INTACT PROTEIN DETECTION, SEPARATION, AND QUANTITATION USING LIQUID CHROMATOGRAPHY - TRIPLE QUADRUPOLE MASS SPECTROMETRY

by

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Abstract

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There is an increasing demand for protein detection and quantitation in biological fluids for disease detection, protein therapeutics monitoring, and drug development response control. Current methods use highly sensitive and specific triple quadrupole mass spectrometry (QqQ-MS) to quantify protein digest peptides to predict original intact protein concentrations from the sample. The bottom-up protein quantitation format requires protein digestion which is often incomplete and can introduce errors into protein quantitation methods. Therefore, absolute protein quantitation is impossible without including expensive isotopically labeled protein standards. In our lab, a method that bypasses the protein digestion step to directly quantify intact protein on QqQ-MS was developed. Myoglobin, cytochrome c, lactalbumin, lysozyme, and ubiquitin were used as protein standards to prove the principle. An intact protein quantitation method was developed on a Shimadzu LCMS-8050 QqQ-MS using multiple reaction monitoring (MRM) mode. MRM transitions for all protein standards were developed and calibration curves were obtained with respectable linearity (R^2>0.99). To address the complex biological matrices, a generic reversed-phase chromatography method was developed. Retention characteristics on C4, C8, C18, Biphenyl, and PFP Propyl stationary phases were evaluated. Prostate specific antigen (PSA) was also included in the study to prove

the feasibility of the method being used for biomarker discovery and quantitation. Specificity of the MRM detection was evaluated for urine and plasma matrices. To more fully investigate gas phase interactions in a triple quadrupole instrument, ion scattering effects, mass resolution filters, and proton transfer were investigated and could be used to optimize future applications. The intact protein quantitation method using QqQ-MS was designed as a model for future development of targeted methods, especially for clinical diagnostic and treatment advancements.

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Chapter 1

Introduction to Dissertation

The demand for protein detection and quantitation has been increasing drastically in the past few decades. Protein level monitoring methods have been required in many scenarios including biomarker discovery in biological fluids, protein therapeutics progress monitoring, and drug development response control during clinical trials. Current methods that use highly sensitive and specific triple quadrupole mass spectrometer (QqQ-MS) require protein digestion steps prior to the analysis. This rout of analyzing peptides from protein digest is also known as the bottom-up approach. However, protein digestion steps are often incomplete and can introduce errors to protein quantitation. Therefore, absolute protein quantitation is impossible without including expensive isotope-labeled protein standards to accompany the target analytes through the digestion process.

In order to make the process of protein quantitation more accessible and applicable to meet the growing demand, we set our goal to eliminate unnecessary errors and resources that relates to the protein digestion steps. By excluding the protein digestion steps, we can make absolute protein quantitation more achievable using triple quadrupole mass spectrometry. We designed a method that bypasses the protein digestion steps and directly quantifies intact protein on QqQ-MS. Protein standards including myoglobin, cytochrome c, lactalbumin, lysozyme, and ubiquitin were used to develop an intact protein quantitation method on a Shimadzu LCMS-8050 QqQ-MS using the multiple reaction monitoring (MRM) operation mode.

Inconsistent and irreproducible fragmentation of intact proteins was initially a challenge when collision energy was maximized to convert precursor multiply-charged protein signals into product ions. Nevertheless, mass spectrometry parameters were

systematically investigated to find the proper condition of fragmentations. A few reproducible and intense product ions were found under a lower collision energy and high collision gas pressure environment. These unique and reproducible product ions were selected for MRM transitions development and optimization. Calibration curves for the standard proteins were obtained with respectable linearity of R^2>0.99. The details in method and results are given in Chapter 3.

Although preliminary data revealed the success in quantifying intact protein on QqQ-MS, it was crucial for us to prove that this method can quantify proteins in biological fluids for relevant applications. To address the complex matrices in biological fluid, chromatography parameters such as gradient slope, flow rate, temperature, additive, mobile phase system, and stationary phase were investigated. The challenges of intact protein separation hinges on the chemical forces governing protein conformation are the same forces governing chromatographic interactions. Therefore, when one parameter was changed to enhance a certain aspect of the separation, the separation could be drastically altered. The effect of all parameters were studied and lessons were learned on behalf of future separation enhancement for different applications. A generic reverse phase chromatography method was developed and separation was performed on widepore Restek Viva C4, C8, C18, Biphenyl, and PFP Propyl (2.1 x 100 mm; 5 µm; 300 Å) columns. A clinically significant protein target, prostate specific antigen (PSA), was also included in the study to prove the feasibility of the method for both of the chromatography and mass spectrometry aspects. Re-evaluation and re-optimization of the intact protein MRM method was performed to enhance detection sensitivity at the specific eluting organic composition from the newly developed chromatographic method. These results are provided in Chapter 4. Specificity of MRM detections were evaluated by analyzing blank and protein spiked urine and plasma matrices.

As the ionization efficiency and detection sensitivity of intact protein is known to be lower than for small peptides, it is crucial for us to investigate possible ways to enhance the sensitivity of the quantitation method. A less than 5% intact protein throughput rate from the first quadrupole to the third quadrupole of the same ion at zero collision energy was observed. While ion scattering was suspected to be responsible, proton transfer to and from the collision gas was also prevalent for a multiply charged intact protein. With the goal of increasing sensitivity of intact protein quantitation using QqQ-MS, collision gases with various characteristics were used to study the effect of proton transfer and ion scattering in the collision cell under zero collision energy. Protein standards, myoglobin and ubiquitin, were observed under various collision induced dissociation (CID) gases and gas pressures. As a comparison, singly charged small molecule reserpine was also monitored at the same settings. Common CID gases with a range of gas phase basicity and proton affinity including nitrogen, helium, and argon were introduced to the second quadruople collision cell. Selected ion monitoring (SIM) in the first quadrupole and multiple reaction monitoring (MRM) were used to monitor ion signal intensity. Possible causes including ion scattering, mass filter, and proton transfer were investigated. The results provide a more fundamental understanding in the use of QqQ-MS for intact protein quantitation and possible sensitivity enhancement parameters that can be optimized for specific applications. The result and specific parameter details are included in Chapter 5.

Chapter 2

Background on Protein Detection, Separation, and Detection

2.1 The significance of protein quantitation

Proteins are vital components of the body. They are involved in nutrient transport, toxin removal, metabolic process control, and defense mechanisms. The analysis of proteins allows one to monitor different signals in the body or as a result of the environment. Tracking such signals may be vital and require a fast response. Most analysis can be categorized into qualitative and quantitative analysis. Qualitative protein analysis provides the information concerning the identity of the proteins, whereas quantitative protein analysis reveals the amount of protein in a sample. In this chapter, we will focus on quantitative protein analysis as it can provide significant insights into processes in our body and impact of environmental stressors, assuming the various targets of interest are known.

