nadja Cech

_____
Date of Final Oral Defense

_____
Date of Acceptance by Committee

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CHAPTER I

INTRODUCTION

Doctors and scientists work to find cures for life threatening diseases by identifying the biomarkers associated with these diseases. Biomarkers are characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention.[1] Proteins that have different concentrations in the normal and diseased states are biomarkers. By examining protein biomarkers, scientists gain the ability to identify the differences in proteins that are expressed in diseased processes. More specifically, biomarkers show the presence and even the extent to which a disease has progressed and are acquired mainly through the blood and urine. As disease research (such as cancer) progresses, disease biomarkers become more and more useful since they contain information such as disease type and stage.[2] By continuing research into these protein biomarkers and pharmaceuticals, scientists also gain insight into disease treatment and prevention by developing proteomic methods, which are the focus of this thesis.

Proteomics is an area of study that has been developed over the past 10 years. The goals of proteomics are to identify: proteins in a system, post translation modifications, and changes in expression levels of the proteins. The last goal will allow for the comparison of normal cells and diseased cells in the human body. With further

investigations using separation techniques, the ability to separate the proteins from complex mixtures such as blood or urine is achievable.

As stated above, biomarkers are proteins, peptides or small molecules that exhibit different concentrations in the diseased and normal states. These differences may be minute and can be difficult to detect therefore requiring state of the art analytical methods. Current analyses are performed by analytical separations, which will be discussed later in this chapter. These separations allow for the identification of multiple biomarkers and acquisition of concentration information in one step.

With such low concentrations and complex sample matrices, such as blood and urine, it is important to employ a method of analysis that incorporates selectivity, efficiency and resolution. Selectivity, in terms of chromatographic separation, is the measure of the retention of one analyte compared to the retention of another analyte. Efficiency is a measure of how uniformly molecules of the same type travel down the column. Efficient methods reduce analyte band broadening leading to better separations. Finally, resolution is a measure of how well two analytes separate. These three important features were examined in all aspects of separation technique development process of this thesis.

Common Separation Techniques

There are two well known techniques, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and high performance liquid chromatography (HPLC), which are currently used to separate biomolecules. Each of these techniques will be discussed

below, as well as aspects of an analysis and difficulties with how it applies to the separation of proteins and amino acids.

Two-dimensional Polyacrylamide Gel Electrophoresis

2D-PAGE combines isoelectric focusing (IEF) with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The first dimension, IEF separates proteins in a mixture according to charge (pI) and SDS-PAGE separates by size in the second.[3] This technique offers the advantage that fluorescence tagging can be used for the detection of post-translational modifications and for measurement of differential protein expression in cellular populations that differ in their physiological, metabolic or disease states.[4] The wide use of 2D-PAGE for the separation of proteins is due to the ability of thousands of proteins to be separated at one time. Currently scientist can ultimately separate 5,000 to 10,000 proteins, producing more information. It is estimated that any given cell type expresses about 10,000 to 20,000 proteins, so the ability to observe a significant portion of the total protein component of a given cell using 2D-PAGE is possible.[3] This achievement has lead to the use of the word proteome, which was first defined by Marc R. Wilkins as the "entire complement of proteins expressed by a genome, cell, tissue or organism."

Although there is a wide recognition of 2D-PAGE for the separation of proteins, there are several technical limitations. Aside from the advancements in the technique, reproducibility continues to be a problem. Also, highly acidic and basic proteins and hydrophobic proteins are generally difficult to detect in 2D-PAGE separations.[4]

Sensitivity is limited, along with the absolute amount of proteins that can be loaded onto the gel (dynamic range).[4] Due to limited dynamic range, proteins which are at low abundance are usually undetected. In a study reported by Shi *et al*., it was estimated on the basis of codon-bias distribution that more than half of all proteins in the yeast proteome are not detectable by 2D-PAGE.[4] Because of these limitations, other methods are needed.

High Performance Liquid Chromatography

Because of its high resolving power, reproducibility and its compatibility with electrospray mass spectrometry (MS), HPLC is an attractive alternative to 2D-PAGE for the separation of both proteins and peptides.[4] HPLC uses high pressure to force solvent through closed columns containing very fine particles that give the high resolution separations.[5]

There are several different types of HPLC, including ion-exchange chromatography, molecular exclusion chromatography, and reversed-phase chromatography. First, ion-exchange chromatography uses cations or anions attached to the stationary phase (usually a resin) to attract oppositely charged particles in the solute moving through in a liquid mobile phase to perform separations, meaning the separations are caused by the difference in charge as compared to the charge of the stationary phase. This process is used for small molecules (MW $\leq$ 500) and large proteins as well.

Second, molecular exclusion chromatography (or gel permeation chromatography) is different from the previous in that this technique separates out the molecules by size. The mobile phase (liquid) must pass through a porous gel. The pores

in the gel are small enough to allow the smaller molecules to enter, but not the larger molecules. The larger molecules move quickly past the gel not entering the pores. The small molecules take longer to pass through the column because they enter the gel and therefore must flow through a larger volume before leaving the column.[5]

Finally, the most commonly used type of HPLC is reversed-phase chromatography which uses a nonpolar stationary phase and a solvent that is more polar. The more polar solvents allow for quicker elution because they have a greater eluent strength, the free energy of salvation. This technique is most commonly used for low molecular weight neutral or charged organic compounds.

Liquid chromatography is a versatile and fundamental part of proteomics because of the wide range of mobile and stationary phases that can be used. The wide range of phases makes this technique very important because high selectivity is achievable and it has the ability to discriminate small differences in polarity, charge and size in a given sample. Unfortunately, different columns are needed to reliably separate molecules on the basis of two of these characteristics.

Capillary Electrochromatography Development

In the late 1980's, capillary electrophoresis (CE) began to gain popularity when it became commercially available. Figure 1 shows a general schematic of the CE instrumentation which has been modified from Harris (reference 5). This instrumentation is very simple and all that is required is a fused-silica capillary with an optical viewing window, a controllable high voltage power supply, two electrode assemblies, two buffer reservoirs, and a detector.[6]

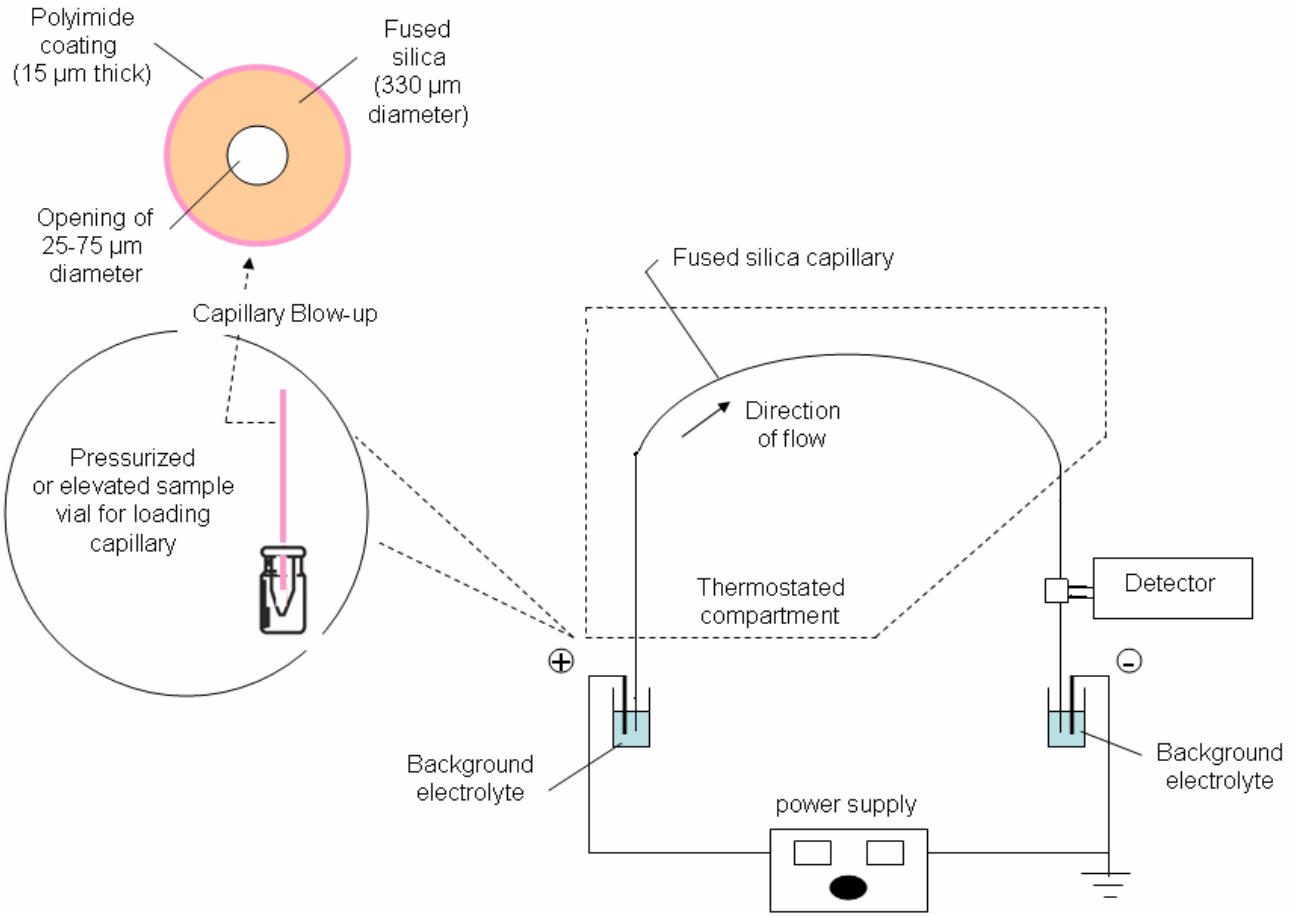


Figure 1. Schematic of general CE instrumentation[5]

CE utilizes the concept of separating compounds inside the fused-silica capillary based on the analyte's charge and mass by applying a high voltage. These charged ions are separated due to the attraction to an electrode of opposite charge and the resistance to movement in the solvent. Injection of the sample is usually performed by applying a small positive pressure to the inlet end of the capillary. The analyte is then in a background electrolyte under the influence of a potential difference between the two

electrodes.[7] The amount of sample injected can be controlled by the amount of pressure being applied and the time it is being applied to the sample vial, however the usual amount of sample that is injected is 1-10 nL.

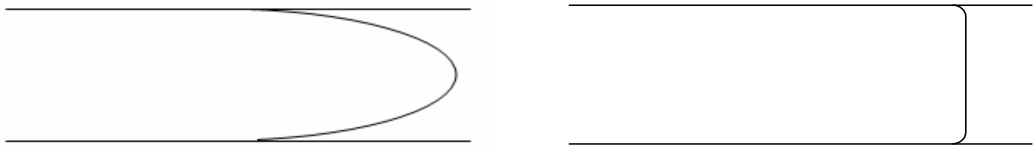
CE is a highly efficient analytical technique that has had a great impact in biomedical research and clinical and forensic practices.[2] This impact is due to the many great advantages CE holds over the leading clinical analytical techniques such as HPLC. These advantages include[2]:

i) small sample volume
ii) little waste
iii) rapid analysis
iv) great resolution
v) low cost

Modes of Operation

Through the development of CE, many different modes of this separation technique have been employed. These different types of CE (such as micellar electrokinetic capillary chromatography, capillary gel electrophoresis and capillary electrochromatography) are readily performed on the same instrumentation.

Capillary electrochromatography (CEC) is based on the effect of interaction of the analyte with the stationary phase on its electophoretic mobility.[7] CEC can be described as a combination of HPLC and capillary electrophoresis (CE). This technique can be used for the separation of molecules ranging in size from small inorganic and organic moieties to peptides and proteins.[8] In CEC, an electric field is used as the force to drive the solutes through the capillary and the stationary phase.



a) Parabolic (laminar) b) Flat (plug-like)

Figure 2. Comparison of EOF in HPLC and CEC: a) Parabolic (laminar) and b) Flat (plug-like) flow

The electric field produces an electroosmotic flow (EOF) which in turn creates a plug-like flow profile.[9] HPLC uses a pressure driven flow which in turn creates the parabolic (laminar) flow profile. As a consequence of parabolic flow, a non-uniform flow profile is created, Figure 2a, occurs in the column, resulting in a flow that is highest in the middle of the capillary and approaches zero at the column walls.[6] This flow profile is the cause of the substantial band broadening observed with separations performed with HPLC. In contrast, the EOF produced by the electric field in CE is uniform throughout the capillary, except very close (~ 10 nm) to the capillary wall, Figure 2b. This change in flow pattern reduces band broadening and increases efficiencies of separation.

Because capillary electrochromatography is a hybrid technique, there are many types of columns used ranging from those commonly used in CE to those described in HPLC.[10] The ones of most interest here are packed columns, monolithic columns and open tubular columns.

Packed columns consist of a capillary tube filled with chromatographic media and can be classified into three categories 1) columns packed with particles, 2) columns containing separation material that has been polymerized *in situ*, creating a "rod-like"

monolithic structure also known as a continuous bed, and 3) columns with entrapped particulate material, which are a combination of the first two groups.[10]

The first classification is one of the most commonly used in CEC. In this method, common HPLC, stationary phase particles are packed into a 100 μm inner diameter or smaller fused silica capillary and are retained by a porous frit. Some problems with packed columns lie in the method by which the capillary is packed. Most packed capillaries consist of two segments 1) packed segment and 2) unpacked (or open) segment. The electroosmotic flow produced in the capillary depends on the fraction of the packed segment to the overall capillary. A poorly packed capillary leads to low efficiency, poor resolution and asymmetric peak shapes.[10] Because of such a small inner diameter, the packing process is elaborate and requires experience. Many problems associated with packed columns are[10]:

i) difficulties packing small particles into a capillary to form a bed producing high efficiency.
ii) failure of frits or generation of bubbles
iii) the inadequate mechanical stability
iv) non-facile operation
v) poor chemical stability
vi) irreproducible EOF

Recently, studies have turned to the modification of capillaries using columns of sol-gels or rigid polymers inside the fused silica capillary, which in turn gives the desired selectivity.[11] In efforts to produce more stable and highly efficient columns, monolithic columns were introduced. Monolithic columns consist of a single network of modified silica or an organic polymer contained within the fused silica capillary.[10] Because monolithic columns possess a one-piece network structure (as seen in Figure 3), they are

able to solve some problems associated with the packed columns (*i-iii* listed above). These types of columns can also solve *iv* (from above) which are associated with retaining frits in CEC. Additionally, monolithic columns can reduce the diffusion path-length and flow resistance due to the small-sized skeletons and large through-pores.

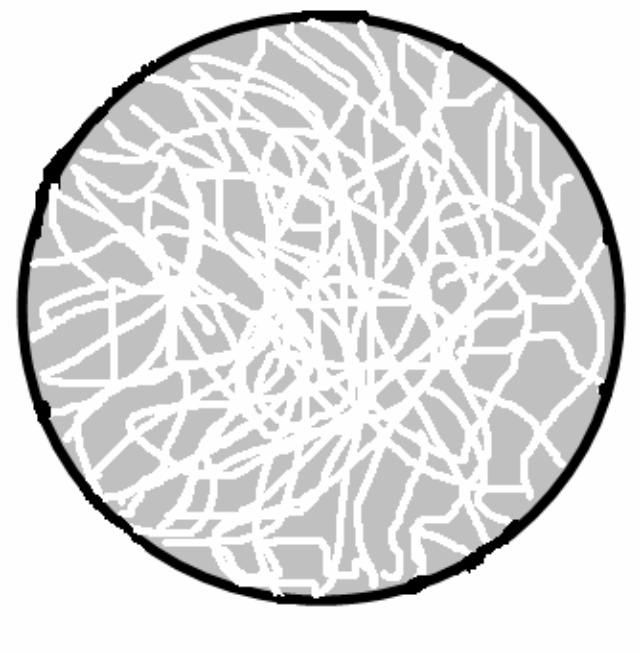


Figure 3. Representation of a monolithic column

Early work in the area of protein separation with capillary electrochromatography focused on techniques that produced high selectivity through the use of capillaries packed with particles; however, many problems with bubble formation and frit stability have limited the use of such techniques.[11] These techniques also showed poor limits of detection and irreproducible retention times. Although some scientists still focus on improvement of monolithic capillaries, some experimental focus turned to open tubular

capillary electrochromatography. In open tubular capillary electrochromatography (OTCEC), the stationary phase is attached to the inner wall of the capillary, (as seen in Figure 4.[9] The stationary phase thickness in this capillary can be anywhere from 1 nm-1μm. The attachment process for open tubular columns is described in detail in Chapter 3 of this thesis.