Quantitative protein analysis has been used in different fields. Nesatyy et al. published a review on protein analysis for environmental studies. In this review, several studies used protein quantitation methods to gauge organismal stress responses caused by the environment.[1] Protein quantitation was also utilized in agricultural applications where researchers observed e.g., the changes in leaf proteome levels in response to salt stress.[2] However, protein quantitation has shown to be most valuable in biopharmaceutical industries. Recently, interest in analyzing biological samples to study therapeutic proteins has increased. Becher et al. published their method of quantifying targeted therapeutic proteins in plasma for an elastase inhibitor EPI-hNE4.[3] Advancement in monitoring therapeutic proteins is closely related to the availability of reference standards, the therapeutic proteins themselves, to enable absolute quantitation.[4] Even without the easily accessible reference standards, protein quantitation

has shown its value for other uses, such as disease biomarker discovery. One can compare the specific or the total protein level in biological fluids from a healthy individual to a sick person to discern the possible disease indicator proteins. Quantitation of cardiovascular biomarkers such as cardiac troponins in plasma was shown to be useful to diagnose myocardial infection.[5] Furthermore, these biomarkers can be essential in detecting acute coronary syndromes including heart attack where blood supplies to the heart muscle is impaired.[6] Proteins such as prostate-specific antigen (PSA), cancer antigen 125 (CA125), carcinoembryonic antigen (CEA), and alpha fetoprotein (AFP) antigen are currently used as biomarkers in the clinical world to detect cancer, to carry-out therapy response control, and to assess cancer reoccurrence.[7]

2.2 Classical protein quantitation

Protein analysis has been around for a long time and protein quantitation has been routinely performed in various labs in different fields. Classical protein quantitation methods can be generalized as the non-mass spectrometry method where different detectors were used. These methods often use dyes, fluorophores, or radioactivity to label the proteins for sensitive detection.[8] The classical methods typically provide good sensitivity, linearity, and dynamic range. Most of these methods were established and used for many years. However, since the classical detectors do not provide protein identity information, the primary disadvantage lies on the requirement of high resolution protein separation with suitable references.

The high resolution protein separation for classical detection methods is normally performed by two dimensional gel electrophoresis (2DGE) technology. In the first dimension, proteins are separated by isoelectric focusing. This separation is typically based on the isoelectric point of the proteins. Proteins are first introduced to polyacrylamide gels or immobilized pH gradient strips with a fixed pH gradient. As the

electric field is applied to the gel, proteins migrate through the pH gradient to their specific pI. Additives including urea, CHAPS as a detergent, dithiothrietol as a reducing agent, and ampholytes are used. Then, proteins will be separated perpendicularly in a gel box from the first dimension (a gel strip) to create the second dimension electropherogram. The second dimension generally complements the first dimension by adding molecular mass separation.[9] The molecular mass separation is often accomplished by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS as an anionic detergent binds to the proteins in proportion to the molecular weight. The negatively charge on SDS brings proteins toward the positively-charge electrode in an electric field. Smaller proteins with higher mobility moves faster and reaches further in the gel. A running buffer often includes trisma, glycin, and SDS. 2DGE reflects the abundance of different proteins by the shape, size, and intensity of the spot. In order to have an objective comparisons to give unbiased conclusion of the result, the gel images are converted into a digital image for data collection.[8] If quantitative data is required, then charge-coupled device camera or a densitometer can be used.[10]

In order to choose the right method for protein quantitation, one must examine whether the application calls for quantitation of total or individual protein in a sample. To quantify individual proteins, immunoassays are often used to target the specific protein of interest with specific antibodies and measure its concentration in sample. Proteins in biological fluids are commonly measured by immunoassays for clinical diagnostic and research purposes.[11] To allow the detection of the antibody or bound protein, various labels are used. Enzyme-linked immunosorbent assay (ELISA) is one of the most commonly used immunoassay and it uses an enzyme as the detection label. ELISA uses antibodies to carry enzymes that convert a substrate from colorless to colored or from non-fluorescent to fluorescent for classical detection.[12] Although the immunoassay route

is relatively simple and widely practiced, reproducibility is low and the specificity can be questionable. Without a detector that directly identifies the protein of interest, errors might occur when co-extraction of similar proteins occurs.

The popular western blot can also be used to compare the abundance of the specific protein in a sample using classical detection. Western blot separates proteins by molecular weight using gel electrophoresis. The separated proteins are then transferred to a membrane and then incubated with specific antibody to bind to the proteins of interest. The unbound proteins could easily be washed off leaving only the protein of interest to be detected.[13]

In some cases, total protein concentration in a sample could provide enough information for the specific application or diagnostic. To measure protein concentration, a simple ultraviolet (UV) absorbance analysis could suffice. UV light can be absorbed by aromatic amino acid residues (i.e., tyrosine, tryptophan, and phenylalanine) and peptide bonds on the proteins. This detection method is nondestructive and can preserve the sample for further analysis. Other total protein concentration quantitation methods mainly involve colorimetric and fluorometric detection. These types of detections rely on noncovalent dye binding or chemical reaction. For example, the Bradford assay measures the binding between proteins and Coomassie Brilliant Blue dye. Basic and aromatic amino acids are responsible for the bindings. When the protein is present, the dye would change its color from brown to blue. This assay is known to be compatible with most buffer, reagent, and sample preparation processes, yet gives accurate result in a short analysis time. It is often used to determine protein concentration before running gel electrophoresis. Lowry assay and bicinchoninic acid (BCA) are known as the protein-copper chelation assays. Lowery assay reduces cupric (Cu^{2+}) to cuprous (Cu^{+}) ions when reacting with peptide. The cuprous ion and the phenolic group, indole group, and the thiol

groups from the amino acids can react with the Folin-Ciocalteau reagent to produce a blue color product that absorbs at 650 nm. Although tryptophan and cysteine contains indole and thiol groups, they are less populated in protein sequences. Therefore, the phenolic group on tyrosine is the main component to deliver protein detection. The BCA assay uses the reduction of copper ion from Cu2+ to Cu+ with the presence of proteins to produce a colored complex that can be analyzed with a spectrophotometer. The reddish-purple color product yields absorbance at around 562 nm. BCA is especially useful in studying protein-protein interactions, protein recovery from cell extracts, and high throughput screening of fusion proteins. Fluorescent methods such as o-phthaldialdehyde (OPA), fluorescamine, and NanoOrange are also used for protein quantitation where a more sensitive method is required.

2.3 Mass spectrometry-based protein quantitation

Mass spectrometry based proteomics has progressed drastically for the past decade from the standpoints of both technology and applications. The notion of mass spectrometry overtaking western blot for routine quantitation will be a reality in the near future. Proteomics has also advanced for both qualitative and quantitative analysis. With the introduction of new mass spectrometers, such as triple quadrupole (QqQ), quadrupole time-of-flight (QTOF) and orbital trap configurations, sensitivity (10-50 times) and data acquisition speeds (5-10 times) have been significantly improved for mass spectrometry based protein quantitation. Mass spectrometry, in conjunction with appropriate sample preparation and separation technologies, has the ability to analyze complex samples and to provide massive multiplexing data with quality information that cannot be achieved by established methods, such as western blots. The ability of mass spectrometry to identify and quantify proteins decreases the need for generating high quality antibodies for methods such as ELISA.[14]

Table 0-1 Comparisons of Commonly Used Mass Spectrometers.

The symbol +,++, and +++ indicate possible or moderate, good or high, and excellent or very high, respectively.

	IT-LIT	Q-Q-ToF	ToF-ToF	FT-ICR	Q-Q-Q	QQ-LIT
Mass accuracy	Low	Good	Good	Excellent	Medium	Medium
Resolving power	Low	Good	High	Very high	Low	Low
Sensitivity (LOD)	Good		High	Medium	High	High
Dynamic range	Low	Medium	Medium	Medium	High	High
ESI	Available	Available		Available	Available	Available
MALDI	Optional	Optional	Available			
MS/MS capabilities	Available	Available	Available	Available	Available	Available
Additional capabilities	Sequential MS/MS			Precursor, Neutral loss, MRM		
Identification	++	++	++	+++	+	+
Quantification	+	+++	++	++	+++	+++
Throughput	+++	++	+++	++	++	++
Detection of modifications	+	+	+	+		+++

Although all mass spectrometers provide the mass-to-charge ratio information of the analyte, different mass analyzer uses different means to separate ions and to obtain this information. Since different mass analyzers have their own characteristics and strong points, it is not surprising that they are preferred in different applications with certain advantages and limitations. Table 1[15] lists the characteristics and performance of some of the commonly used MS instruments for protein analysis. In the following paragraphs, several mass spectrometers that are particularly beneficial to protein quantitation work will be reviewed.

Before considering the mass analyzers at the heart of the mass spectrometers, it is worth noting that the ionization source is an essential part of the mass spectrometry-based protein quantitation process. Electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) drastically changed the biomolecule analysis world to make polypeptides accessible to mass spectrometry detection. MALDI (Figure 1) involves

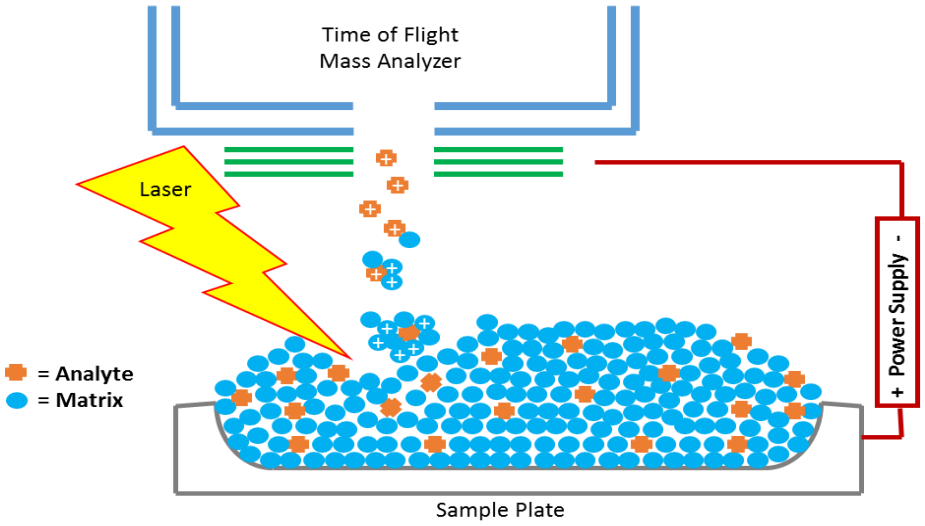


Figure 2-1 Matrix-Assisted Laser Desorption Ionization Diagram

the co-crystallization of proteins of interest with an appropriate small molecule matrix. When the high energy (usually UV) laser beam hits the analyte-matrix mixture, the mixture is ablated and desorbed into the gas phase above the sample plate. During this process, matrix acts as proton donor and receptor to ionize the protein of interest, and then the ions are accelerated into the mass analyzer.[16] MALDI provides nondestructive vaporization and ionization of the biomolecule allowing the analysis of intact analyte with high accuracy and sensitive detection. The attractiveness of the MALDI technology to protein analysis is the capability of providing accurate molecular weight information. The highly accurate mass can be particularly useful for qualitative protein analysis.[17] Electrospray ionization (Figure 2) is also a soft ionization technique that preserves the

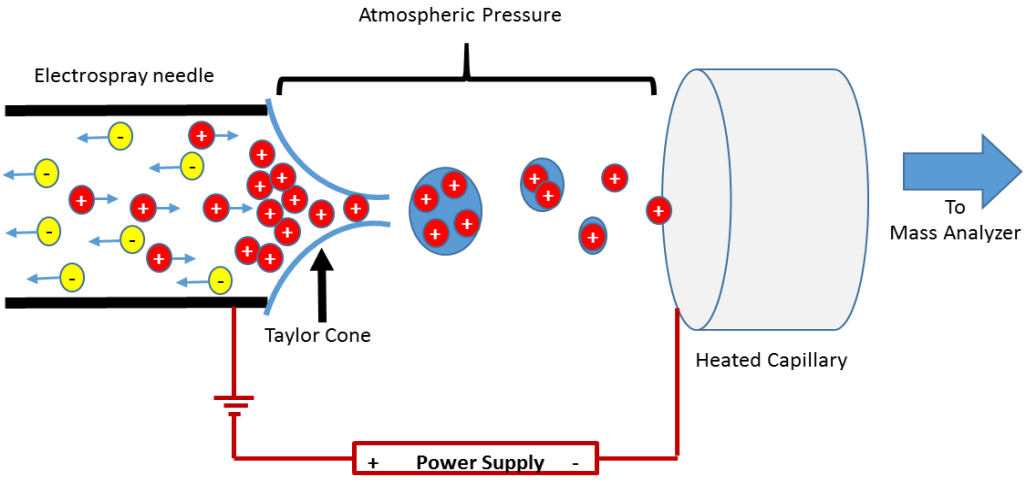


Figure 2-2 Electrospray Ionization Diagram

macromolecule intact through the ionization process. ESI produces multiply charged protein ions to bring the mass-to-charge ratio to a lower mass region.[18] This capability accommodates mass spectrometers with limited mass range. ESI can be coupled with liquid chromatography system to allow on-line separation whereas MALDI requires off-

line sample preparation. MALDI, however, has higher tolerance to salts and reagents in a protein sample, compared to ESI.

Time-of-flight (TOF) and hybrid TOF instruments are commonly used as the mass analyzer for protein analysis.[19] A time-of-flight mass analyzer analyzes the mass-to-charge ratio of an analyte by measuring the flight time of the ion of interest under vacuum from the source to the detector. This measuring method requires a well-defined start and end flight time. Therefore, it is often paired with the pulsed ionization method such as matrix assisted laser desorption ionization (MALDI) or rapid electric field switching. Theoretically speaking, when all ions are subjected to the same kinetic energy, different masses will move in different velocities. Therefore, TOF can use the different flight time to determine the differences in mass. TOF mass analyzer is known for its high mass resolution and mass accuracy. It is one of the fastest MS analyzer in the market and can be combined with both the ESI and MALDI ionization source. It can also provide protein mass-to-charge information at high mass range, which is particularly useful in studying large proteins.[20] TOF being one of the moderately high mass resolution instrument can be more expensive than unit resolution systems. Importantly, the high mass resolution capability is easily affected by the change in temperature and can requires constant tuning or the incorporation of internal standards to adjust mass accuracy during processing of data. Experience and well trained specialists are also required for instrument operation and data analysis.