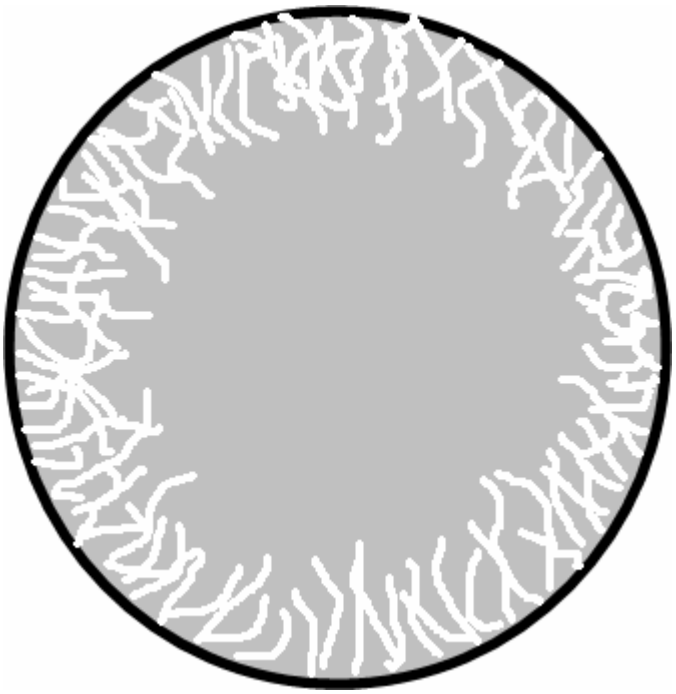


Figure 4. Representation of a modified open tubular capillary

In one configuration the small inner diameter capillary is etched to increase its surface area 1000 fold. By increasing the surface area of the capillary, there is an increase in the phase ratio and a small positive effect on loadability of phase. Generally, capillary

based separations can be applied to the small amounts (picomoles) of sample that are needed for the analysis in biological arenas where sample volumes are limited.[11] In addition to the above, OTCEC, with its high efficiency and reproducibility, is beginning to prove itself as a promising method for the separation of proteins, peptides and pharmaceuticals. Specifically, in a study by Matyska and Pesek, stability was observed for capillaries modified by the silanization/ hydrosilation process used in CE for the separations of proteins.[8] After repeated runs (approximately 150) on the same cyanopentoxy capillary, there was no statistically discernible variation in migration times (relative standard deviation, RSD <2.0%).

Modes of Detection

Depending on the nature of the analytes being separated, CEC is compatible with many different modes of detection including ultraviolet-visible absorption (UV-Vis), amperometry, mass spectrometry (MS) and laser induced fluorescence (LIF). The most common modes of detection, however, are UV-Vis absorption and fluorescence.

UV-Vis absorption detection is the most common means to examine separations in CEC because the majority of analytes studied absorb in the UV-visible region which means that no derivatization of the analyte is necessary. The primary disadvantage to absorbance detection for CEC is the relatively poor minimum detectable concentration (MDC), generally in the 10^{-6} M range. The poor MDC is caused by geometric constraints imposed by the small internal diameter of the capillary (path-length) and the inherently insensitive nature of absorbance detection.[14]

Optical techniques are dependent upon the volume of analyte available, whereas electrochemical techniques are mass sensitive (involve the direct contact with an electrode surface). Amperometric detection techniques generally provide good sensitivity and can be selectively tuned to a certain class of compounds.[14] The most common electrochemical detection technique is amperometry which involves the charge-transfer type electrode reaction (oxidation or reduction) for the analysis of compounds at a solid electrode under the influence of an externally-applied DC voltage.[14] Amperometry is, as stated above, sensitive and selective, but the analytes must be electroactive in the background electrolyte. In order for this technique to achieve the level of sensitivity and selectivity, special electronic instrumentation to decouple separation and detection voltages and capillary modifications are required. These realities have prevented the commercial availability of amperometric detection for CE and CEC.

Mass spectrometry (MS) when coupled to CEC can prove to be a great source of structural information. The advantages of mass spectrometry are that it has the capability to determine molecular weight and provides structural information.[14] Mass spectrometry can be coupled to CEC by means of electrospray ionization. In electrospray ionization, droplets containing analyte are formed when an electric field is applied to the sample at the end of the capillary entering into the MS system. The MS provides qualitative data as well as quantitative data though its limit of quantitation is on par with that of amperometry.[14]

Finally, as research continues in the field of separations by CEC for proteins continue, detection methods with lower limits of detection must be explored. Studies

have shown that when CEC is coupled to fluorescence detection, limits of detection could be reduced to concentrations below 10^{-13} M.[14] In a study performed by Yan *et al.*, CEC was coupled to laser induced fluorescence for the study 16 polycyclic aromatic hydrocarbons (PAHs) classified by the EPA as priority pollutants. In this case, the combination of the two techniques yielded subattomole detection limits.[15] Therefore, laser induced fluorescence detection is said to be the most sensitive detection method available for CEC. Below is a detailed schematic of a laser induced fluorescence detector that would be used for the purposes of CEC (modified from the Picometrics manual).

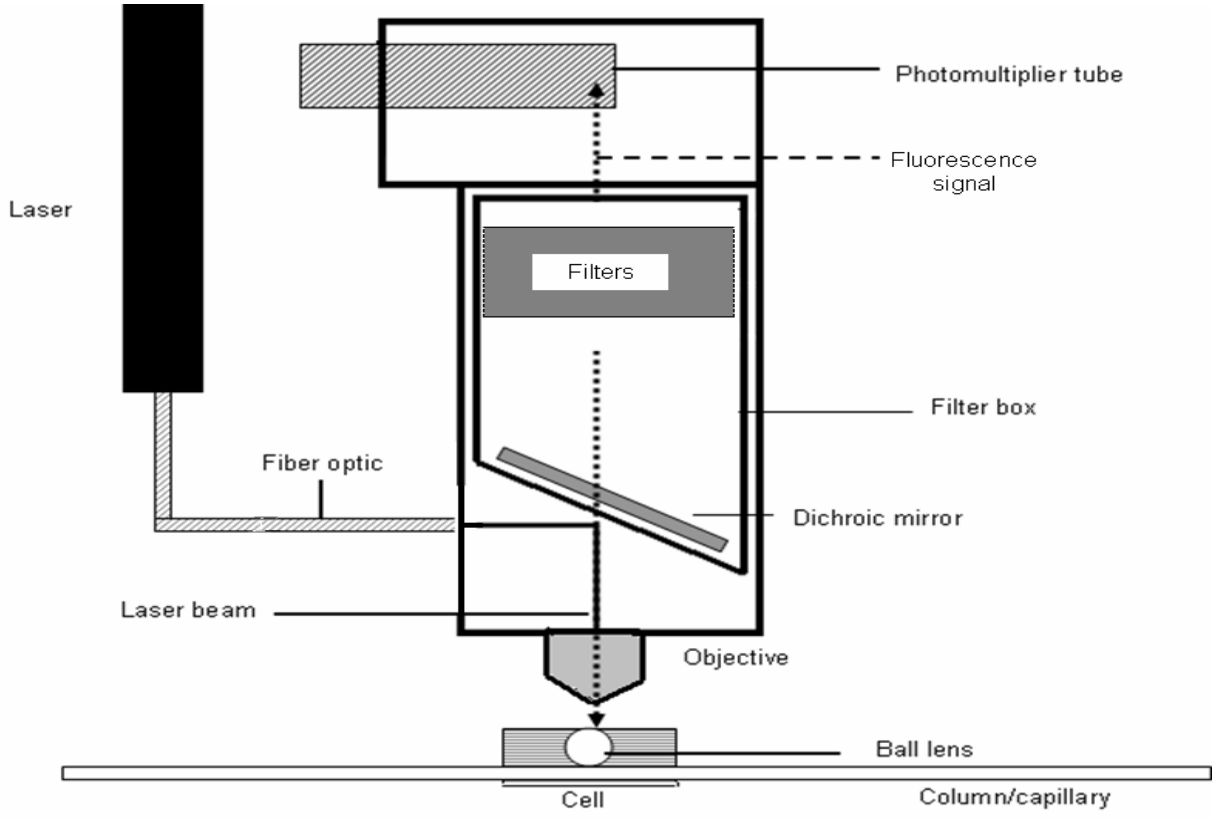


Figure 5. Schematic of a ZETALIF fluorescent detector

The construction of the LIF detector is central to its success, as with any instrument. When LIF is coupled to another instrument (such as CEC), the operation is going to be dependent on the analyte being determined. When the laser is turned on, it passes through the optical fiber which in turn carries the laser beam to the optical bench. Located within the bench is the dichroic mirror which reflects the laser beam through the objective and focuses on the sample using the ball lens (the process is described in Chapter 2). More specifically the dichroic mirror reflects light of wavelengths less than 500 nm and transmits light longer than 500 nm. The sample will absorb the laser light (at 488 nm) and emit light at longer wavelengths. Once the sample passes through the window of the capillary (located in the sample cell), this fluorescence signal is passed through the mirror and travels through a series of filters. These filters help to filter out wavelengths shorter than 520 nm and noise due to the Raman scattering of water. After filtration, the signal reaches the photomultiplier tube amplifies the light signal through a series of dynodes, and converts the fluorescent signal into an electrical signal.

With fluorescence detection, it is often necessary to label (derivatize) samples with a fluorophore in order to make them available for detection. Because there is an introduction of a new substance to the analyte, it is important to understand certain analytical parameters that accompany this label (fluorophore). The most important parameters that allow for the analytical use of a particular fluorophore are: absorptivity, fluorescence quantum yield and photostability. Unfortunately, these parameters can be difficult to optimize simultaneously. High absorptivity means the molecule is more likely to be excited at a given illumination intensity, meaning it will be considered a good

chromophore.[14] Fluorescence quantum yield is equal to the fraction of excited molecules emitting photons. For the most sensitive fluorophores, the yield can approach unity.[14] Two dyes which add strong absorptivity and good quantum yields to protein molecules are fluorescein-based dyes (fluorescein isothiocyanate, FITC) and Atto-tag FQ (3-(2-furoyl)quinoline-2-carboxaldehyde).

The amine-reactive fluorescein derivatives have been the most common fluorescent derivatization reagents for covalently labeling proteins.[16] Fluorescein derivatives have relatively high absorptivity, excellent fluorescence quantum yield, and good water solubility. Also, the excitation wavelength for fluorescein (494 nm) is relatively close to the excitation wavelength of an argon-ion laser (488 nm) making it a good choice for CE separations.[16] However, fluorescein-based dyes and their conjugates have several drawbacks:[16]

i) relatively high rate of photobleaching
ii) pH-sensitive fluorescence that is significantly reduced below pH 7
iii) relatively broad fluorescence emission spectrum, limiting their utility in some multicolor applications
iv) tendency toward quenching of their fluorescence upon conjugation to biopolymers, particularly at high degrees of substitution.

The first two drawbacks, photobleaching and pH sensitivity, make it very difficult to obtain quantitative data. Also, photobleaching limits the sensitivity that can be obtained, which is a significant disadvantage to separations requiring ultrasensitive detection. These limitations encouraged researchers to explore other options available, and another option is Atto-tag FQ.

Atto-tag FQ is very useful because of its ultrasensitive detection of primary amines (10^{-15} moles), the use of visible-wavelength excitation (480 nm or 488 nm with an argon-ion laser), and freedom from background fluorescence.[16] In other words, the reaction between Atto-tag FQ and primary amines goes to completion or near completion allowing for high sensitivity and detectability. Atto-tag FQ is only fluorescent when it has successfully reacted with a primary amine, therefore, it can be successfully applied to on-column and post-column derivatization, which are discussed in the next section. The principal limitation to obtaining ultrasensitive detection (between 10^{-13} and 10^{-10} M) is the relatively high concentration of the derivatizing reagent required for the adequate kinetics and sufficient modification of the analyte.[16] This reaction is further discussed in Chapter 2.

Unfortunately, with fluorescence, there are three important problems concerning background noise: Rayleigh scattering, Raman scattering and background luminescence. Rayleigh scattering refers to light scattered at the excitation wavelength. It is removed using appropriate filters or monochromators. Raman scattering of water is weak, but is considered one of the more serious background sources at low fluorophore concentration[14] because it can interfere with the sample signals. Other background sources can include luminescence given from the capillary walls and impurities in the separation medium (buffers). Even with the best water filtration systems, trace impurities are often the limiting background source for extremely low concentration fluorescence detection.[14] At the fluorescence detection limit, the background luminescence is often orders of magnitude stronger than the signal given from the sample. Since the detection

limit is determined by distinguishing between the small signal sample and large background; it is important to further examine method optimization.

Literature Review and Applications

Researchers examine the changes in proteins/ peptides throughout the duration of diseases, such as cancer, in order to identify the disease earlier. Biological changes, such as these, are most commonly studied by gel electrophoresis and high-performance liquid chromatography, which are discussed thoroughly in the earlier part of this chapter. Recently capillary electrochromatography has been used for protein analysis including some biomarkers. Biospecific interactions in CEC allow for the capturing of minute and important biochemicals from small sample sizes.[17] The conventional separation techniques do not incorporate high resolution and sensitivity with small sample sizes as is found in CEC.

Many reviews have started to focus on more specific modes of operation for CEC so scientists have the ability to examine the developments in CEC technology. In a review on biochemical analyses performed using CEC, many experiments were discussed using multiple modes of CEC when studying amino acids, peptides and proteins. For studies of amino acids, Mueller *et al.* investigated N-terminal sequencing using packed-column CEC. In this experiment, resolution was obtained in 13 minutes, as opposed to 30 minutes required for gradient elution HPLC, with repeatability over 3 columns within 1.9% except for Leu and His.[18] An experiment using open tubular capillary electrochromatography (OTCEC) was performed by Lin and Liu using a proline-coated

capillary. This yielded nine amino acid separations within 40 minutes. After continuous use for over 2 months, this capillary was able to be used with no significant change in EOF.

As for the separations of peptides, monolithic capillaries are the significant focus; however, advancements in OT-CEC have also been explored. Fu *et al.* used a monolithic column with a zwitterionic stationary phase, which allowed for different separation selectivity. This selectivity was achieved because the stationary phase could generate an anodic or a cathodic EOF depending on the pH of the mobile phase.[19] OTCEC was explored by Pesek *et al.* using a modified capillary with different surface moieties.[11, 20-21] This experiment depended highly upon temperature optimization for the separations. Once the temperature was optimized, baseline separations of peptides were almost fully observed.

Protein separations, just as those of peptides, focus on monoliths and modified open tubular capillary methodologies. Bedair and El Rassi studied the use of a monolithic capillary for the study of proteins and peptides. In their experiment a cationic stearyl-acrylate monolith for the separation of water-soluble and membrane proteins was studied on CEC.[22] This type of monolithic capillary allowed for the 5 minute separation of crude extracts of the membrane proteins. Bandilla and Skinner also studied the effects of the use of a monolithic capillary for the separation of some standard proteins.[23] This separation used a butylacrylate monolithic column which in turn yielded selectivity due to the interactions with the stationary phase. Pesek *et al.* developed an OTCEC method for the separation of PEG-modified proteins using cholesterol and octadecyl-coated

capillaries.[24] This experiment yielded an electrochromatogram with multiple peaks as a result of the number of sites available for interaction and the number of amino acid residues that were modified.

Extending past actual separation modes for proteins and amino acids, one must examine the modes of detection. As discussed earlier in this chapter, there are three common modes of detection: UV absorption, LIF, and MS. Václav Kašička reported that peptides separated by CEC can be detected at a concentration detection limit in the micromolar range when UV-absorption detection of a peptide bond at 200-220 nm wavelength. Though when using such wavelengths, one can be limited to the buffers that are used. At these wavelengths, phosphate or borate buffers are considered to work best because they absorb very small amounts of radiation. However, if organic buffers are used they do absorb radiation at these wavelengths. Although UV absorption is the most common of the three techniques, LIF is the most sensitive detection mode in CEC with potential detection limit of a few or even a single molecule.[26-30] However, the LIF must incorporate the derivatization of the protein or peptide with a fluorescent label. As for MS, this mode of detection is considered to be ideal for separations performed by CEC. This method is universal, sensitive and selective and has been summarized in reviews of both early and resent developments of CE-MS. This method is also allows for the coupling to electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) for further analysis of the separations. The use of MS detection enables for the detection and characterization of peptidic and nonpeptidic parts of peptides and proteins.

The focal point of this thesis is open tubular capillary electrochromatography using a capillary that has been etched and derivatized with a cyano pentoxy biphenyl stationary phase. With the aid of LIF detection, it is hoped that OTCEC will provide a solution to the current analytical problems, for separation of proteins, peptides and amino acids, accompanying conventional analytical techniques by combining the use of small sample size, providing high resolution, and giving high efficiency.

In order to achieve results that are compatible with the above statement, parameters for derivatization and separation had to be determined. In the process of determining what parameters to use, manipulation of standard procedures were used. For instance, during separations, multiple buffers were used, multiple pH values and different acetonitrile to buffer ratios were employed.

CHAPTER II

DERIVATIZATION ANALYSIS AND SEPARATION OPTIMIZATION OF PROTEINS AND AMINO ACIDS USING OTCEC-LIF

Introduction of the Derivatization Analysis

Derivatization methods used to enable the fluorescence detection of proteins and amino acids must be examined due to the coupling of open tubular electrochromatography (OTCEC) with laser induced fluorescence (LIF). As discussed in the previous chapter, proteins and amino acids are not fluorescent, when excited with visible light, so a derivatization process must be incorporated into the overall experimental procedure. It is important to first examine the structure of amino acids and proteins because it will play a role in the attachment of the fluorophore. Because the structure of a protein is affected by the changes in pH and temperature, these aspects will also be discussed in this section.

In the derivatization process, the fluorophores (FITC and Atto-tag FQ) will attach to all primary amines (Lys or the N-terminal). If the fluorophore does not have the same pKa as the primary amine (which it usually does not); the derivatized amine will have a different electrophoretic behavior than the underivatized one. In analytes containing multiple amines, such as proteins, if derivatization is not complete (i.e. some primary amines remain underivatized), multiple sample zones will be present in the capillary each

of which contains a fraction of the total analyte and give a fraction of the potential fluorescent signal. To narrow the distribution of multiply labeled proteins, short reaction times are used to take advantage of processes that are kinetically controlled. In these reactions more collisions between reactants must take place before products are formed. Only kinetically favorable reactions will take place during short reaction times.

The fluorophores are electrophilic in nature and will combine with an unprotonated nucleophilic amines. By controlling pH and temperature, it is expected that the same number of primary amines will react on each of the analyte molecules, thus giving one sample zone of maximum signal. The goal of this portion of the research was to determine if Atto-tag FQ was a better label for amino acids and proteins compared to FITC.

Introduction of the Separation Optimization

To determine if proteins and amino acids were labeled effectively, separation techniques were used. Separation optimization was another focus of this study and therefore is introduced in this chapter as well. One of the major reasons for studying proteins is to gain the ability to identify differences in normal and diseased conditions to better understand diseases. Once these differences are identified and understood better ways to treat the diseases can be developed. As was previously mentioned, these differences that are observed, in normal and diseased process, are very small. Current methods of analysis are performed by analytical separations. The problem with current methods is that no one technique maximizes all of the important parameters: selectivity,

efficiency and resolution at such low concentrations. The goal here was to find conditions that optimize these parameters for derivatized proteins and amino acids.

The work described in this chapter used a stationary phase attached to the wall of an etched capillary. This stationary phase is a monolayer thick coating which is attached via a silanization/ hydrosilation process. Silanization covalently attaches a silicon hydride layer to the reactive silanol groups of the capillary walls (shown in Figure 6), and then during hydrosilation the hydride layer reacts with a variety of reagents to form the stationary phase (shown in Figure 7).[5] In these figures, Y stands for an adjacent stationary phase ligand. In both of these figures a catalyst is represented by the abbreviation cat. In Figure 6 the catalyst was hydrochloric acid (HCl) and in Figure 7 it was t-butyl peroxide.

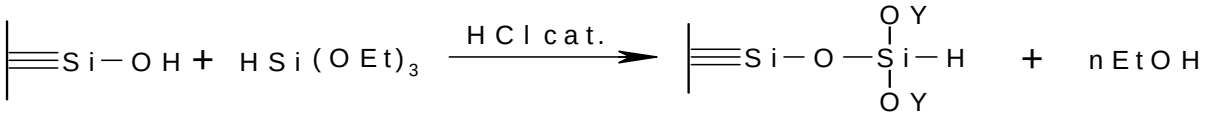
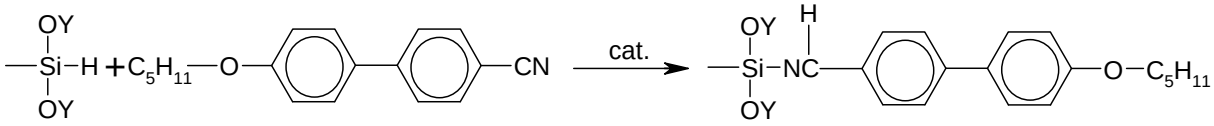


Figure 6: Versatile method for coating capillaries



cat. = t-butyl peroxide

Figure 7: Attachment mechanism for capillary modification[3]

A major point of separation optimization is, of course, the stationary phase. However, the mobile phase is another very important aspect to be discussed in terms of separation optimization. The buffers used as the mobile phase in this experiment varied in pH and acetonitrile content. The concentrations of the acetonitrile were changed to find optimal buffer:acetonitrile ratios along with the pH. With the adjustments, maximum resolution of amino acids and proteins were obtained.

In any separation, it is important to understand how the analytes move through the capillary. Both the stationary phase and the mobile phase contribute to this process. The analytes move through the capillary based on size and charge. Depending on the pH, the analytes will be protonated or deprontated, in turn affecting migration. Also, it is important to remember that size will also play a factor; the slower moving analytes will be larger.

Experimental

Materials and Instrumentation

Peptides and proteins were obtained from Sigma (St. Louis, MO). Fluorescein isothiocyanate, FITC, was obtained from Acros Organics (Geel, Belgium). Atto-tag FQ amine-derivatization kit was obtained from Invitrogen (Carlsbad, CA). All derivatizations discussed, at temperatures higher than room temperature (25^{o}C), were performed using an Isotemp 202S from Fisher Scientific (Pittsburgh, PA). Capillaries, inner diameter of 75 μm, were purchased from Polymicro Technologies (Phoenix, AZ). Capillary modification at high temperatures were done using a Hewlett Packard 5890A GC oven

and examined with a microscope from LW Scientific, INC. (Atlanta, GA). All separations were performed on a Hewlett Packard 3D capillary electrophoresis system and detected using a laser induced fluorescence detector from Picometrics (Cambridge, MA). All data was processed on ChemStations computer software from Agilent Technologies (Santa Clara, CA).

Buffer Preparation

Buffers were prepared from empirically determined recipes that gave the appropriate conductivity and absorbance values.[5] Running buffers used were diluted to the desired concentration from the stock solutions. Buffers that were used are pH 2.00, 50 mM phosphate; pH 7.00, 100 mM phosphate; pH 4.00, 50 mM tris formate; pH 9.5, 100 mM carbonate.

Capillary Preparation

The inner surface of the capillary was etched with a 5% w/v solution of ammonium bifluoride in methanol and reacted at an elevated temperature in a gas chromatographic oven. The etched surface was then reacted with triethoxysilane in the presence of an acid catalyst (a mixture of Dioxane, TES, and 2.3 M HCl). Then the biphenyl layer was attached to the hydride layer through a free radical initiated process using *t*-butyl peroxide. This solution was passed through the capillary and heated for 24 hours at 100^{o}C. This process was repeated for a total of 5 days. After the 5 days, the capillary was rinsed prior to use. The capillary was prepared for instrumentation use by

removing the polyimide coating to create a window for the laser detection. The window was created to measure between 3-5 mm wide using an open flame or capillary window burning device. Argon gas was flushed through the capillary while the window was being formed to prevent charring of the inside coating.

Derivatization

Amino Acid (dry FQ)[31]: Stock solutions of 0.1 mg/mL of each of the following amino acids in deionized water were prepared: GABA, glutamate, and L-homoserine. Atto-tag FQ was placed in a vial and the methanol was removed using nitrogen gas. Once methanol was gone, KCN in 10 mM borate buffer was added. Then 0.1 mg/mL of an amino acid solution was placed in the vial and allowed to react at 65^{o}C. While waiting, a mixture of 50:50 of 50 mM phosphate buffer (pH 2.00) and methanol was made. After reaction completion, the buffer/methanol mixture was placed in a vial and vortexed.

Protein (FITC)[32]: A 100 mM FITC stock solution was prepared by dissolving FITC in anhydrous DMF. A 10 mM substock was made by performing dilutions with the anhydrous DMF. A 10 mM solution of EDTA was made by dissolving EDTA in 100 mM phosphate buffer (pH 7.00). This solution was diluted to a concentration of 400 μM EDTA in 100 mM phosphate buffer (pH 7.00). Conjugations were initiated by adding 10 mM FITC to a solution containing 100 mM phosphate buffer (pH 7.00)/ 400 μM EDTA and 10^{-4} -10^{-6} M solution of protein. Upon addition of FITC, reaction tubes were

protected from the light and mixed, and then allowed to react in the dark overnight at room temperature.

Protein (FQ): To have rapid and complete derivatization of amino acids in the protein, there must be at least a six-fold molar excess of 3-(2-furoyl)quinoline-2-carboxaldehyde (Atto-tag FQ) and a fivefold molar excess of KCN. The derivatization process, seen in Figure 8, was carried out by mixing a 10^{-4} to 10^{-6} M protein (made in a high pH borate buffer) with a 10 mM KCN solution and a 10 mM FQ solution, and allowed to react for at least one hour at room temperature. Procedure is from the Invitrogen Fluorescent Dye Handbook.

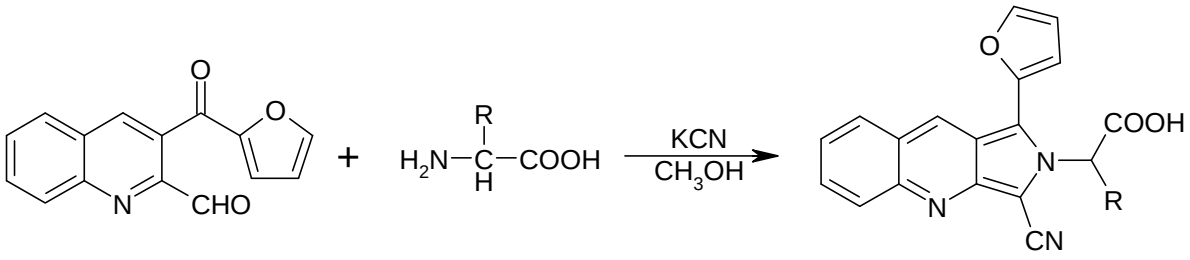


Figure 8: Amino acid derivatization reaction

Instrumentation Preparation/Laser focusing

Before any sample injection, the laser of the LIF detector was focused on the capillary in use. A 10^{-7} M solution of a fluorescein-based dye (fluorescein isothiocyanate, FITC) was used in a carbonate buffer solution (pH 9.5). This solution was allowed to pass through the capillary for 1 minute. After such time, a signal on the fluorescent detector was taken while adjustments were made to the microscope objective in the

vertical and horizontal directions. Optimum signal was reached when the highest possible fluorescent signal was observed continuously. Once this signal was obtained, the capillary was flushed with filtered acetone and filtered deionized water until the fluorescent signal had returned to a baseline value.

Separation Specifications

Amino Acid: A 10^{-6} M Atto-tag Fq GABA, glutamate, and L-homoserine derivative was injected for 10 seconds at 50 mB on an etched cyano pentoxy biphenyl capillary. The capillary length for this analysis was 70 cm, and the effective capillary length (the length from the inlet to the window) being 64 cm. A positive electrical current was applied at 25 kV with a background electrolyte of a 80:20 acetonitrile: 50 mM phosphate buffer (at pH 2.00). LIF detection specifications are an excitation wavelength of 488 nm, emission wavelength of 520 nm.

Protein (FITC): A 10^{-6} M FITC β-galactosidase, carbonic anhydrase, and protein mixture (a mix of β-galactosidase and carbonic anhydrase) derivative was injected for 10 seconds at 50 mB on an etched cyano pentoxy biphenyl capillary. The capillary length for this analysis was 70 cm, and the effective capillary length (the length from the inlet to the window) being 64 cm. A positive electrical current was applied at 25 kV with a background electrolyte of a 100 mM phosphate buffer (pH 7.00)/400 μM EDTA. LIF detection specifications are an excitation wavelength of 488 nm, emission wavelength of 520 nm.

Protein (FQ): A 10^{-8} M Atto-tag Fq albumin, β-galactosidase, carbonic anhydrase, phophorylase B, and myosin derivative was injected for 10 seconds at 50 mB on an etched cyano pentoxy biphenyl capillary. The capillary length for this analysis was 70 cm, and the effective capillary length (the length from the inlet to the window) being 60 cm. A positive 25 kV electrical current was applied. A background electrolyte of a 80:20 acetonitrile: 50 mM phosphate buffer (at pH 2.00). LIF detection specifications are an excitation wavelength of 488 nm, emission wavelength of 520 nm.

Results and Discussion of the Derivatization Analysis

In this step of the investigation, the derivatization procedures were explored, and the differences between two fluorophores examined. Proteins and amino acids themselves are not fluorescent; therefore, without derivitization of the amino group within the protein, peptide or amino acid, detection by LIF would be impossible. In order for the derivatization reaction to be successful, the amine must be deprotonated. Therefore, if the amines have different pKa values and the reaction pH is in the middle of these pKa values then deprotonated amines will react, but protonated ones will not. The N-terminus has a lower pKa than most lysines and should react at a lower pH, such as 7.00. The secondary and tertiary structure of the protein will also influence the pKa values of the lysines.

It is also important to consider the length of the reaction per derivatization process used. A much shorter reaction time is needed when Atto-tag FQ is used as opposed to

FITC. Atto-tag FQ is ready for separation preparation after one hour of reaction time, but FITC requires that the sample react overnight and in the dark. Not only is reaction time important when deciding which fluorophore is better but the problems relating to stability of the derivatized species. Atto-tag FQ derivatives are stable for approximately 24 hours after the reaction is complete. If kept at low temperatures, it is possible to sustain longer stability. As far as FITC derivatives, those of primary and secondary amines are the only stable derivatives.

For the purposes of this study the following amino acids (seen in Figure 9) were used: GABA, glutamate, and L-homoserine.

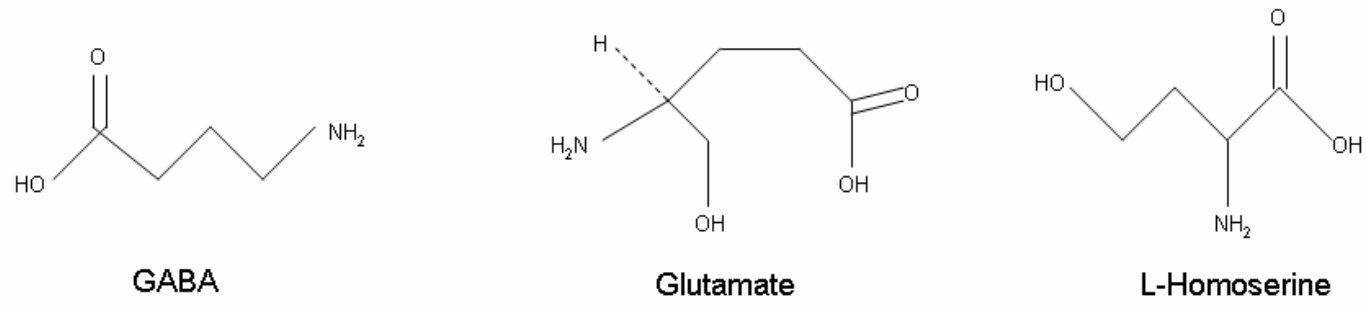


Figure 9: Amino acid chemical structures

Table 1 identifies the number of approximate available amino groups for derivatization of the proteins. Each amino acid used in this study only contains one available amine group.

Table 1: Molecular weight and amino group analysis of proteins

Proteins		
Sample	**Molecular Weight** (kDa)	**Available number of amino groups**
Albumin	45	20
β-galactosidase	116	31
Carbonic Anhydrase	29	20
Myosin	205	190
Phosphorylase B	97	48

In order to determine whether the fluorescent labeling reaction worked correctly, each amino acid was analyzed separately with open tubular capillary electrochromatography on a cyano pentoxy biphenyl capillary. Figures 10-12 show the individual separations for the amino acids after each was derivatized with the Atto-tag FQ. The main peak for each amino acid shows a relative fluorescence of greater than 19, which can be seen in Table 2 along with the migration time of each peak.

Table 2: Atto-tag FQ derivatized amino acid electropherogram analysis

Amino Acids		
Sample	**Relative Fluorescence (rFu)**	**Migration Time (min)**
GABA	1.4; > 19	6.8; 7.1
Glutamate	> 19	10.5
L-Homoserine	> 19	10.4

Each amino acid analysis showed one main peak (Figures 10-12) because only one amino acid is present. These peaks were extremely well defined, as expected. More specifically, Figure 10 shows a small peak which most likely represents an impurity. The early migration time that is seen is due to the molecule's positive charge, due to the quinoline having a pKa of 1.55 and the carboxylic acid group having pKa of around 4. The fluorescent derivative is expected to be positively charged at this pH. As for Figure 11, the migration time recorded for glutamate, when compared to GABA, is later due to the presence of 2 carboxylic acid groups; one having a pKa ~ 2.3 and the other having a pKa ~ 4. The same can be seen for L-homoserine, Figure 12, compared with GABA, it also has a carboxylic acid group with a lower pKa.

Table 3: pKa values of the carboxylic acid groups within the amino acids

Amino Acid	pKa 1	pKa 2
GABA	4.44	----------
Glutamate	2.10	4.07
L-Homoserine	2.21	----------

The significance of this amino acid analysis was to show the ideal elution results for amino acids. The single distinct peak that is seen in each electropherogram is representative of the single amino acid. The intensities of the peaks show that the derivatization process worked for the amino acids, even at 10^{-6} M. The derivatization procedure was performed at a relatively high pH (above pH 7.00). This is due to the fact that the amino group must be deprotonated in order for the reaction to go to completion. In this portion of the experiment, no other derivatization pH changes were employed, but

if performed at a low pH, the amino group within the amino acid would be protonated making it a poor nucleophile.