TOF-TOF as a hybrid mass analyzer can provide high mass resolution MS/MS data and is often paired with the MALDI ionization source.[21] It uses high energy collision to cleave the peptidic bonds and side chains to create fragments. Quadrupole - time-of-flight (QTOF) mass spectrometers enjoy the high mass resolution and mass accuracy from TOF, yet also attains the MS/MS capability from two tandem quadrupoles. As the

precursor ions are selected in the first quadrupole, the second quadrupole acts as a collision-induced dissociation cell to fragment the selected ions. The following TOF then provides identification for the fragmented product peptide ions to tighten the search parameters and increase the confidence of the result. QTOF is thus a good instrument of choice for both qualitative and quantitative protein analysis.[22]

Ion trapping mass analyzers are also used for proteomic analysis. The main mechanism of action of the ion trapping mass analyzers involves storing ions in the trap and then manipulation of the ions for selective trapping, excitation, and ejection by electric fields.[23] Since ions can be kept in the trap for a significant time, introducing collisional activation in the trap offers the capability for higher order tandem mass spectrometry (MSn) experiments. Different types of ion traps offer different levels of resolution and sensitivity. However, there are some drawbacks. Unimolecular decomposition can occur where ions fall apart in the trap after an extended period of time. A small trap space also encourages molecule interactions to occur that can result in space charge effects that decrease mass accuracy and sensitivity, as well as limited dynamic ranges. Ion traps also have longer duty cycles, which can sometimes hurt compatibility of mass analysis with fast, high efficiency separations.

Commonly used ion trapping mass analyzer for protein analysis includes quadrupole ion trap, linear ion trap, ion cyclotron resonance, and orbital trap. Quadrupole ion trap relies on the manipulation of a radio frequency (RF) voltage on a ring electrode to trap ions of interest in three dimensions. Direct current (DC) on endcap electrodes is then used to excite and eject ions in various ways. Lin et al. has shown the ability to quantify proteins in serum using a quadrupole ion trap in a multiple reaction monitoring mode.[24] A linear ion trap operates in a similar principle to the quadrupole ion trap, but ions are trapped in a two-dimensional space. This allows for an improvement in sensitivity,

capacity, and mass resolution compared to 3-D traps. Ion cyclotron resonance operates by moving ions in a circular path in a magnetic field. Since the frequency of the ion motion is mass dependent, one can measure the cyclotron frequency to determine the mass of an ion.[25] This process involves the use of fast Fourier transformation. Ion cyclotron resonance systems can attain the highest mass resolution of all mass analyzers. It can be used for MS/MS experiments and can also be paired with various different ionization sources. The detection is non-destructive so another detector could be paired after it. However, like all trap based mass analyzers, ion cyclotron resonance and quadrupole ion trap have limited dynamic range, which can make quantitative analysis challenging. The orbital trap is a relatively new ion trap mass analyzer. It has drawn attention for its high resolution, mass accuracy, space charge capacity, and reasonable linear range. The barrel shaped orbital trap traps the ions to cycle around a central spindle electrode while oscillating on the horizontal axis. All ion traps involve the manipulation of pulses of ion packets, though they can be paired with a continuous ion source as long as an ion gating device is accessible.[26]

The triple quadrupole (QqQ) mass spectrometer (Figure 3) is the most widely used for small molecule and protein quantitation. Traditionally, proteins are digested into constituent peptides that represent the target protein prior to the analysis.[27] A quadrupole mass analyzer uses combined DC and RF potentials on the quadrupole rods to select and filter out ions with certain mass-to-charge ratio. Only the selected mass-to-charge ratio ions can pass through the quadrupole. All other ions will fall out of the trajectory and never reach the detector. With the different combination of the RF and DC potential, quadrupole analyzer can act as a mass filter to select specific ions or as an RF only ion

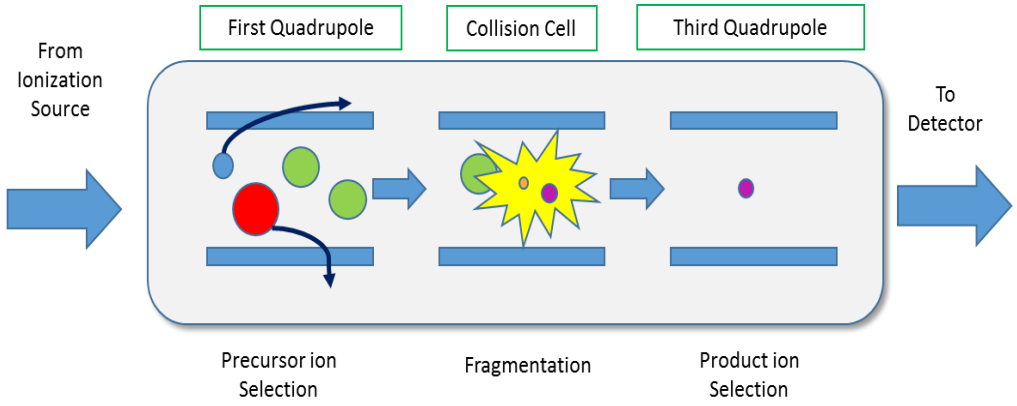


Figure 2-3 Triple Quadrupole Mass Spectrometry Diagram

bridge to pass all ions with a stable trajectory.[28] The different modes of analysis allows the QqQ mass spectrometer to be a powerful instrument for quantitation in terms of both sensitivity and specificity. By using the multiple reaction monitoring (MRM), specific precursor ions can be selected to be fragmented into product ions that are unique to the analyte of interest. MRM significantly increases the signal-to-noise ratio to achieve high quantitation sensitivity. The benefits of a quadrupole analyzer system includes good reproducibility, relatively low cost, and allowing specific precursor to product ion method to be developed in a triple quadrupole mass spectrometer system. The greatest disadvantages of the triple quadrupole mass spectrometer is the low mass resolution (unit resolution).

Mass spectrometry has now became the modern protein quantitation method. It provides the ability to measure hundreds of proteins and posttranslational modifications in parallel.[14] A large amount of data is obtained from both qualitative and quantitative information of protein analysis using mass spectrometry. Therefore, appropriate software is just as important as the hardware. Commercial and public software have been developed to meet the demand. Nonetheless, there is still a gap between the information

mass spectrometry provide and the amount of data being processed and analysis even with the constant development of new software.

2.4 Top-down vs. bottom-up approach for protein analysis

Mass spectrometry based protein analysis can be roughly categorized into top-down and bottom-up approaches. Although sometimes, the terms top-down and bottom-up can be used to refer to the separation of entities,[29] according to Reid and McLuckey, top-down and bottom-up are used to refer to entities introduced into the mass spectrometer.[30] Since the second definition is more widely accepted in the proteomic world, we will abide with this description for this dissertation.

Bottom-up proteomics is widely used for protein identification and characterization as well as quantitation. An enzyme such as trypsin will cleave the specific sites of proteins in a sample to create a mixture of peptides. These peptides are usually separated by liquid chromatography prior to the mass spectrometry analysis. Methods based on the bottom-up approach often use reversed-phase chromatography and conventional or nano-electrospray ionization (ESI) MS/MS.[29] The nano ESI is beneficial since it requires less amount of sample; it can be prone to clogging and is more difficult to maintain and optimize. The instrument of choice is typically the QqQ mass spectrometer for protein quantitation. It offers extraordinary sensitivity especially when quantifying digested proteins. The limitation of bottom-up proteomics include incomplete digestion step, which makes absolute protein quantitation impossible without expensive isotope-labeled protein standards. Since the protein sequence is only partially obtained, information including protein isoforms and post-translational modifications (PTMs) can easily be lost.