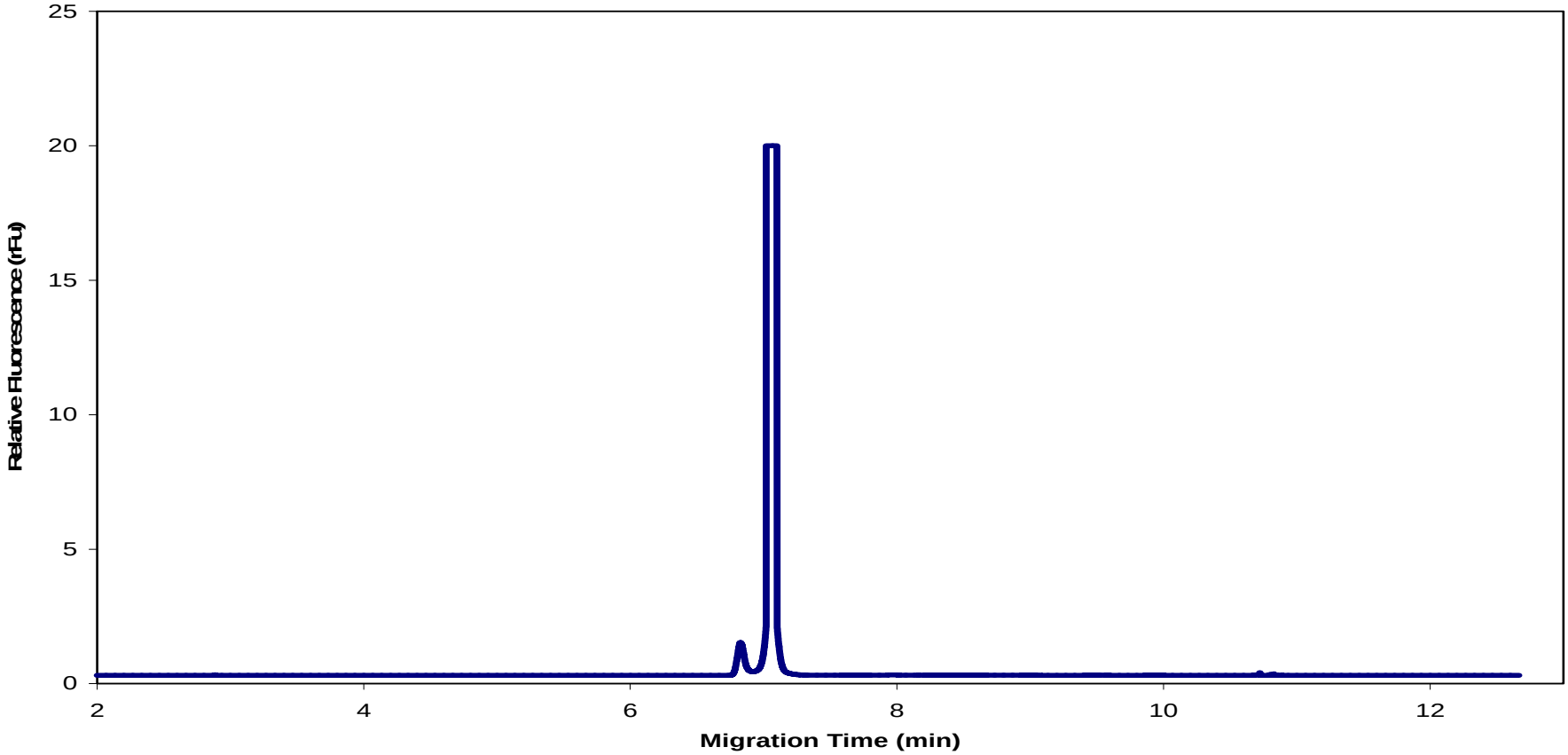


Figure 10. Electropherogram of 10^{-6} M AttoTag Fq GABA. Showing efficient labeling of amino acids. See Chapter 2 Separation Specifications for details.

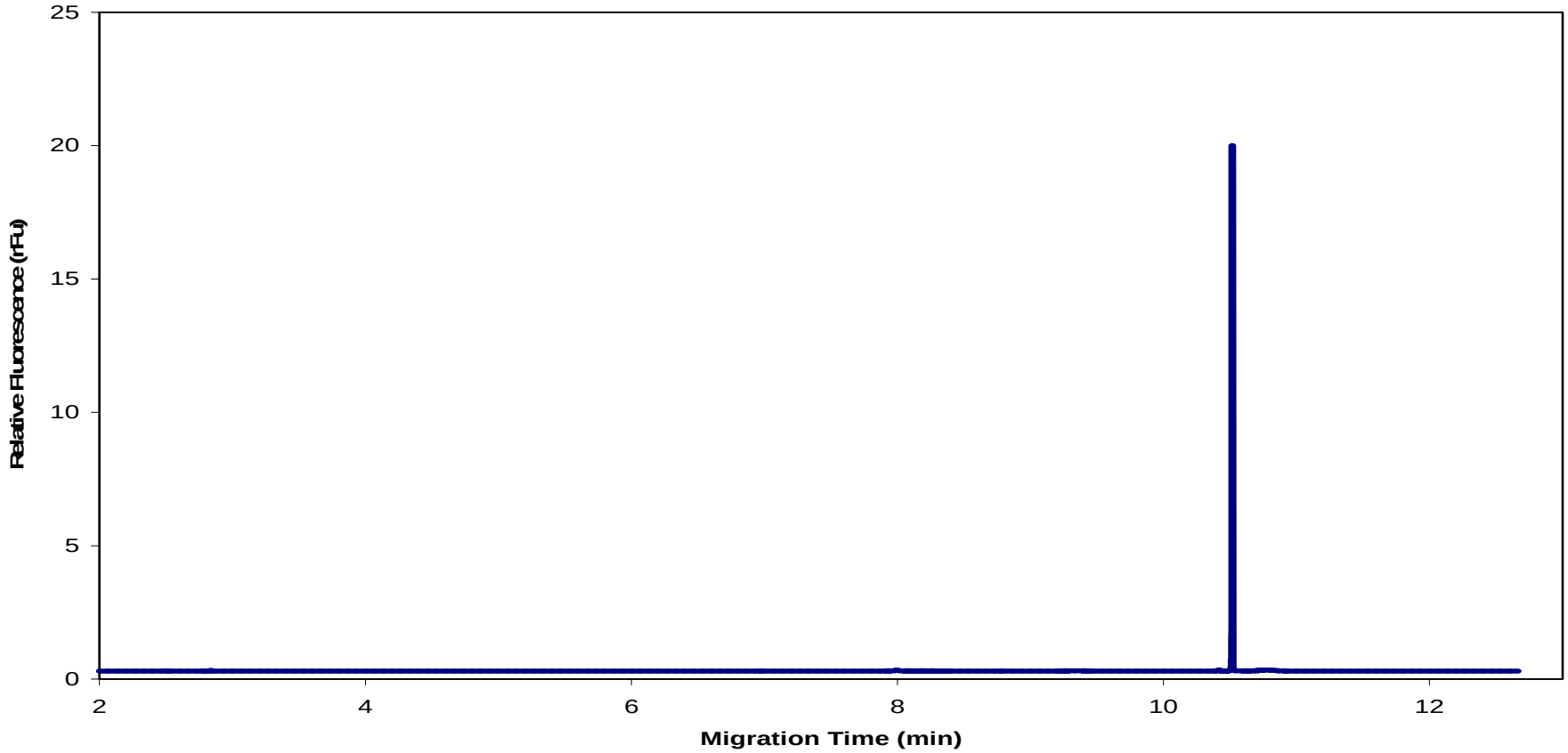


Figure 11. Electropherogram of 10^{-6} M AttoTag Fq Glutamate. Showing efficient labeling of amino acid. See Chapter 2 Separation Specifications for details.

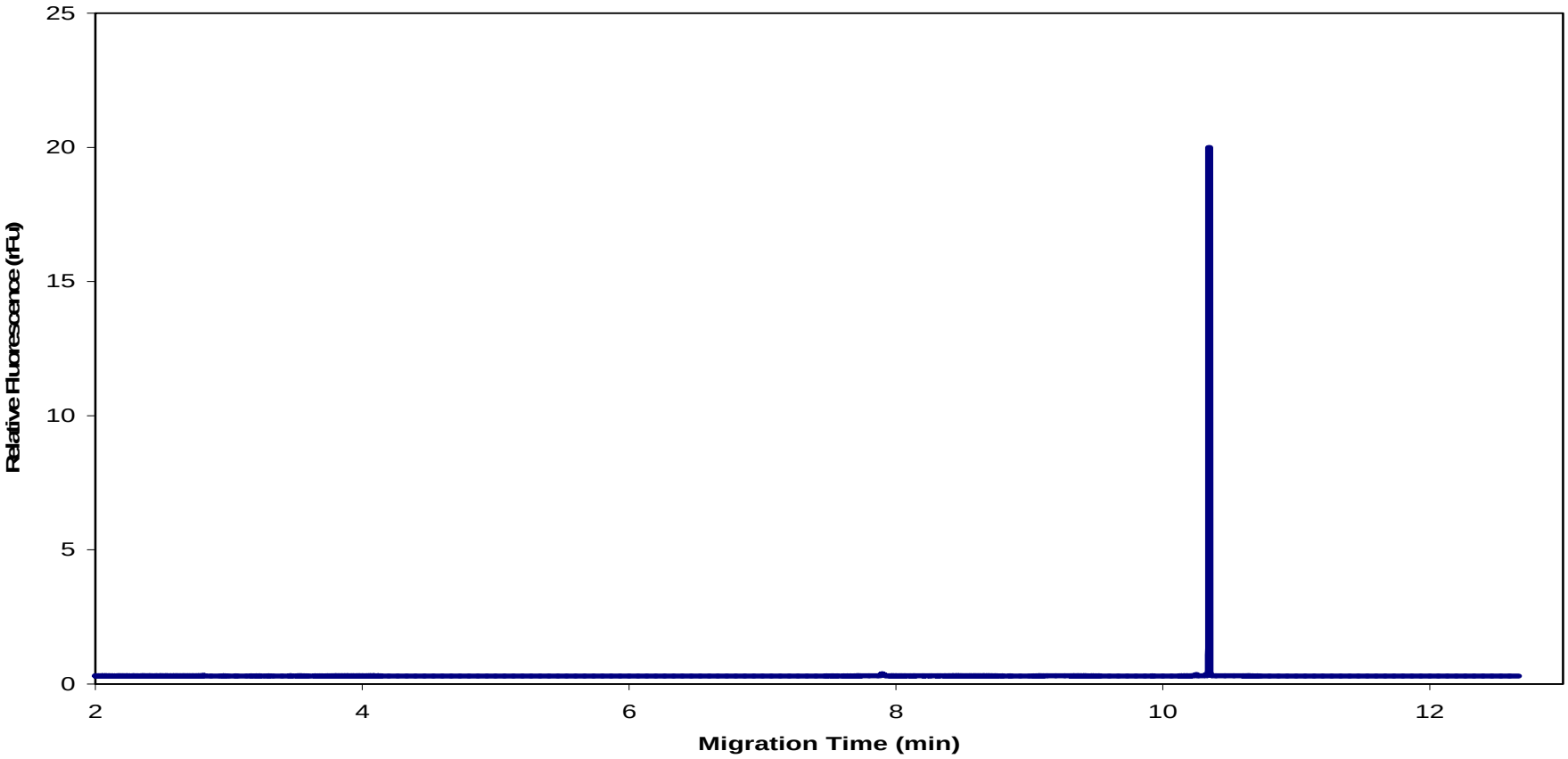


Figure 12. Electropherogram of 10^{-6} M AttoTag Fq L-Homoserine. Showing efficient labeling of amino acid. See Chapter 2 Separation Specifications for details.

In another portion of this study, derivatized proteins (with FITC and Atto-tag FQ) were individually studied using the cyano pentoxy biphenyl capillary. The proteins that were used are: albumin, β-galactosidase, carbonic anhydrase, myosin, and phosphorylase B.

First, proteins were derivatized with the FITC fluorophore. These samples were analyzed and the data can be seen in Figures 13-15. Detailed analysis data can be viewed in Table 4 for closer examination. The relative fluorescence (rFu) that is listed in this table represents the overall intensity of the peaks seen in the figures. Proteins have multiple derivatization sites; therefore, more than one peak should be observed in an electropherogram. These figures show this trend for each individual protein.

Table 4: FITC derivatized protein electropherogram analysis

Proteins		
Sample	**Relative Fluorescence (rFu)**	**Migration Time (min)**
β-galactosidase	0.449; 0.485; 0.512; 0.595; 0.472; 1.176; 0.602; 0.673	4.643; 4.991; 12.051; 12.388; 12.486; 13.195; 14.091; 14.431
Carbonic Anhydrase	0.572; 0.436; 0.459; 1.175; 0.959; 0.970; 2.015	4.639; 4.772; 5.138; 12.781; 13.740; 14.350; 14.557
Protein Mix	0.483; 1.728; 1.514; 0.716	4.81; 11.44;11.748; 12.662

In Figure 13, peaks occurring in the region of 12-14.5 minutes are due to β-galactosidase having different numbers of labels, because of the high quantity of lysines on the protein. The earlier migrating peaks are due to the less extensively labeled proteins

while later migrating peaks are due to the more extensively labeled ones. It is not clear whether each derivative was resolved, but from the distribution it is apparent that proteins with an intermediate number of labels were most abundant. The high efficiency (or sharpness) of the peaks is most likely due to the lack of organic solvent in the buffer and the protein remaining in the same conformation throughout the course of the separation. The presence of protein dimers, however has not been excluded and could account for these later migrating peaks. Peaks seen around 4.5 minutes are due to the unreacted FITC within the sample.

Similar results are seen for carbonic anhydrase in Figure 14. The peaks occurring in the region of 12-14.5 minutes are due to the protein having different numbers of labels, and the peaks seen around 4.5 minutes are due to the unreacted FITC.

As for Figure 15, which was a mixture of β-galactosidase and carbonic anhydrase, there are only three very distinct peaks towards the latter part of the electropherogram for the protein mixture (around 11-12.5 minutes). There are, however, the small peaks seen around 4.5 minutes that are also seen in the other electropherograms. This mixture was expected to represent both the β-galactosidase and carbonic anhydrases' individual analyses. However, the electropherogram for the mixture was simpler and the peaks do not correspond to the individual protein analyses. The simplicity of this electropherogram, compared to the two individual electropherograms, could be explained by interactions of the two proteins (i.e formation of heterodimers) that decreases the number of distinct analyte zones in the sample.

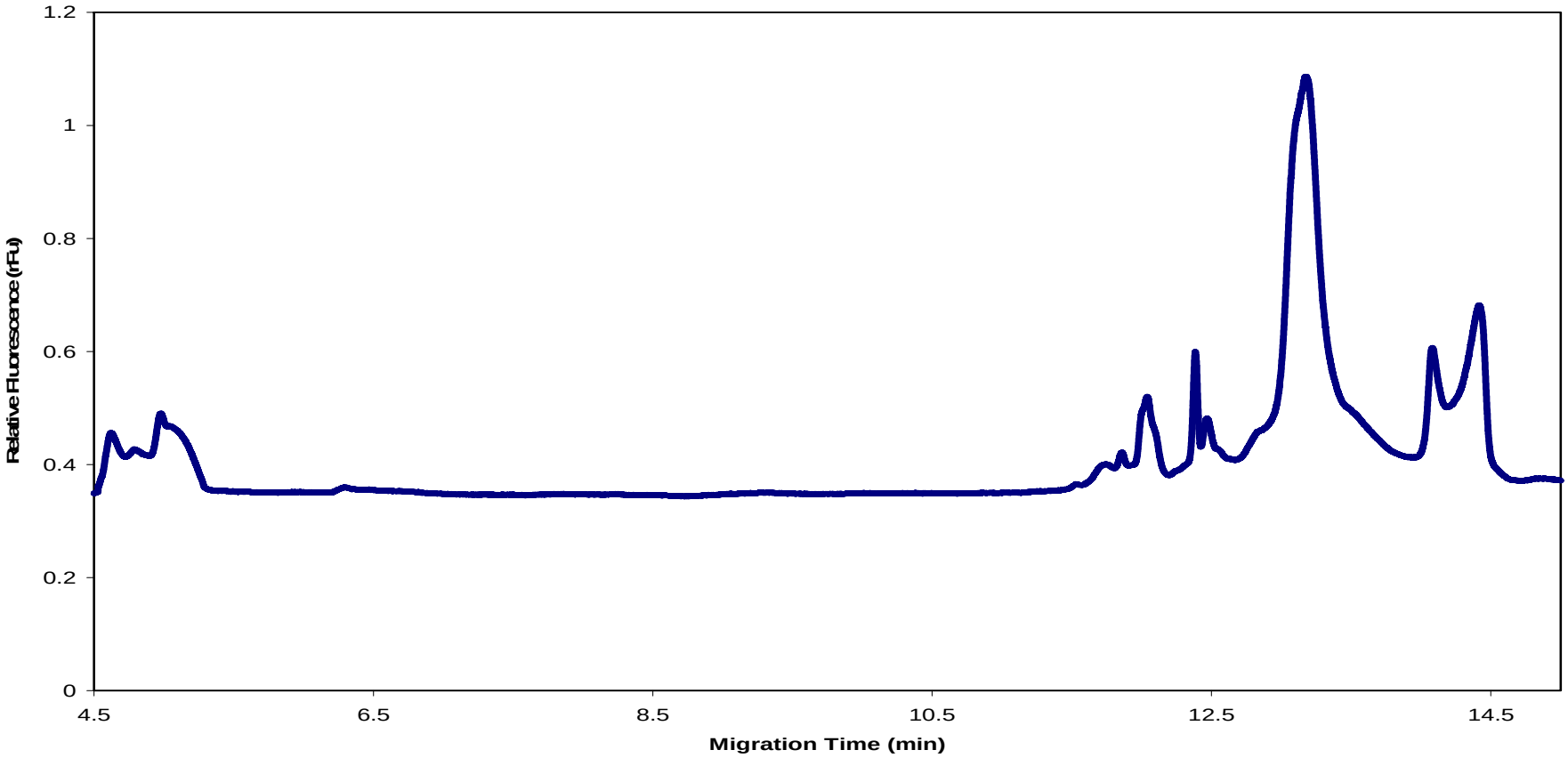


Figure 13. Electropherogram of 10^{-6} M FITC β-galactosidase. Showing multiply labeled sites for the protein. See Chapter 2 Separation Specification for details.

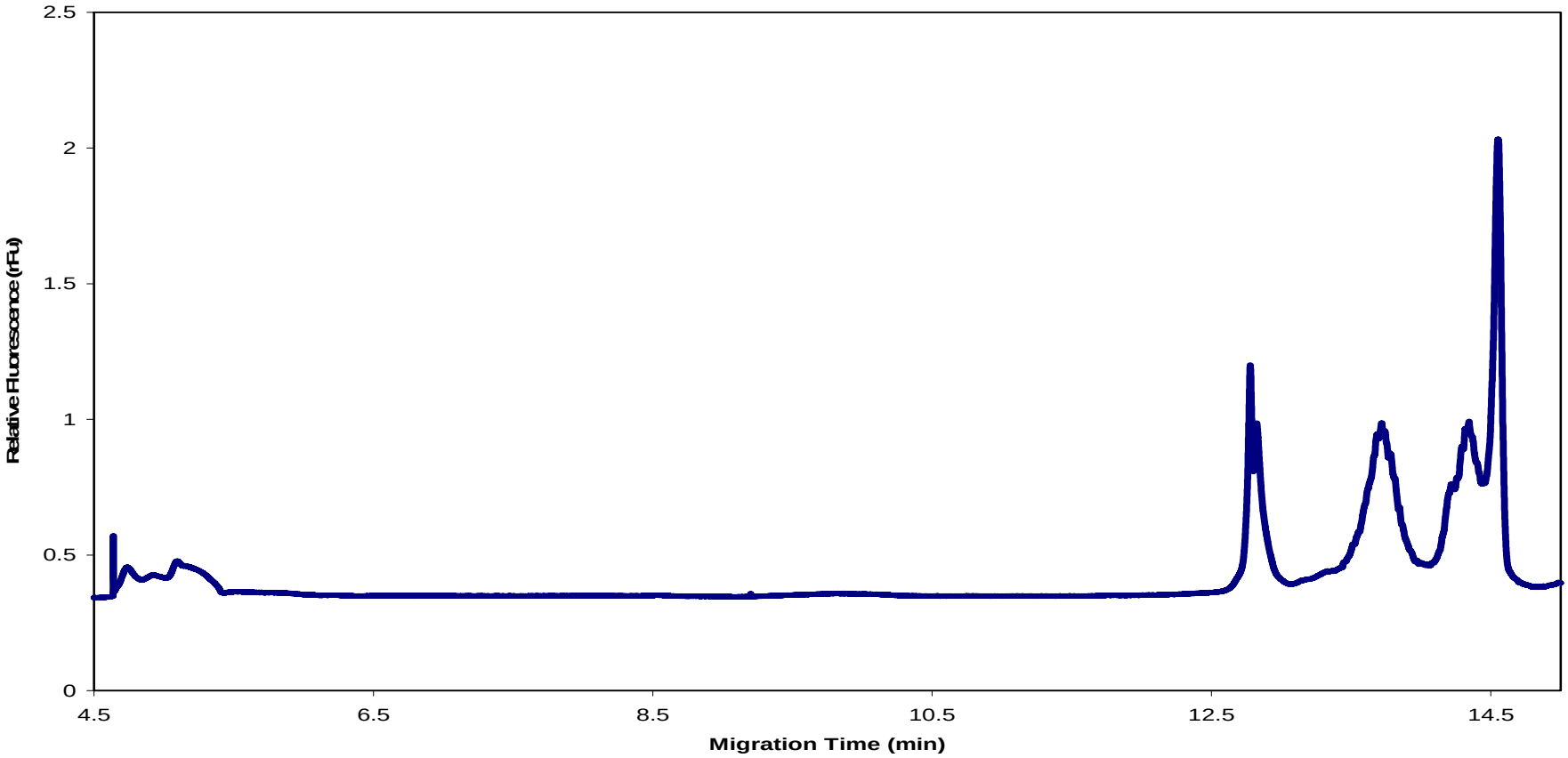


Figure 14. Electropherogram of 10^{-6} M FITC Carbonic Anhydrase. Showing multiply labeled sites for the protein. See Chapter 2 Separation Specification for details.

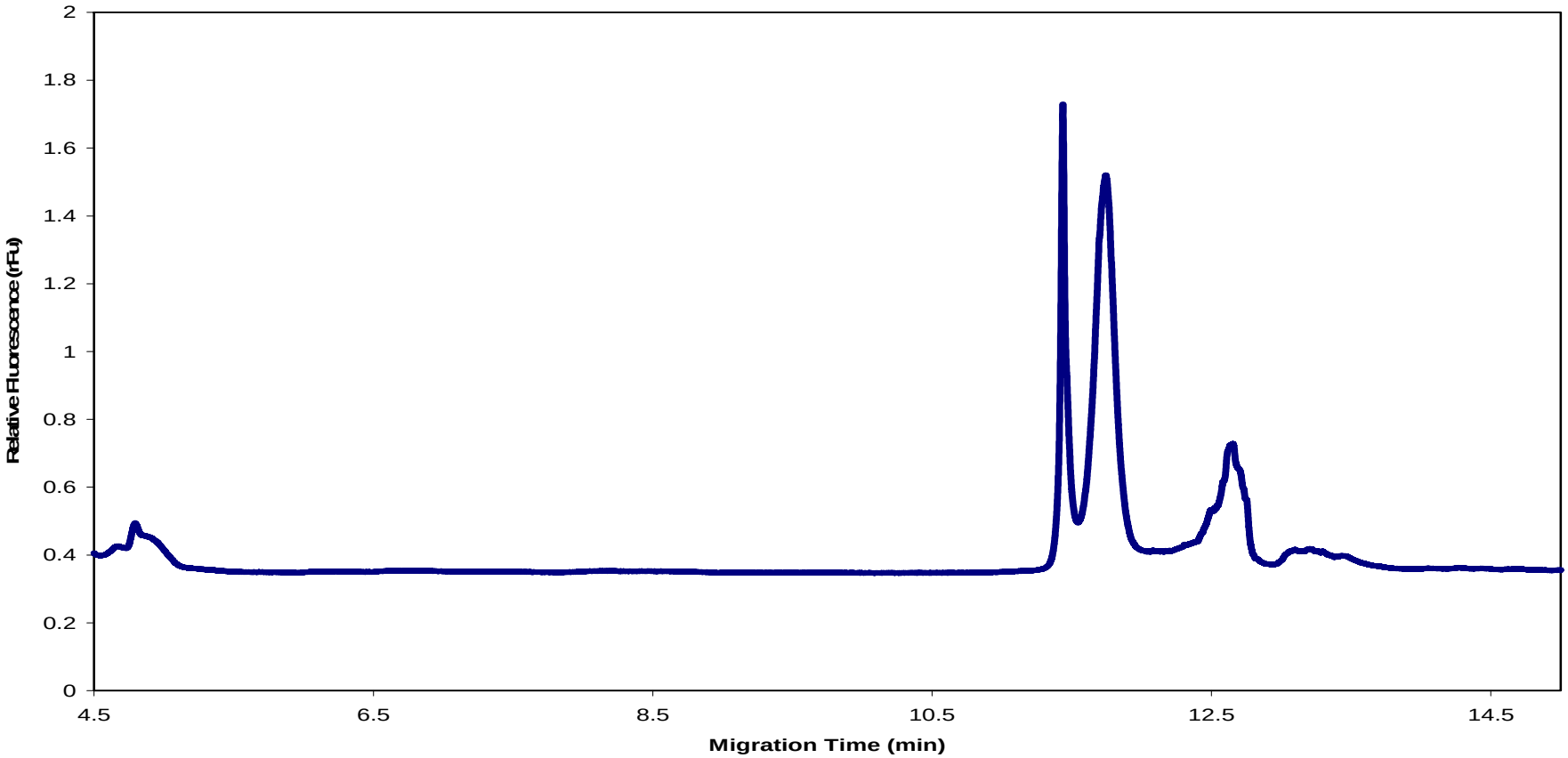


Figure 15. Electropherogram of 10^{-6} M FITC Protein Mix. Showing multiply labeled β-galactosidase and Carbonic Anhydrase mixture. See Chapter 2 Separation Specification for details.

Second, proteins were derivatized with Atto-tag FQ fluorophore. Each electropherogram can be seen in Figures 16-20 and detailed analysis in Table 5. Again the multiple peaks are seen showing that there is more than one labeled site within the protein as seen with the FITC derivatized proteins.

Table 5: Atto-tag FQ derivatized protein electropherogram analysis

Proteins		
Sample	**Relative Fluorescence (rFu)**	**Migration Time (min)**
Albumin	0.527; 0.568; 0.946; 0.752	6.35; 9.62; 12.36; 12.43
β-galactosidase	0.377; 0.477; 0.480; 0.426	6.71; 10.05; 12.87; 12.91
Carbonic Anhydrase	0.362; 0.478; 0.515; 0.478	6.87; 10.20; 13.32; 13.38
Phosphorylase B	0.340; 0.721; 0.873; 0.446; 0.384	7.56; 10.95; 14.07; 14.36; 14.55
Myosin	0.352; 0.485; 0.455; 0.425	7.17; 10.90; 14.38; 14.46

In Figure 16, peaks occurring at 12.36 and 12.43 minutes are due to the albumin. The other peaks are from amine-containing impurities present in the sample. The narrowness of the peaks for the albumin indicates that the protein is not changing conformation during the analysis. Often proteins do change conformation during the course of a separation using reversed phase HPLC due to the presence of a high concentration of organic solvent and high surface area of the stationary phase. In Figures 17-20, as with Figure 16, peaks occurring at later migration times are due to the proteins: β-galactosidase-12.87, 12.91 minutes; carbonic anhydrase-13.32, 13.38 minutes; phosphorylase B-14.36, 14.55; and myosin-14.38, 14.46. The presence of two peaks is

contributed to only two predominant forms of the derivatized protein that are present. Although, Figure 19 shows there are three distinct forms, the one with the fewest number of labels showed the highest fluorescence. This could be due to a high concentration of this derivative or less self-quenching within each molecule. Self-quenching occurs when multiple fluorescent labels on one protein quench each other.

For all of these protein separations, the peaks with similar migration times indicate a similarity in charge to size ratio under the conditions used.

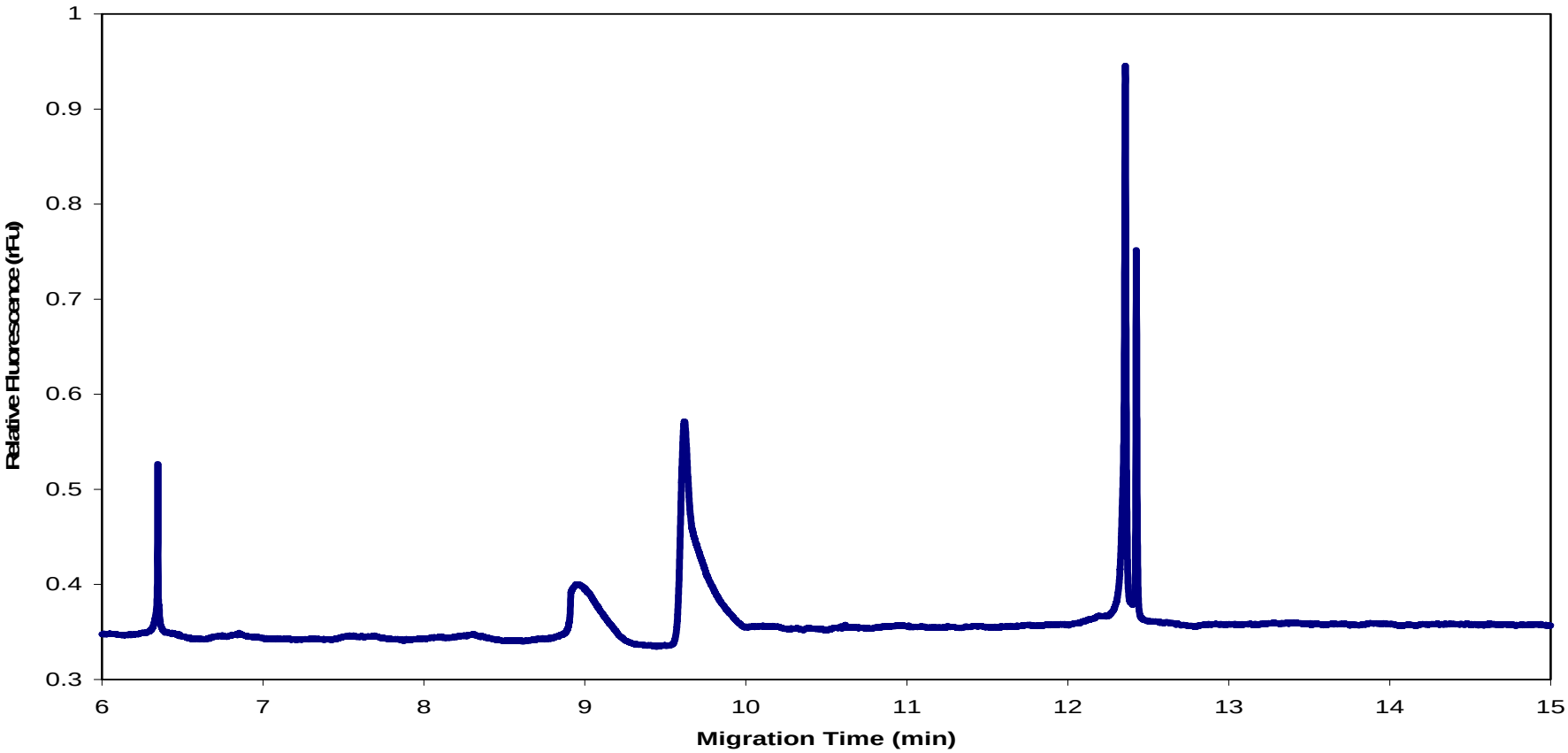


Figure 16: Electropherogram of 10^{-8} M AttoTag Fq Albumin. Showing efficient multiply labeled sites for the protein. See Chapter 2 Separation Specification for details.

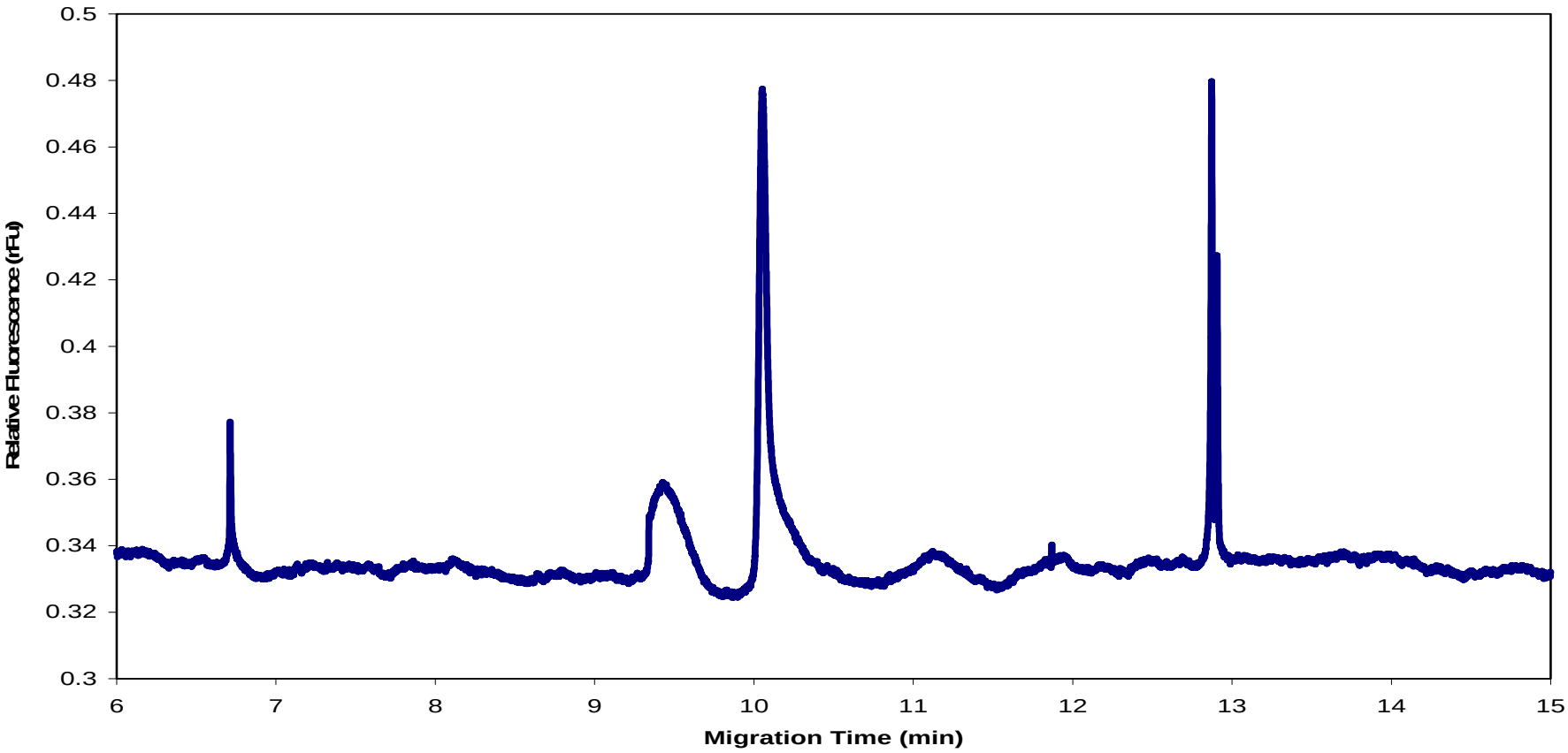


Figure 17: Electropherogram of 10^{-8} M AttoTag Fq β-galactosidase. Showing efficient multiply labeled sites for the protein. See Chapter 2 Separation Specification for details.