Top-down proteomics allows intact protein ions to be generated by ESI or MALDI. A normal top-down proteomic experiment will consist of intact protein

introduction, ionization, fragmentation, fragment separation, and data interpretation of intact protein molecular weight and protein fragmentation ladders.[29] Since the top-down strategy preserves the intact protein, information including protein sequence and PTM localization can be obtained. Top-down is less widespread as bottom-up as it requires high accuracy mass spectrometry such as Fourier transform ion cyclotron resonance (FTICR) or orbital trap systems.[31] High accuracy mass spectrometry like FTICR has the capability to separate multi-charged isotopic cluster of proteins greater than 200 kDa.[32] Not only is the availability of these instruments limited compared to less expensive systems, the sensitivity and throughput of intact protein analysis can also be a challenge. Analyzable protein size and dynamic range will also pose a limitation on application. In ESI based methods, multiple charge states and a large number of isotopic peaks can dilute the signal of the intact proteins. The low relative abundance PTMs are also difficult to localize.[33] Although the top-down strategy provided immense information, data interpretation of the potentially ambiguous MS and MS/MS spectra could be challenging. Various software were developed to simplify this task; however, they are not powerful enough at this state to reconstruct protein identity effortlessly.[29] Compared to the bottom-up approach, the top-down approach for protein quantitation is not nearly as common.[34] However, more and more studies have investigated the option of using high resolution mass spectrometry for protein quantitation. Gordon et al. used a high resolution FTICR mass spectrometer to quantify cytochrome c by measuring the relative ion intensities.[35] Du et al. also used the top-down method to quantify intact proteins with the help of 15N isotopic-labeled yeast proteins.[36]

2.5 Mass Spectrometry-based protein quantitation strategies

With the increasing demand of protein quantitation, strategies that aid the protein quantitation process are being developed. For the past decade, several mass

spectrometry based protein quantitation strategies were shown to be particularly valuable and were widely used. (Figure 4) The two category of protein quantitation can be divided as relative protein quantitation and absolute protein quantitation. Although relative quantitation are widely used for various applications to compare two samples, absolute protein quantitation allows data to be compared from different batches and different laboratories.

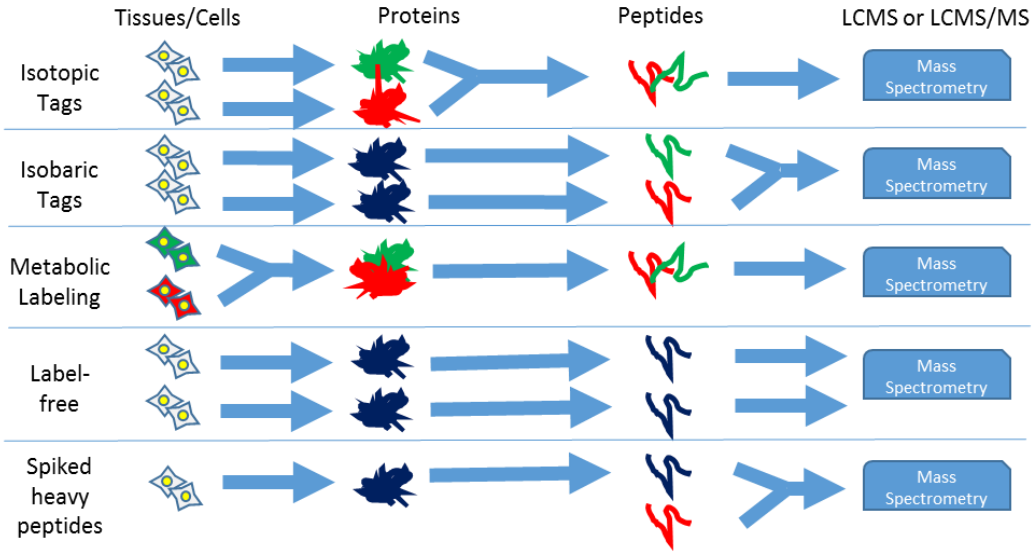


Figure 2-4 Overview of Major Protein Quantitation Workflows

Isotope-coded affinity tags (ICAT) reagent modifies proteins containing cysteine. The ICAT reagent first activate the alkylation of cysteine, then replace the eight hydrogen to deuterium atoms. A predetermined ratio of the isotopically heavy and light ICAT reagents are used to treat two different samples. The two samples are then combined and often digested to be quantified in a relative scale using LC/MS-MS. Since the peptides from the two samples are only different in the H/D ratio, it could be challenging for reversed-phase chromatography the separate the two. Also, the differences in 8 Da

could be difficult to differentiate when the protein or peptide ion is multiply charged by ESI. The alkylation to the thiol group is specific in ICAT strategy. However, it cannot be used for cysteine-free protein and post-translational modified cysteine samples. Since one out of seven protein does not contain cysteine, it can majorly limit the applicability of this strategy. Mass-coded abundance tagging (MCAT) is also useful in performing relative quantitation for proteins. Similar to ICAT, MCAT tags a specific amino acid residue. Instead of cysteine, MCAT tags the C-terminal lysine of the digested peptides. It uses the guanidination reaction to create a 42 Da differences between the two sample groups for relative quantitation. Although guanidination reaction is known to be slow when reacting with intact proteins, it is relatively fast with digested peptides (~1hr). Granting that partial guanidination is one of the great disadvantages for protein quantitation analysis, it provides qualitative information for protein identification. Different from ICAT and MCAT that target specific amino acid residue, isotope-labeling strategy uses stable isotope-labeled internal standards such as ^{2}H, ^{13}C, ^{15}N, ^{18}O for protein quantitation. Isotope-labeling strategy can be used on intact protein and digested peptides. However, to accurately analyze and differentiate the small differences with the isotope-labeled internal standards, high-resolution MS is often required.[37] Stable isotope labeling with amino acids in cell culture (SILAC) is one of the most widely use method were ^{13}C and ^{15}N labeled arginine and lysine are added into the culture medium. Relative quantitation can be achieved by comparing the treated and untreated samples.