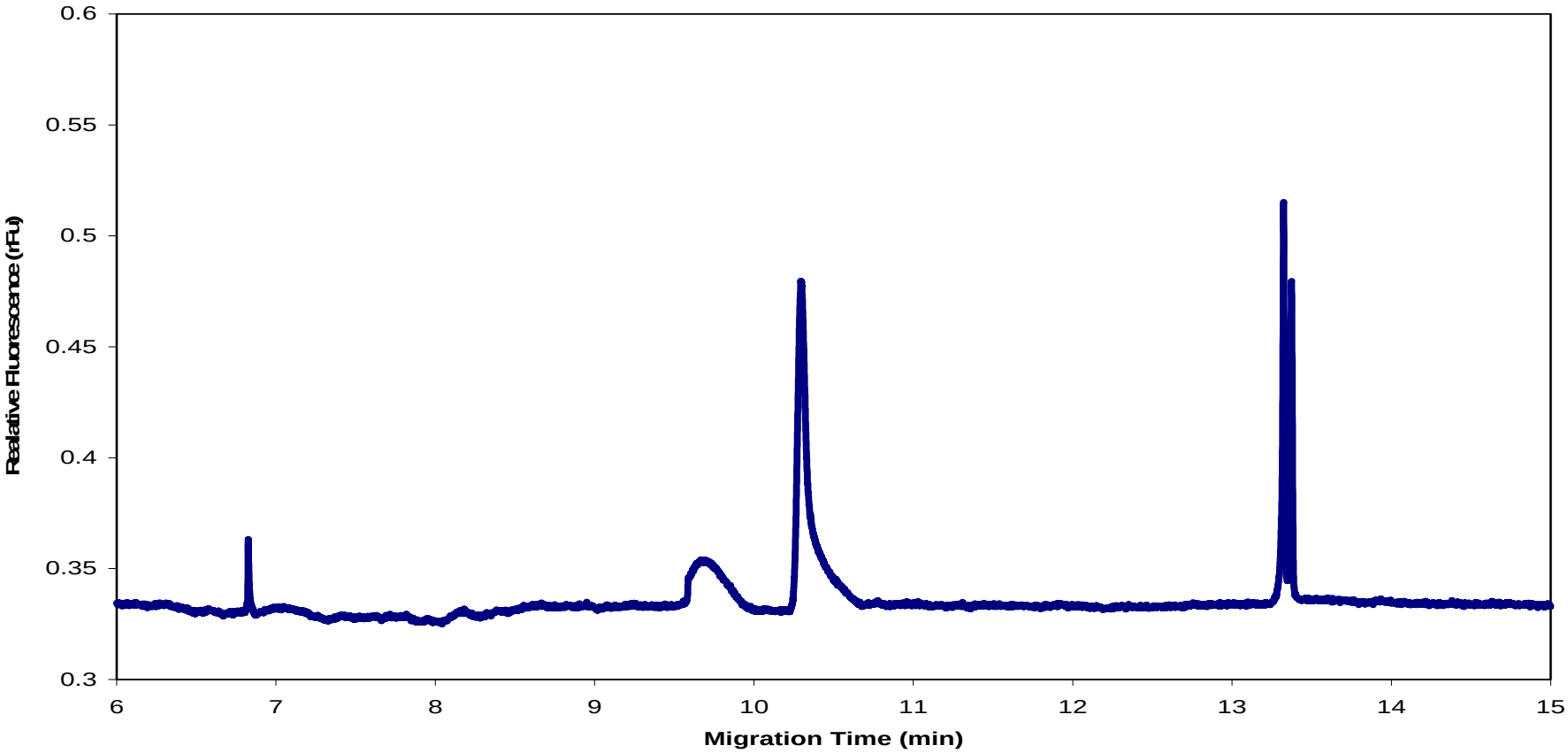


Figure 18: Electropherogram of 10^{-8} M AttoTag Fq Carbonic Anhydrase. Showing efficient multiply labeled sites for the protein. See Chapter 2 Separation Specification for details.

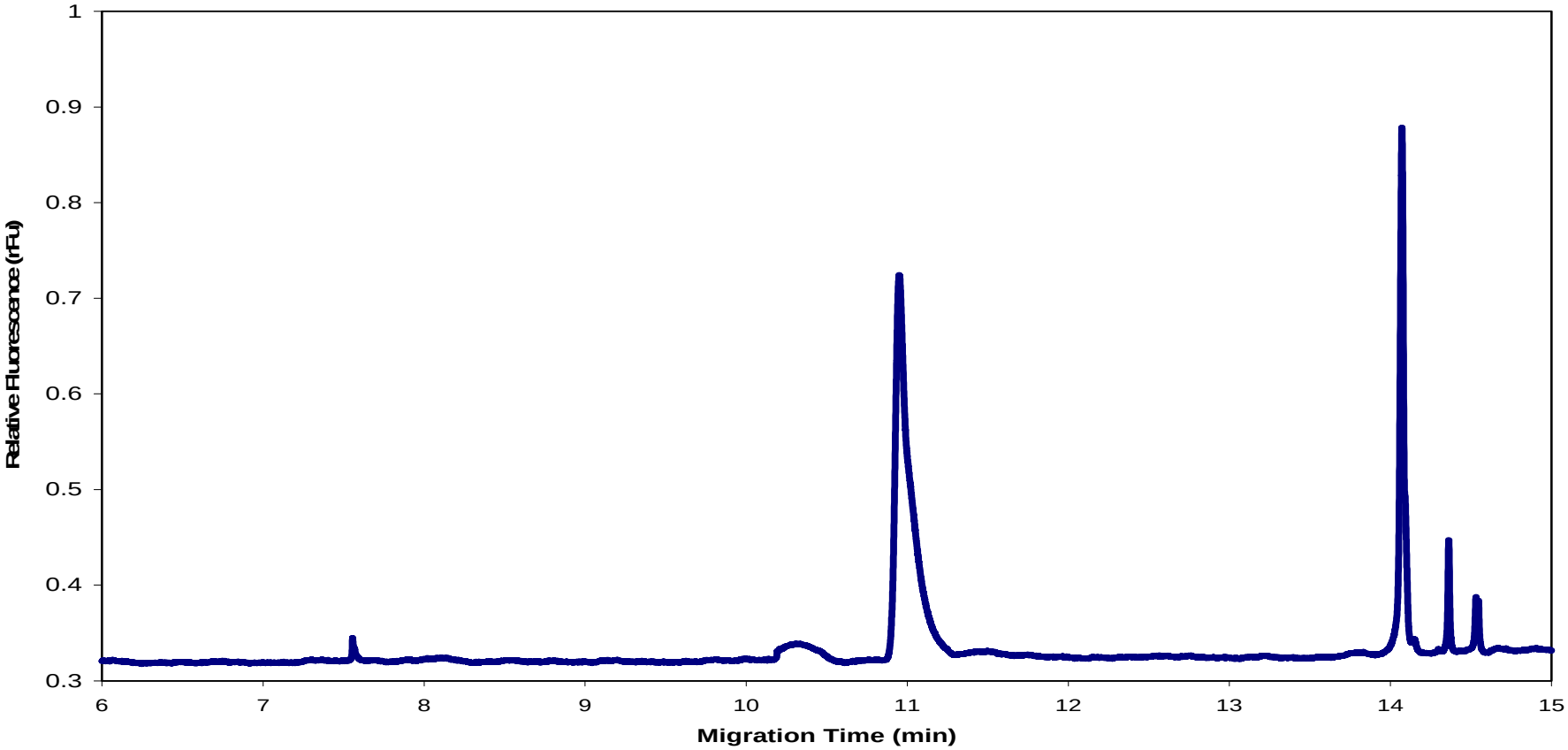


Figure 19: Electropherogram of 10^{-8} M AttoTag Fq Phosphorylase B. Showing efficient multiply labeled sites for the protein. See Chapter 2 Separation Specification for details.

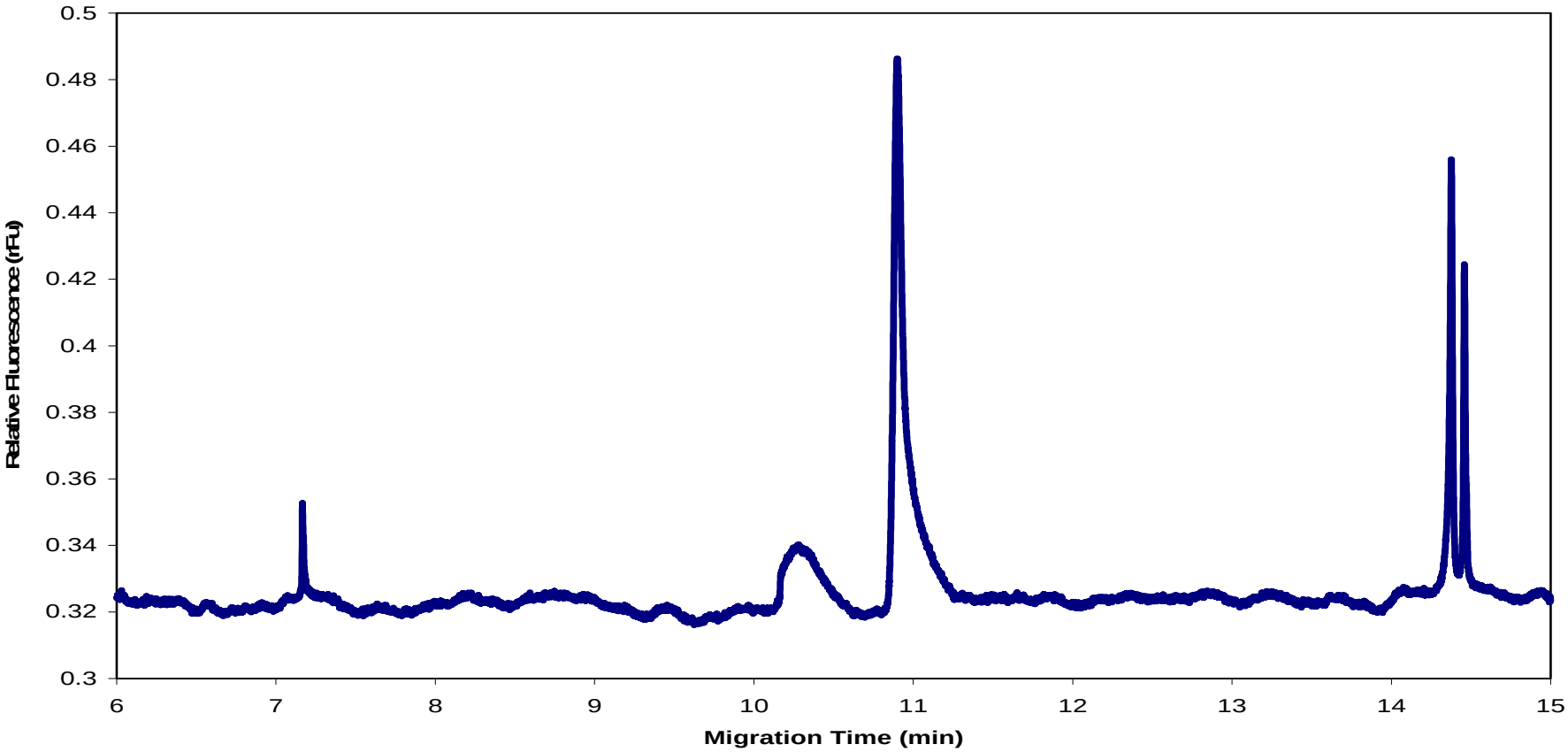


Figure 20: Electropherogram of 10^{-8} M AttoTag Fq Myosin. Showing efficient multiply labeled sites for the protein. See Chapter 2 Separation Specification for details.

Results and Discussion of Separation Optimization

After the optimization of the derivatization process of the amino acid and proteins were developed, the separations were then studied to find the optimal parameters for each. It is important to remember that proteins have multiple derivatization sites which can present multiple derivatives with different electrophoretic mobilities. Because of this fact, multiple peaks may be obtained; which is one disadvantage to the derivatization process.

The migration order is determined by the number of fluorophores attached to the molecule. The more neutral fluorophores attached to the molecule the longer the migration time of the molecule. Each attached fluorophore takes the place of a lysine that will be protonated under the separation conditions. Additionally, the small change in size of the protein, due to the attachment of the fluorophore, will be negligible and will not greatly affect the charge to size ratio. This technique looks only at fluorescence signals; therefore no positive identification of the individual signal is possible.

In the first stages of separation optimization, the electropherograms did not produce reliable data. In the case of amino acids, a 50:50 acetonitrile:phosphate buffer (pH 2.00) produced single peaks (in most instances); however, the baseline was not consistent. Figure 21 shows the analysis for glutamate when separated using a 50:50 acetonitrile:phosphate buffer (pH 2.00) background electrolyte. The baseline is very noisy with dips, somewhat like a valley. These dips most likely are caused by changes in the Raman scattering of the solution throughout the separation, usually ranging from 2 minutes to 4 minutes in length. Also, the signal for glutamate is weak; less than 0.5 rFu

for the tallest peak. When the 80:20 acetonitrile:phosphate buffer (pH 2.00) background electrolyte was used, each peak was well defined and has a strong fluorescence signal with constant baseline (as seen in Figures 10-12, 22).

Each amino acid, GABA, glutamate or L-homoserine, has been previously analyzed separately under identical conditions (see Figure 10-12 for individual separation electropherogram). When the electropherograms are overlapped for a visual comparison (Figure 22), the results show that each amino acid's electropherogram had a different retention time (seen in Table 2). In this comparison, the differences in the chemical composition of the amino acids can be seen by the differences in the migration time. Recall, the analytes are separated by size and charge. With the difference in chemical composition, the size of the amino acid is going to be different based on the compounds that form it. Also, the composition of the amino acid is going to determine the overall charge that is observed at the separation pH. The single peaks that are seen in these figures prove this separation technique works well with amino acids.

If we now examine the proteins that were derivatized by FITC and Atto-tag FQ we can compare how well the separation works with each fluorophore. For the FITC derivatized proteins, an attempt to use the acetonitrile:buffer ratio as a background electrolyte did not produce expected results as seen with the amino acids. These attempts produced wide peaks or had a very noisy baseline. The baseline was also not consistent. It continuously increases as the separation continues. Figure 23 shows carbonic anhydrase using 80:20 acetonitrile:phosphate buffer as the background electrolyte. The peaks shown are very weak, less than 0.5 rFu; and the entire process took 60 minutes.

The reason for this migration time is the low electrophoretic mobility of carbonic anhydrase using the 80:20 acetonitrile:phosphate buffer background electrolyte or increased interaction with the pentoxy biphenyl coating. When the phosphate:EDTA mixture was used, separations were better. Figure 24 examines all of the FITC derivatized proteins, β-galactosidase, carbonic anhydrase, and a mixture of the two proteins, run under the same conditions; by overlapping the electropherograms. The proteins exhibit different peaks with different migration times, but with the FITC derivatized proteins, the peaks are wide and asymmetric.

As for the Atto-tag Fq derivatized proteins, at first a phosphate buffer pH 7.00 was used as a background electrolyte. This did not produce any structured peaks and a very noisy baseline was present. When a 50:50 acetonitrile:tris formate buffer (pH 4.00) was used peaks could be seen, however, the valleys that were observed in the 50:50 mixture for the amino acids were present. These valleys occur directly before the fluorescence signal. Figure 25 shows carbonic anhydrase using this 50:50 mixture. The same was seen for the 70:30 acetonitrile:tris formate background electrolyte. It was decided that with the use of the acetonitrile, structured peaks were appearing; so possible use of the original phosphate buffer and the acetonitrile would produce the desired outcome. With a 50:50 acetonitrile:phosphate buffer (pH 2.08), a very noisy baseline was observed, but a single very sharp peak was seen in each protein separation. This can be seen in Figure 26 which shows a myosin separation using this 50:50 mixture. In both figure 25 and 26, the protein signal is very low, less than 0.4 rFu. The 80:20 acetonitrile: phosphate buffer (pH 2.00) yielded desirable results since it did produce multiple peaks

and distinct separations (Figures 16-19, 27). This high peak symmetry could be due to the less concentrated analyte bands in the Atto-tag Fq samples. The distinct peaks show that increasing ability to analyze the proteins is achieved on a biphenyl coated capillary using the Atto-tag Fq fluorophore.

When examining the analysis (Figure 27), each protein analysis yielded multiple peaks. Each peak is expected to represent one of the multiple labeling sites of that protein. The number of peaks seen is most likely a result of multiple labeling sites, and proves that some amines react, but some will not. Some of the peaks also show a small peak area and others show a larger peak area. This increase in peak area is due to the concentration difference of each derivative.

As for the reproducibility of this technique, one can turn to the data shown in Figure 28. This figure shows overlapping myosin separations derivatized with Atto-tag FQ, each performed on a cyano pentoxy biphenyl capillary under identical conditions. This result was obtained using a 50:50 pH 4.00 tris formate buffer: acetonitrile background, before optimization of separation conditions. For the purposes of discussing reproducibility, this analysis is beneficial. The structure of the peaks are relatively the same as well as the retention times for each separation. The retention time of the peaks are around 13.18 ± 0.0163 min. The only distinct difference in the peaks is the relative fluorescence. The relative fluorescence decreases from the first run at 0.40 rFu to 0.37 rFu in the second and third runs. Aside from this difference, it is evident that the reproducibility of this technique can be achieved with continuation of the separation optimization.

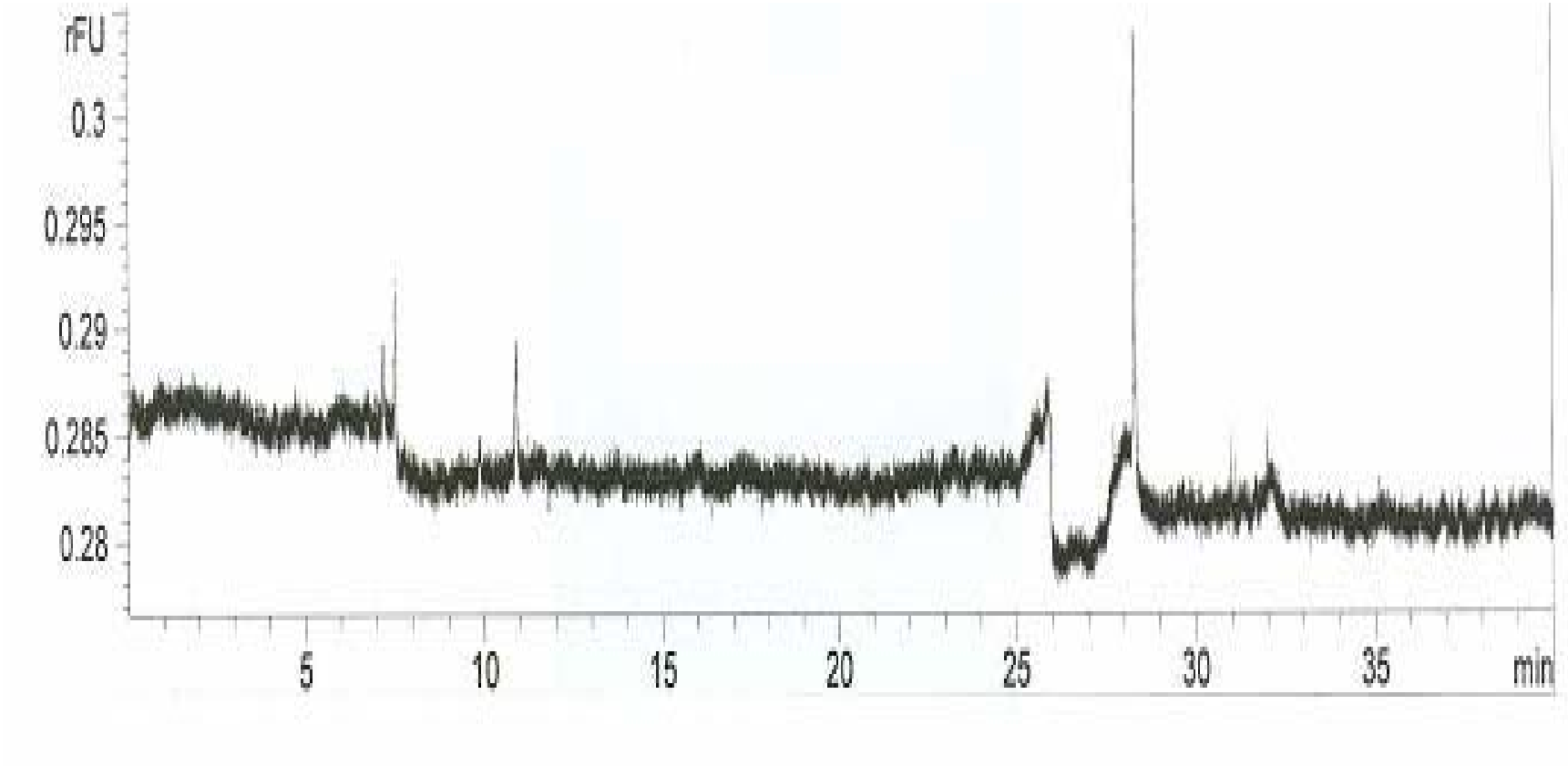


Figure 21. Electropherogram of 10^{-6} M AttoTag Fq Glutamate 50:50 Acetonitrile:phosphate buffer (pH 2.00). Showing initial separation of Atto Tag Fq labeled amino acid. See Chapter 2 Separation Specification for other details.

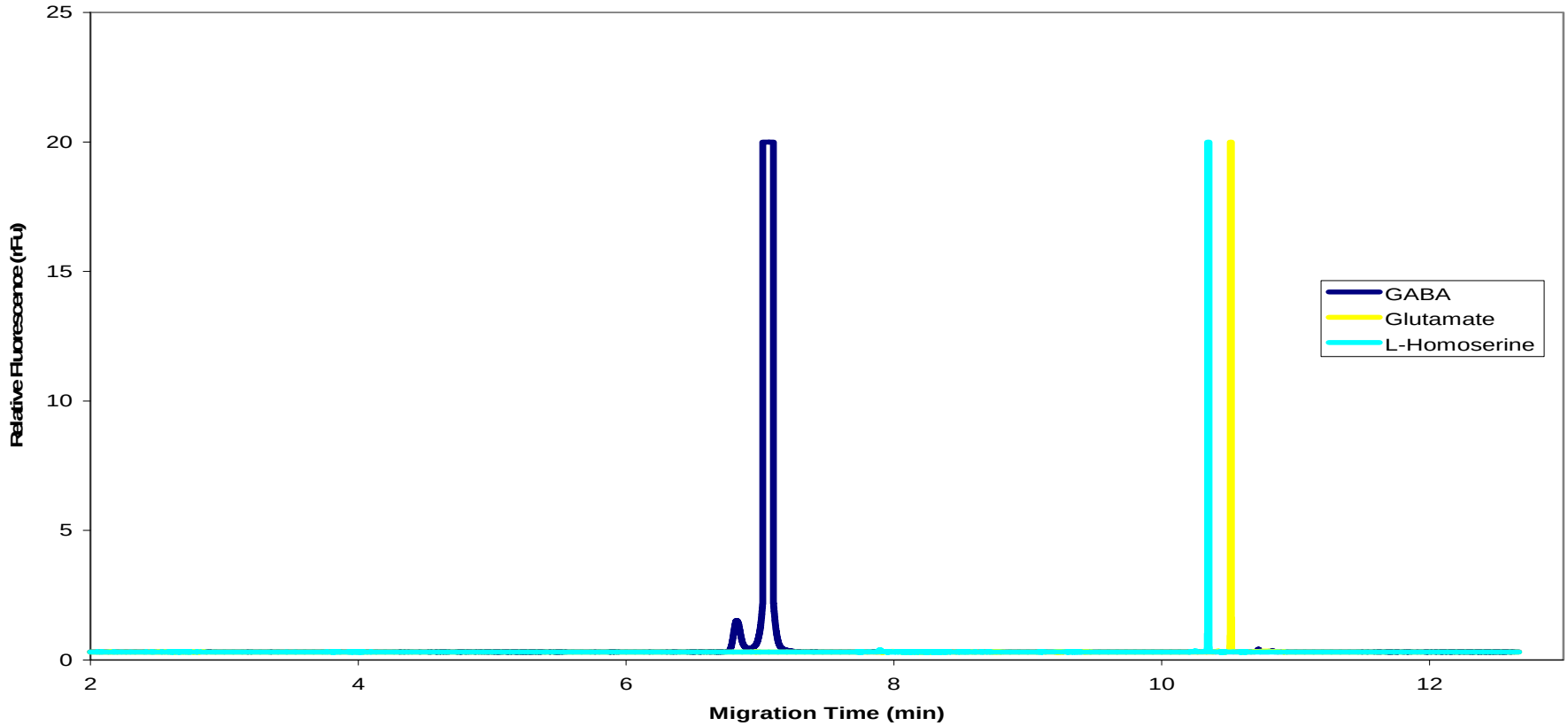


Figure 22. Electropherogram of overlapping 10^{-6} M AttoTag Fq GABA, Glutamate and L-Homoserine. Showing efficient individual separations of Atto Tag Fq labeled amino acids. See Chapter 2 Separation Specification for details.

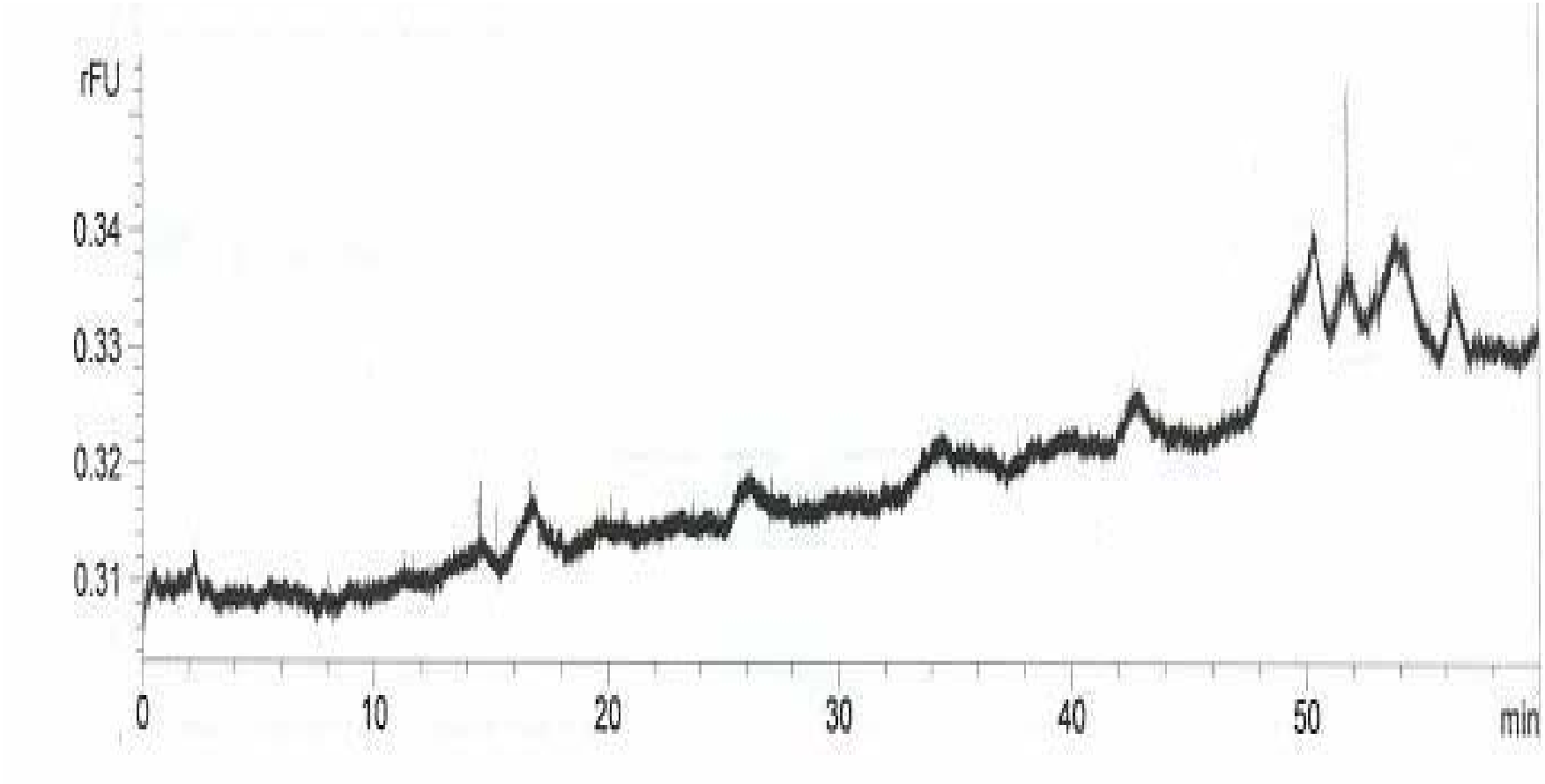


Figure 23. Electropherogram of 10^{-6} M FITC Carbonic Anhydrase 80:20 acetonitrile: phosphate buffer (pH 7.00). Showing initial separation of FITC labeled protein. See Chapter 2 Separation Specification for other details.

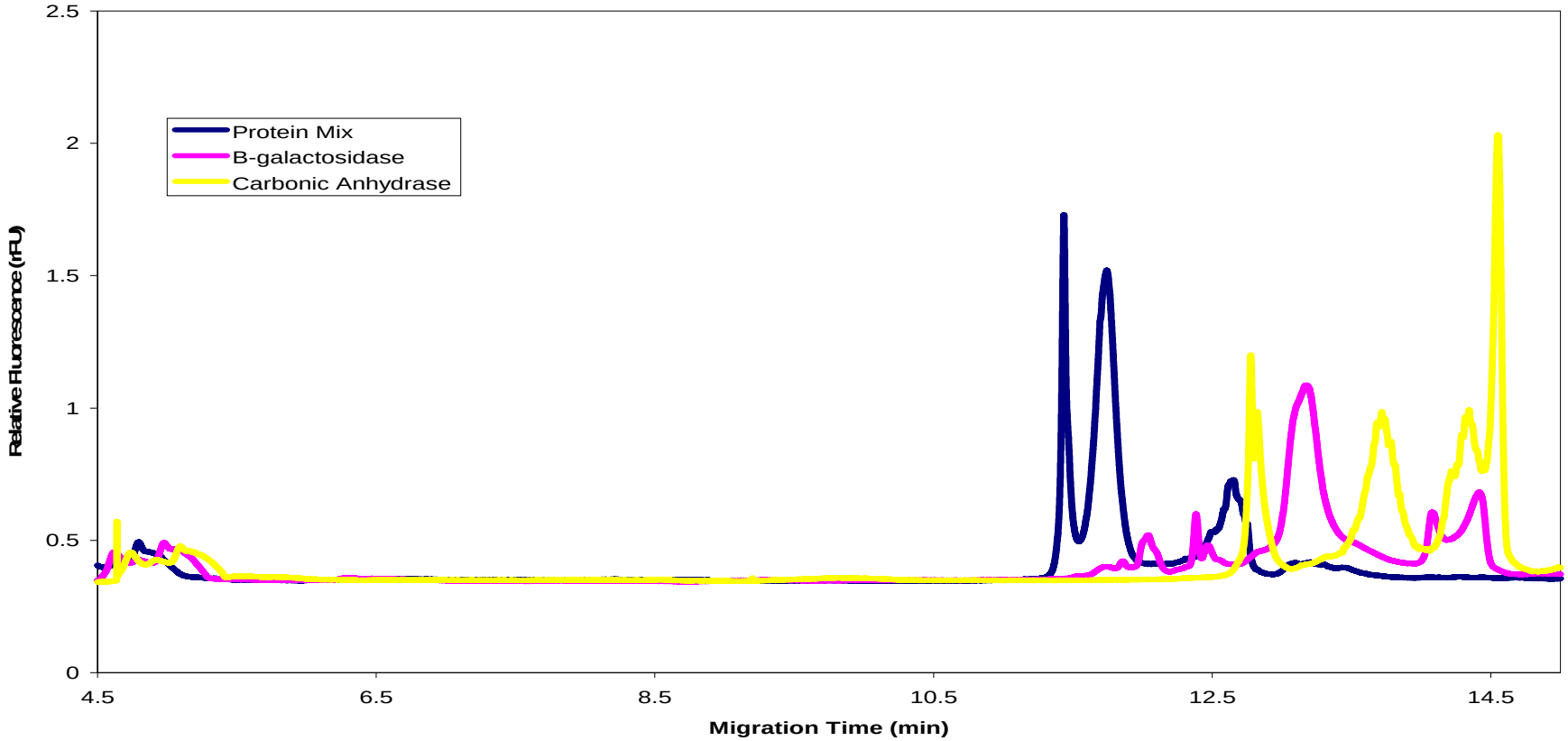


Figure 24. Electropherogram of overlapping 10^{-6} M FITC β-galactosidase, Carbonic Anhydrase and Protein Mix. Showing comparison of individual separations of FITC labeled proteins. Also showing inconsistency in mixture separation. See Chapter 2 Separation Specification for details.

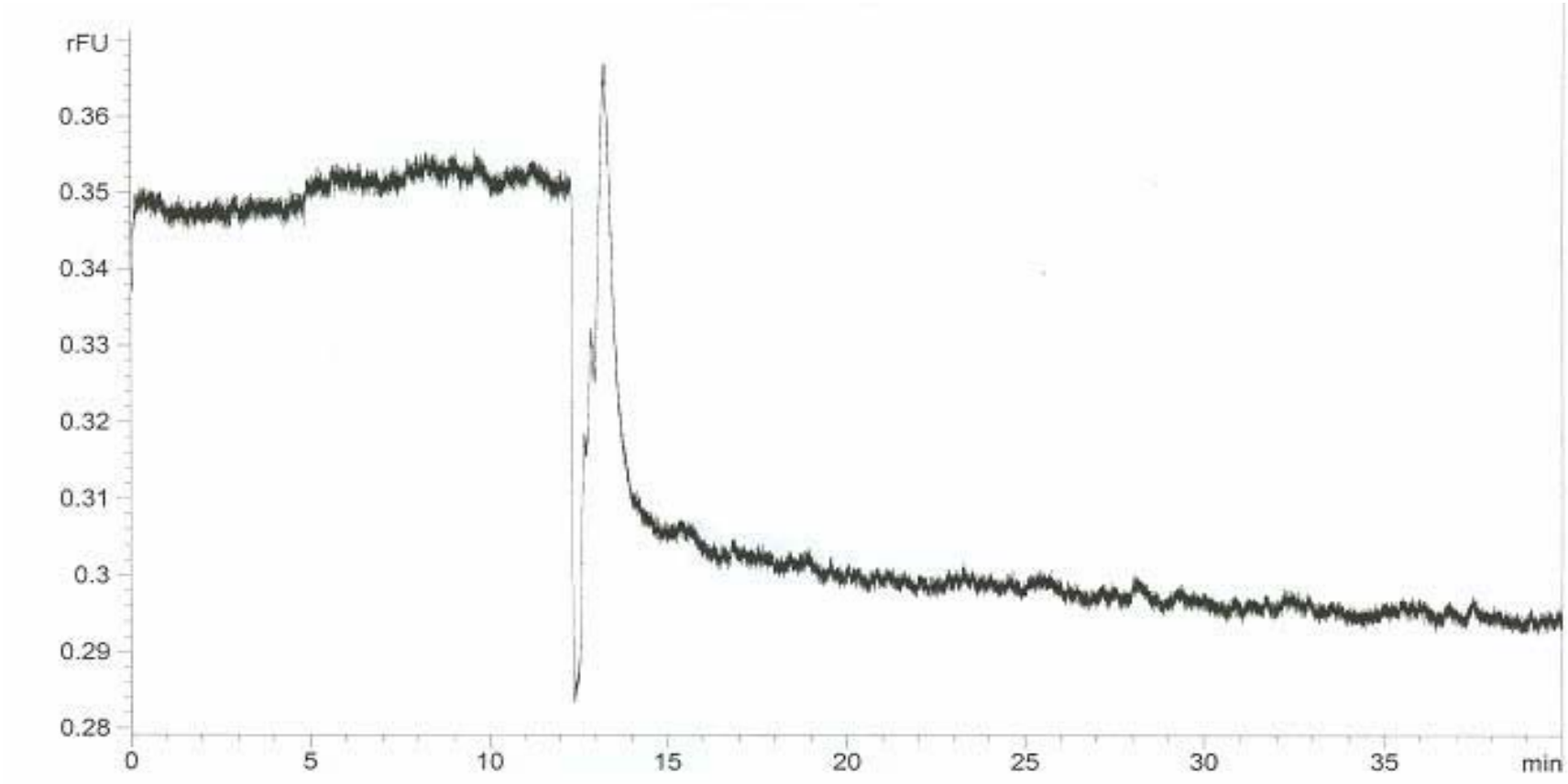


Figure 25: Electropherogram of 10^{-8} M AttoTag Fq Carbonic Anhydrase 50:50 acetonitrile: tris formate buffer (pH 4.00). Showing initial separation of Atto Tag Fq labeled protein. See Chapter 2 Separation Specification for other details.