Global internal standard strategy (GIST) was designed to be a universal label for all peptides disregarding their amino acid sequence and composition. In the early 2000, acrylation with N-acetoxysuccinimide or N-acetoxy-[2H3]succinimide were introduced. After the trypsin digestion, one of the reagent above was added to either the control or the experiment sample. Each peptide then increased its mass by 42 Da. However,

peptides contained c-terminal argining and lysine would get acetylated twice with a mass increase of 84 Da.[37] The most popular chemical labeling strategies are tandem mass tags (TMTs)[38] and isobaric tags for absolute and relative quantitation (iTRAQ)[39] that target the primary amines. They are also known as isobaric tags as the peptides from different sample would have identical mass but can be differentiate by the isotope encoded reporter ions after fragmentation. The isobaric labeling method does not increase the complexity of separation or the mass spectra. Although analyzing the low *m/z* ion reporter was initially a challenge using mass spectrometer such as ion trap, the introduction of linear ion trap and orbitrap has shown to eliminate this concern. Other than labeling the proteins and peptides, label-free strategies can also be used for quantitative analysis. One of the way to achieve label-free is by the spectrum count approach. Protein quantitation information can be obtained by using the peptide response intensity directly or the peptide-to-spectrum matches (PSMs). By comparing relative intensity, a label-free method can be easily applied for relative quantitation.[14]

While numerous strategies were developed for relative protein quantitation, only a few strategies for absolute protein quantitation can be utilized. This is likely caused by the high level of difficulty in achieving absolute protein quantitation. A chance of obtaining different digestion character, fragmentation patterns, or signal response is always there when modifying a part of a protein or peptide. Reaction completion can also hinder the accuracy of the absolute quantitation method. In most cases, absolute quantitation are achieved by tagging the intact proteins or digested peptides. AQUA, for example, creates standards by attaching stable-isotope-containing tags to the peptide that is unique for the protein of interest to be added in the sample for analysis.[40] Strategies such as protein standard absolute quantitation (PSAQ), absolute SILAC, and FlexiQuant label intact protein metabolically and introduce to the sample before digestion and sample

preparation to achieve absolute quantitation.[14] If one wishes to avoid using expensive labeling standards, yet reach absolute quantitation, an algorithm (APEX) was develop to predict the number of peptides from a digested protein.[41] This prediction, however, will not compensate the errors and inconsistent percent completion of the digestion process.

2.6 Protein analysis in complex matrices

For almost all the applications that require protein analysis, protein samples exist in complex matrices that require separation for reasonable detection accuracy, specificity, and sensitivity. In a highly specific mass spectrometry based detection, pairing an atmospheric pressure ionization such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) has shown to provide satisfactory results in some matrices. Yet, in most cases, sample preparation, purification, and/or separation steps prior to analysis can only be simplified but not completely eliminated.[42]

In a mass spectrometry based analysis, a common problem caused by the interfering compounds from the complex matrix is the suppression or the enhancement of the ion of interest.[43] Despite all the efforts that were put in to explain the phenomenon, ion suppression and enhancement occurrences in ESI are still not fully understood. Until now, all suggestions stayed as hypothesis. The suppression and enhancement of ionization efficiency is likely to be caused by: 1) The interfering compounds compete for the charges and the access to the surface of the droplet; 2) the increase of liquid phase viscosity interferes with the evaporation process and gas phase ion formation; 3) non-volatile additives form solid particles; and 4) mobile phase additives act as ion pairing reagents creating pre-formed analyte ions or neutral complexes.[42] Matrix effects thus limit the quantitative analysis accuracy, sensitivity, and reproducibility. It is important to properly manage the sample matrix to obtain useable data. However, it is rather challenging since it is unpredictable and changes from matrix to matrix, and from batch to

batch, may occur. Also, while addressing the matrix, purifying the sample can lead to sample losses.[42] Therefore, to choose a suitable separation method where interfering compounds can be eluted at different time from the analyte of interest is extremely beneficial.

2.7 Protein separation techniques

There are a vast number of techniques that are used to separates proteins for different applications and based on the detector of choice.[44] In this chapter, we mainly focus on the most widely used protein separation techniques.

Ion exchange is one of the most widely used liquid chromatography modes for protein separation. During ion exchange chromatography, proteins interact with either positively (anion exchange) or negatively charged (cation exchange) functionalized groups on the stationary phase. Proteins can be eluted by altering the pH or the ionic strength of the mobile phase. The charged amino acid residues on the surface of the protein are responsible of binding and retention. Although the constitution of the protein does not change during ion exchange chromatography, the protein behavior still varies. This is due to the pH value and the additives of the mobile phase alternating the charge density and accessibility of the surface charged residues.[44]

When ion exchange chromatography was first used for protein purification, the stationary phase was cellulose with diethylaminoethyl (DEAE) or carboxymethyl (CM) groups attached.[45] Today, most people use natural polymers dextran agarose and synthetic polymer resins.[44] Since proteins can expose negatively or positively charged groups on their surface, anion exchanger or cation exchangers were both used in different application for better protein purification results. In most cases, the decision of choosing one over another is based on empirical tests.

Ion exchange chromatography can be applied for most of the known proteins. Yet, some enzymes from halophilic bacteria require high ionic strength to be stable.[46] In cases like that, this method is either not possible or requires major modifications.[47] Other than for the proteins that require extreme condition for elution, ion exchange is known to have higher intact protein yield than other liquid chromatographic modes. For analyzing membrane proteins, which requires detergents in the separation environment, ion exchange chromatography is compatible. Non-ionic and zwitterionic detergents can be added to maintain the structure and function of the protein of interest during ion exchange chromatography. Ionic detergents, on the other hand, can interfere with the ion exchange process to decrease binding capacity, increase back pressure, and create irreproducible elution. Overall, ion exchange chromatography provides high protein binding capacity and moderate to high resolution for chromatography. It can also hold a large volume of sample, thus reducing the need for excessive pre-concentration steps. Furthermore, concentrated protein fractions can be obtained from diluted samples using ion exchange chromatography.

Although ion exchange chromatography is widely used for protein purification and separation, it is not generally paired with mass spectrometry. The high salt concentration that is used for the elution of the compounds from the ion-exchange column is not compatible with mass spectrometry. While efforts has been made to develop techniques that substitute out the sodium or potassium with H+ to convert the eluent into pure water, ion exchange is still mostly paired with a conductivity detection.[48]

Proteins are separated according to their size in size exclusion chromatography. The concept of separation based on the size of the molecules was first reported by Synge and Tiselius in the 1950s.[49] The first report of using size exclusion separation for biomolecules was by Lindqvist and Storgårds five years later.[50] They used a starch

column to separate peptides from amino acids. Later, Lathe and Ruthven reported their finding of using potato and maize starch to separate proteins and peptides by the size exclusion effect.[51] Throughout many years of improvement on the stationary phase of the size exclusion, silica became predominate stationary phase of its mechanical strength, and stable and inert nature.[52] For organic and aqueous solvent based size-exclusion separation, styrene-divinylbenzene copolymers and cross linked glycidoxymethacrylate polymers are often used, respectively. Size exclusion chromatography separates smaller molecules from larger molecules by passing them through a porous stationary phase. While smaller molecules can enter into the pores, larger molecules pass by or only partially enter the pores due to their size (hydrodynamic volume) and elute out of the column faster. It is important to note that in size exclusion chromatography the analyte does not interact with the stationary phase. However, the surface silanols can introduce strong ionic interaction with proteins. Diol functional group are commonly used now to minimize hydrophobic interaction.[53] However, diol groups could not provide complete coverage of the surface silanols groups.[54] Consequently, high ionic strength mobile phase are required to diminish ionic interactions.