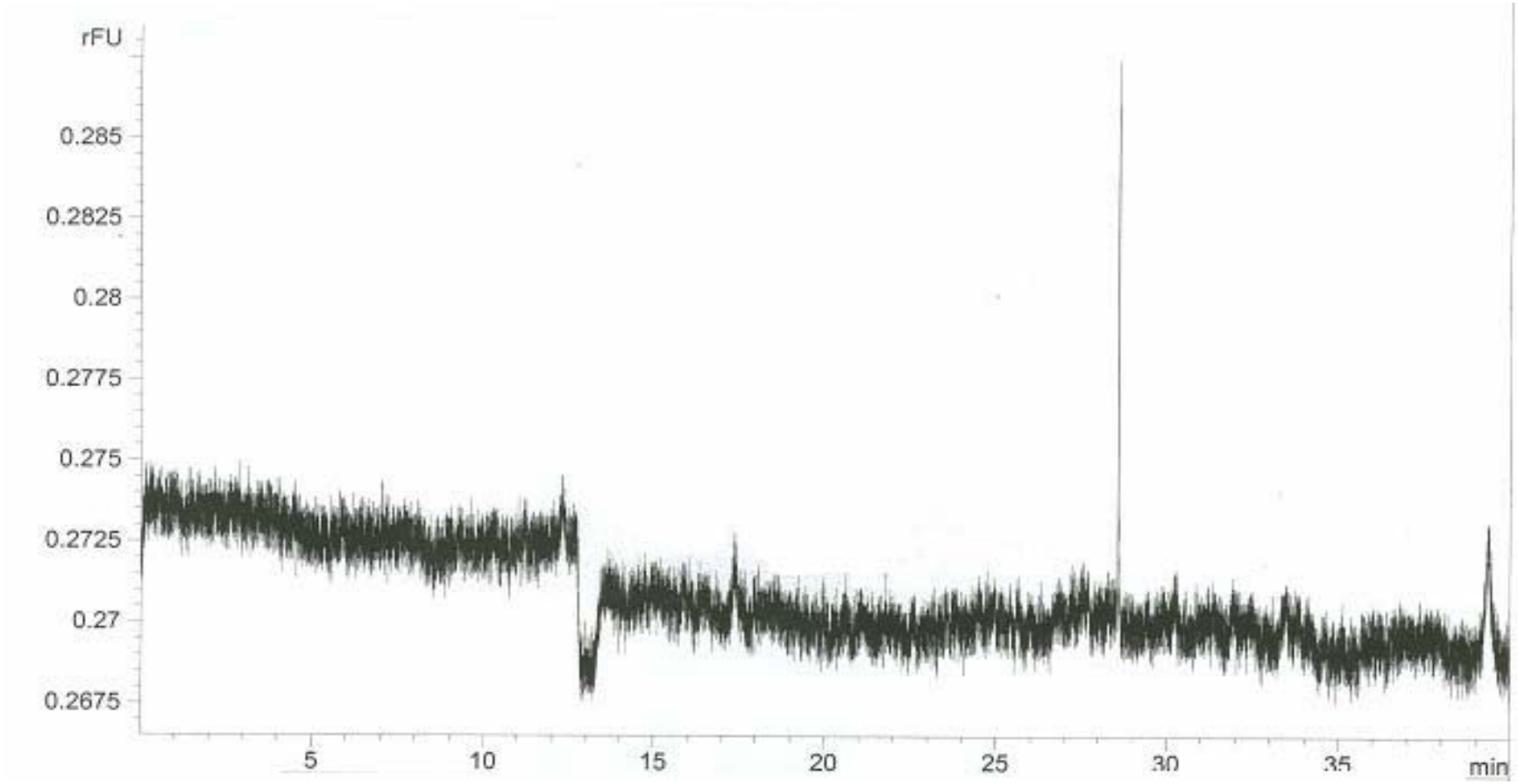


Figure 26: Electropherogram of 10^{-8} M AttoTag Fq Myosin 50:50 acetonitrile:phosphate buffer (pH 2.08). Showing second stage for separation of Atto Tag Fq labeled protein. See Chapter 2 Separation Specification for other details.

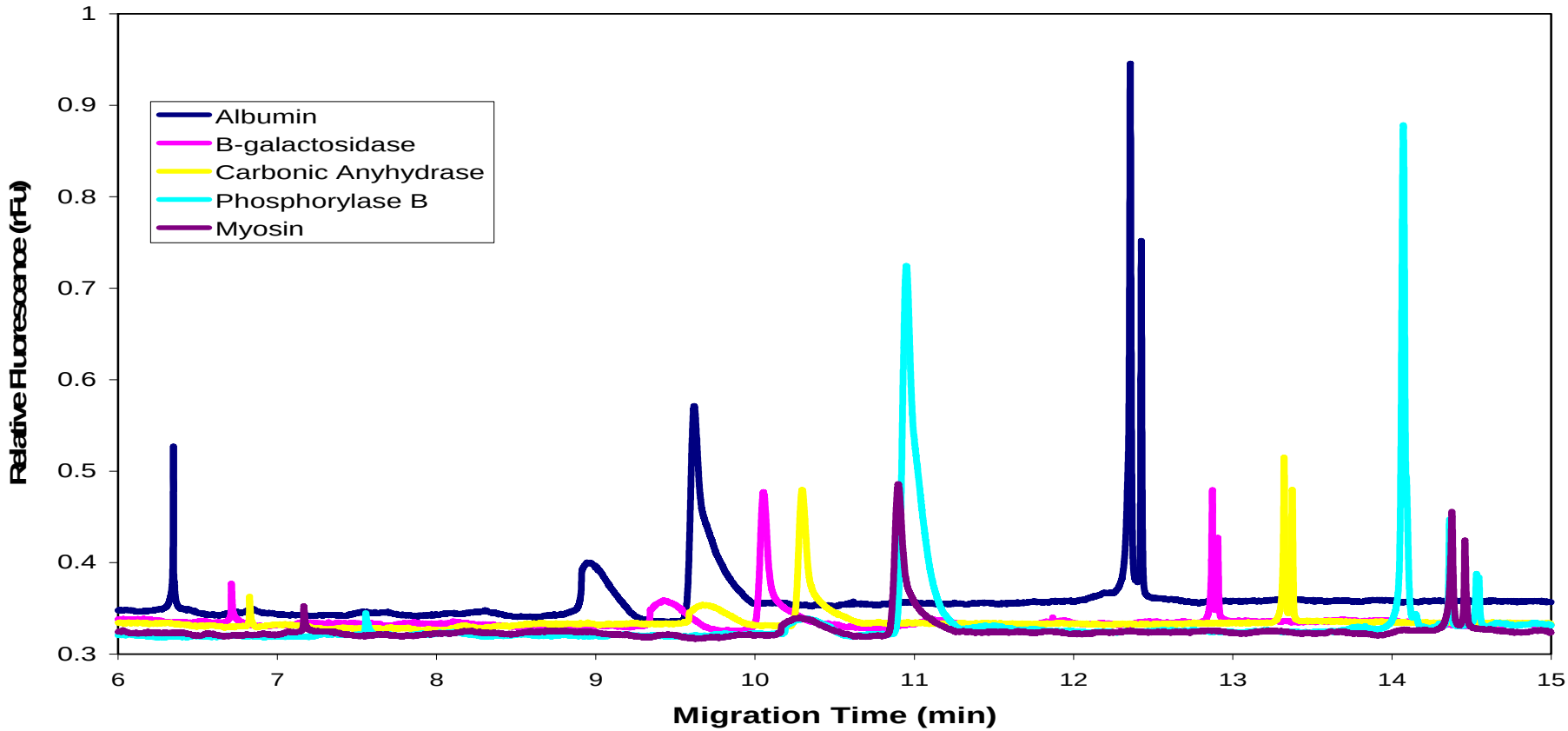


Figure 27: Electropherogram of overlapping 10^{-8} M AttoTag Fq Albumin, β-galactosidase, Carbonic Anhydrase, Phosphorylase B, Myosin. Showing comparison of individual separations of multiply labeled Atto Tag Fq proteins having different migration times. See Chapter 2 Separation Specification for details.

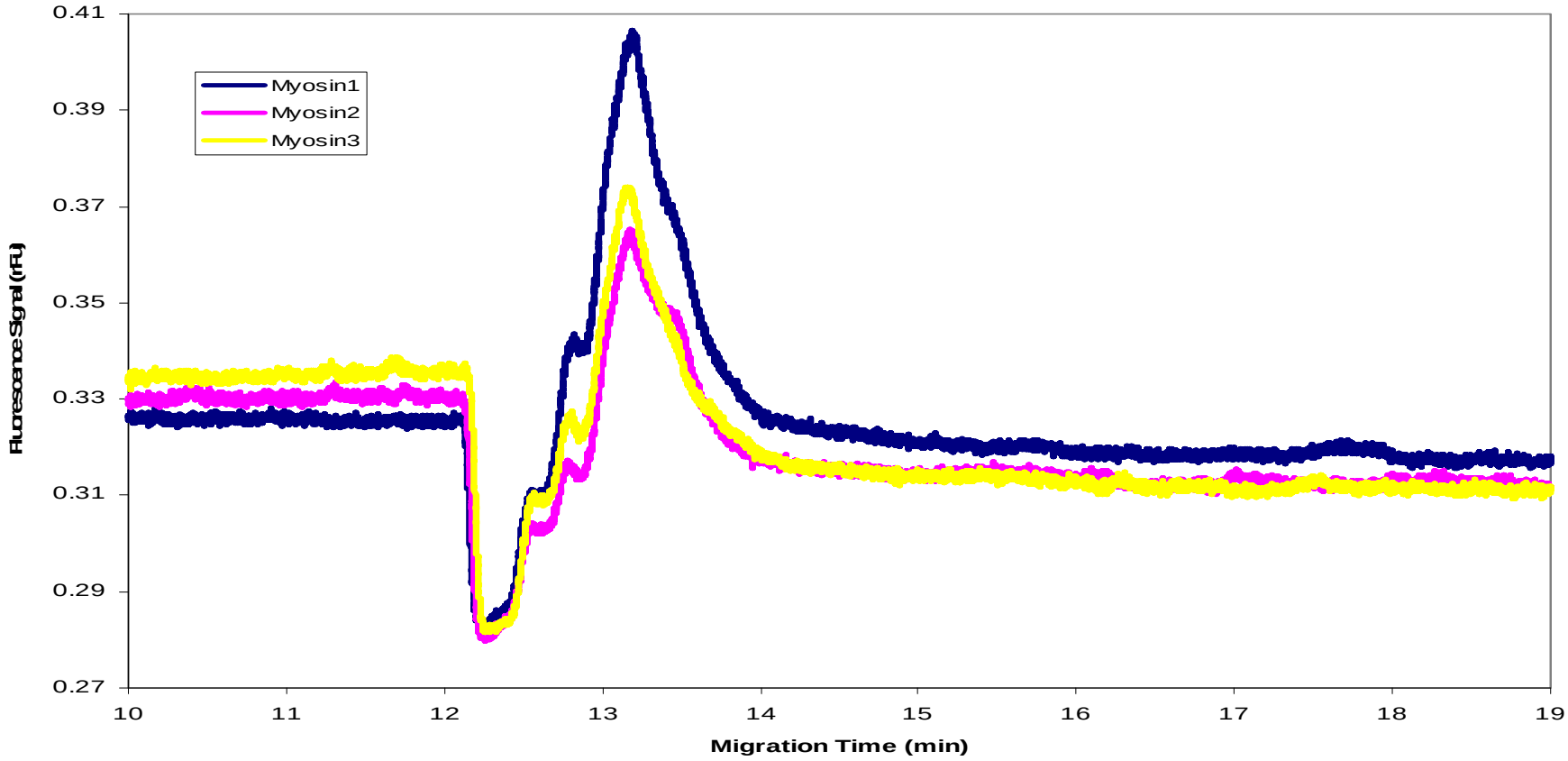


Figure 28: Electropherogram of overlapping 10^{-8} M AttoTag Fq Myosin separations. Separations with 50:50 tris formate: Acetonitrile. Showing comparison of individually separated AttoTag Fq labeled myosin. Reproducibility of separation. See Chapter 2 Separation Specification for details.

Conclusion

The derivatization of proteins and amino acids is essential for the detection by LIF. The purpose of this project was to optimize the separation and detection of derivatized proteins and amino acids. Both FITC and Atto-tag FQ were used in the derivatization process. The importance of the derivatization procedure is to make sure that the amino groups in the protein or amino acid are labeled with the appropriate fluorophore.

When examining the effects of each type of fluorophore, it is important to remember that FITC is fluorescent without having to be reacted with an amine, unlike Atto-tag FQ. Therefore, when separations are performed using FITC, some residual fluorescence is observed. The residual fluorescence is observed at the same retention time throughout the electropherograms.

Peak identification entails the use of other modes of detection coupled to CEC-LIF, such as MS or MALDI-TOF-MS (Matrix-assisted laser desorption/ionization-Time of flight-Mass spectrometry). MS will provide structural information to identify unknowns in solution and also provide molecular weight determinations. MALDI-TOF-MS will allow for mass ratio determination and primary sequencing of the structures. With these different types of methods, there is the ability to identify the individual peaks seen on the electropherograms.

The overall expectations of this project were to determine 1) which of the two fluorophores used (Atto-tag FQ or FITC) would be the better fluorophore for the derivatization of proteins and amino acids; 2) if OTCEC would to prove to be useful for

the separation of proteins and amino acids; 3) if the biphenyl stationary phase was going to work as the stationary phase in this separation. Here in this chapter, the analysis of proteins and amino acids provide evidence to support this technique.

The amino acid analysis using the Atto-tag FQ derivatization process, proves to work with this technique. The comparison of Figures 21 and 22 shows the resolution, asymmetry and efficiency were improved by using the Atto-tag FQ reagent. Moreover, the excess reagent from the derivatization was not fluorescent and did not add to the complexity of the electropherogram. Electrophoretic analysis of proteins derivatized with this reagent showed strongly intense and narrow peaks at different retention times which is easily seen in this figure.

Capillaries modified with biphenyl stationary phases were capable of resolving amino acids and proteins derivatized with the fluorophores. The recorded peaks were highly symmetrical indicating a low degree of wall adsorption. Additionally, the open tubular method showed a high reproducibility for the migration time of myosin.

OTCEC is proving to be a strong competitor in the separation of amino acids and proteins. Etched capillaries coated with a biphenyl stationary phase show promise for the resolution of fluorescent derivatives of proteins and amino acids. However the data retrieved from performing separations on a protein mixture did not produce results that to contribute to the use of this technique for the separations. This could be further explored through further optimization of the derivatization and separation technique so that only one label is attached and only one peak is visible in the electropherograms. Recall that this technique does not allow for the exact identification of an individual peak. With the

ability to attach a single label, thereby simplifying the electropherograms, will allow for a better comparison of the individual proteins.

The use of OTCEC using the modified capillary with biphenyl crystals, along with LIF, does proved many promising aspects that allow for the use of this technique for the study of proteins and amino acids. This technique allows for a low detection limit that is needed. Also, the improvement of the efficiency of the separations contributes to a uniform separation allowing for a better comparison.

CHAPTER III

CONCLUSION

The purpose of analyzing proteins and amino acids is to gain insight into the information they carry. More specifically, proteins are analyzed in the hope of gaining knowledge into disease processes. The ability to detect these small changes seen in disease processes, as compared to the original protein, in the early stages of a disease allows scientists to better treat these diseases.

In the work described in this thesis, the main focus is to optimize the separation process to better detect proteins at very low concentration. Here OTCEC-LIF using a modified etched capillary with a cyano pentoxy biphenyl stationary phase was employed. To start the optimization process, the derivatization process was performed. In performing these tests, two different fluorophores were used. However, both fluorophores are amino reactive and react with the lysines and the N-terminus of proteins. As for Atto-tag FQ, this fluorophore is not fluorescent until it reacts with the amine, and therefore, no residual fluorescence should be observed. This was the case, and Atto-tag FQ proved to be a better choice for the derivatization process, over FITC, because of the intense and narrow peaks.

The actual separations using OTCEC-LIF using the Atto-tag FQ derivatized proteins and amino acids were the last and final stage of this work. As was stated very early on, chapter 1, there are three important parameters that must be utilitized. The

separations of the proteins show high efficiency, good retention and provide resolution of the derivatized proteins and amino acids.

In the future, the issue of minimal qualitative information in the technique will be addressed. This information will be gained through coupling the separation with another analytical method, electrospray or MALDI mass spectrometry. Although much information can be gained through the coupling with MALDI mass spectrometry; it does present its set backs. Recall mass spectrometry requires a higher concentration for analyses. Field-amplified sample injection could be used to pre-concentrate the protein or amino acid sample prior to analysis.[33-34] When CEC is coupled to mass spectrometry molecular weight and structural information can be obtained allowing for the exact determination of each individual peak in a separation. This contributes to the continuing efforts in making OTCEC-LIF a widely used proteomics technique.

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