For size exclusion chromatography, ultra-violet and refractive index detectors have been most commonly used.[55] The near UV or longer wavelength region can detect aromatic amino acids.[56] Low wavelength region can also be used to detect the amide peptide bonds. While the lower wavelengths provide good sensitivity and the higher wavelengths support greater linear dynamic range, scattering and interferences from other chromophores can challenge the quantitation accuracy.[57] Fluorescence detection is also used as a detector for size exclusion chromatography. It is especially useful in detecting proteins in low level as in many case the fluorescence detector has shown to improve sensitivity.[58] Multi-light scattering detector was also used to determine the

property of the proteins including the size and the shape. It enables better molecular weight determination and confirmation. However, since multi-light scattering detection is not concentration dependent, refractive index and UV detectors are needed to be coupled with the system for concentration determination.[59] Although mass spectrometry can be considered as a detector for size exclusion chromatography, the high nonvolatile salt concentration in the mobile phase makes it an extremely challenging coupling. The high salt and non-denaturing aqueous mobile phase at the physiological pH results in ion suppression and contamination in a mass spectrometer. Although volatile adducts such as ammonium formate and ammonium acetate can be used, the physiological environment cannot always be adequately achieved.[60]

Reversed-phase chromatography separates molecules based on their relative degrees of hydrophobicity. It is the most popular HPLC technique by a large margin. Reversed-phase chromatography is especially dominating in the small to intermediate molecule separation. Its use in large molecule separations is now increasing due to the high separation method demand.[61] The name reversed-phase was initiated based on its relation to the normal phase separation mode. Normal phase chromatography uses silica as the stationary phase to retain less polar molecules based on their differing degrees of polarity. Elution is achieved when a polar organic solvent is flowing through the column. Reversed-phase separation is the opposite of normal phase separation. Alkyl chains or other hydrophobic functional groups are covalently bonded to the silica support. Compounds that are hydrophobic in character tends to interact with the reversed-phase stationary phase, and compounds that are more hydrophilic in nature will elute out first. By decreasing the polarity of the mobile phase by adding organic solvents, polar compounds can elute out of the column depending on their degree of hydrophobicity.

According to the hydrophobicity of the specific analyte, different percentages of organic solvents will be required for elution. Thus, a variety of compounds can be separated.

Since reversed-phase chromatography is exceptionally popular and is used in many different fields, column companies have made significant progress on attaching different functional groups on different solid supports for different applications. The variation of the stationary phase provides a wide range of selectivity and has become one of the major advantages of reversed-phase separations. Other than varying alkyl chain length to provide different degrees of retention, different groups such as phenyl and polar groups can also be attached to provide different selectivity. The previous hurdle on surface silanol-group interaction with the analyte that can cause peak asymmetry have been drastically reduced with silanol interaction blocking endcap technology. Superficially porous particles have also been introduced for reversed-phase chromatography. The non-porous core with the thin layer of porous shell has shown drastic improvements in separation efficiency by reducing the diffusion path.[62] Furthermore, column companies and researchers are working to develop new functional groups and technologies to further optimize reversed-phase chromatography.

Although reversed-phase chromatography is gaining popularity in protein separation method in recent years, the organic mobile phase and various modifiers can denature intact proteins. Denatured proteins expose numerous hydrophobic residues from the core and can lead to peak broadening, low recovery, and irreversible retention.[63] If the separation can be optimized, reversed-phase chromatography can be an attractive separation technique for mass spectrometry based detection. The solvents and additives are generally compatible with mass spectrometry, with some exceptions. The various stationary phases reversed-phase chromatography offer might resolve similar proteins by

the different interactions between the stationary phase and the protein surface functional groups.

HILIC is the term given for hydrophilic interaction liquid chromatography. It uses the hydrophilic stationary phase, like a normal phase chromatography stationary phase, and a predominantly polar organic mobile phase.[64] Although the mobile phase are similar to a reversed-phase chromatography (including the use of water), the elution order is reversed to a typical reversed-phase separation. This is caused by the method where the separation started from high organic with the successive addition of water as the strong solvent to effect elution. Therefore, hydrophilic compounds will exhibit longer retention than the hydrophobic compounds. The mechanism of HILIC is believed to involve the formation of an aqueous layer around the polar-functionalized particle surfaces, so hydrophilic analyte can partition between the aqueous layer and the polar organic mobile phase. The increasing water content in the mobile phase will thus encourages elution.[65]

HILIC was first introduced in the 70's[66] but did not gain momentum until the 90's.[67] The increasing popularity is driven by the demand of analyzing polar compounds that have little retention on reversed-phase chromatography in a complex sample (e.g., in metabolite analysis).[68] HILIC stationary phases are typically made from a silica base with siloxanes, silanols with or without some metals,[69] derivatized silica with cation or anion exchanger,[70] amines and amides, zwitterionic groups,[70d] and click saccharides.[71] The different functional groups provide different retention mechanism that can be used to optimize different applications, but it can be a complex task requiring significant empirical tests to determine the optimal separation conditions.

The buffer and mobile phase required by HILIC is highly compatible with mass spectrometry. Even more, HILIC typically operates in high organic composition and can increase sensitivity in ESI-MS by providing a mobile phase that will evaporate more

easily upon electrostatic nebulization.[72] HILIC has also been used for targeted analysis of protein and peptide post-translational modifications (PTMs) including glycosylation,[73] N-acetylation,[74] and phosphorylation.

Hydrophobic interaction chromatography (HIC) separates proteins based on the interaction between the hydrophobic surface of the proteins and the functional groups on the stationary phase. Although this interaction can appear to be very similar to reversed-phase chromatography, the major differences lies on the interaction of hydrophobic interaction being heavily influenced by the salt in the running buffer.[75] Although the exact mechanism of retention is still unknown, some theories have emerged pointing to the role of water carrying out the separation process. Since the mobile phase is highly aqueous, water molecules cannot wet the surface of the stationary phase with the hydrophobic functional groups. Instead, water forms a highly ordered shell around the analyte driven by the hydrogen bond interaction.[76] When the mobile phase contains more salt, which reduces the solvation of the analyte, interactions between the proteins and the hydrophobic groups are enhanced. Using lower salt concentration lowers the ionic strength to allow more solvation of the analyte, thus weakening the interaction with the stationary phase and allowing the proteins to elute. A typical HIC analysis uses high salt buffer as the starting mobile phase and a low salt buffer as the eluting mobile phase.[77] The mobile phase is directly controlling the relative solvation of the analytes, which in turn alters the degree of hydrophobic interactions that can occur between proteins and hydrophobic functional groups on the stationary phase surface.

The solid support of choice for HIC stationary phases is hydrophilic carbohydrates, silica, or synthetic copolymer materials. The widely used functional groups are linear alkanes and in some cases with a terminal amino group in the end. Phenyl and other aromatic groups can also be used to induce π-π interactions. The

hydrophobic stationary phase is very similar to reversed-phase chromatography. However, as discussed above, the stationary phase is not the only contributor to hydrophobic interaction chromatography. Other parameters such as salt concentration, pH of the mobile phase, temperature, and additives also play a major role.[78]

HIC is versatile as it can be used after protein precipitation,[79] in combination with gel filtration,[80] or with ion-exchange chromatography.[81] However, some disadvantages including irreversible binding of some proteins. To elute such proteins, organic solvents, detergents, or other additives may be required but can also denature proteins.[82] When coupling with mass spectrometry, the high aqueous content can hinder the solvent evaporation process thus decrease sensitivity.[83] The normally used non-volatile salt (sulfate, phosphate, or citrate) will also limit the compatibility.[84] Although volatile ammonium acetate can be used as the salt, it is reported to be inadequate in retaining proteins in a typical hydrophobic interaction method.[85]

Immobilized metal affinity chromatography (IMAC) uses the interaction between transition metals such as Zn(II), Cu(II), Ni(II), and Co(II) with amino acids in proteins surface such as cysteine, histidine, and tryptophan. To elute the target protein, a low pH mobile phase, displacement agents, and/or chelating agents (i.e. EDTA) can be used. Traditionally, agarose and cellulose were used as the stationary phase support for their biological compatibility but their low mechanical strength limits their use in a high pressure systems. Recently, silica has been used for the mechanical strength but the irreversible adsorption for protein creates a challenge. Since the immobilized metal affinity chromatography relies heavily on the metal protein interaction, it is important to choose the proper metal to achieve appropriate affinity for the application. Horvath et al. have shown the ranking of the affinities of the metal in the following order: Cu(II)> Ni(II)> Zn(II)> Co(II).[86] It is common to use sodium chloride (0.3 to 1.0 M) in the method to

reduce ionic interactions between proteins and resin.[87] Glycerol and ethanol was also added to prevent the hydrophobic interactions between proteins.[88] Phosphate and acetate buffers are also used during the analysis.[89]

As expected, immobilized metal affinity chromatography will not be useful on separating proteins without metal affinity. In other words, if the protein surface does not contain cysteine, histidine, or tryptophan, no retention or separation will be able to achieved. Also, if chelating agents such as EDTA are used and these strip off the metal ions from the resin, metal regeneration steps will need to be included in the method. In complex samples, which contain other chelating groups (i.e., ammonium salts, arginine, cysteine, glutamine, glycine, and histidine), the affinity separation efficiency will be greatly decreased.[90] It is also possible that immobilized metal affinity chromatography will destroy both the side chains and the backbone of proteins.[91] The high salt concentration and other additives are not MS compatible. A desalting steps is required and can be seen when pairing with the MALDI-MS system.[92]

Immobilized artificial membrane technology is another technology that utilizes the stationary phase as a support to bind specific functional groups for separation. It often involves the binding of a monolayer of membrane-forming lipids to a silica substrate. It is designed to mimic the physiochemical environment of the membrane and is useful in predicting cell membrane drug transport and purification of proteins.[93] Immobilized artificial membranes have been used to study solute partitioning into the membrane, drug permeability through cells, drug absorption, amino acid uptake, salt-membrane interaction, and skin permeability.[94] A typical mobile phase consists of phosphate buffer, glycerol, EDTA, and sodium cholate. These nonvolatile salts and additives are not suitable for mass spectrometry based detection.[95]

Dye-ligand affinity involves the immobilization of reactive dyes such as Cibacrone blue to interact with proteins of interest. The versatility of the Cibacrone blue comes from its structure, which contains both non polar aromatic and ionic sulfonate groups. Due to its structural and stereochemical characteristics, it can offer “pseudospecific” interactions with many proteins. The binding mechanism of the immobilized dye to protein has been described as nucleotide-specific, ionic, and hydrophobic. Although these description may sound to be controversial to each other, it is very likely that the dye interacts with proteins by all these interactions in different settings.[96] Since the interactions vary in different applications, the elution of proteins also varies. In many cases, high chaotropic salt concentration and organic modifier can be used to disrupt hydrophobic and ionic bindings. Changing the pH can also aid the elution of protein if it is above their pI point.[97] Since dye-ligand affinity has different modes of retention, it may offer very different chromatographic selectivity. However, the heavy use of salt and additives might not be compatible with mass spectrometry.

Liquid-liquid chromatography is also known as partition chromatography. Liquid-liquid partition is based on the sample distribution in two immiscible solvents, posed as the stationary and mobile phases, respectively. For conventional liquid-liquid chromatography, stationary phases are held to a medium by adsorption. Liquid-liquid chromatography offers another dimension of chromatographic selectivity since liquid phases can be used as the stationary phase. Different combinations of the aqueous and organic solvents can be used for various applications. Liquid-liquid chromatography is useful in differentiating proteins based on their surface properties. This capability has allowed researchers to differentiate polyclonal IgG antibodies from patients with autoimmune disease from the healthy individuals.[98]

In a typical liquid-liquid partition chromatography for proteins and enzymes, water soluble polymers such as polyethylene glycol and dextran are used with potassium phosphate salts.[99] This method calls for a considerate amount of buffer salt and ion pairing reagent to be added. These non-volatile substances will certainly be problematic if the coupling with mass spectrometry is considered.[98b, 99]

Displacement chromatography uses the concept of one competitor being stronger to replace than the other; a stronger binding compound is used to displace the previously bound compound. Displacement chromatography can be broken down into loading, displacement, and regeneration steps. The proteins are first loaded onto the column, then being displaced by a displacer that has higher binding affinity to the stationary phase to push out the proteins.[100] In a mixture where multiple proteins exist, a tighter binding protein can act as a displacer of a more weakly bound protein. After the run is complete, which is observed by the breakthrough of the displacer, a regeneration step is made by running a buffer through the column to remove the displacer.[101] Biopolymers, protamine sulfate, and heparin were proven to be effective candidates as displacers in cation and anion exchange displacement chromatography systems.[102] Low molecular weight antibiotic Streptomycin A can also be used as a displacer for proteins.[101]

Displacement chromatography is beneficial in its ability to simultaneously concentrate and purify proteins. Both high molecular weight and low molecular weight displacers have been used for protein displacement chromatography. In displacement chromatography, salt (ie. KCl, KOH, and NaCl) is generally used to regenerate the column. UV absorbance is typically used to observe the displacer breakthrough. If mass spectrometry detection is desired, prior desalting steps will need to be carried out.[103]

Chromatofocusing is a variant of ion exchange chromatography as it uses ion exchange resins. However, instead of enhancing elution with increasing ionic strength of the mobile phase, chromatofocusing enhances elution by the change of pH. As the pH of the mobile phase is changed, the molecule will elute out once the net charge on protein surface became zero. Since the protein was bound to the ion exchange resin through Coulombic forces, when net charges becomes zero at pH = pI, elution will occur.[104] A down side of this method is possible protein aggregation to obtain zero net surface charge; the aggregated proteins can block the column and create problems.

The unique part of chromatofocusing chromatography is that it separates proteins according to their differences in isoelectric point. Since the isoelectric point is crucial for the separation, carefully manipulated pH is essential for optimizing the separation and analyzing result.[105] It can be used as an off-line fractionation step before reverse phase chromatography.[106] As the mobile phase is generally aqueous with various acid and base, evaporation process required by the mass spectrometry will be compromised and ionization efficiency can be rather challenging.

Electrophoresis is a separation technique that separates molecules based on their mobility under an electric field, which is heavily influenced by a molecule's charge and size. Although the electric field mainly enhances the movement by charges, the size of the molecule determines how easily it can move through the pores of the gel. This technique is commonly used to separate proteins.

A gel medium is used for proteins to move through and is placed in an electrophoresis chamber with alkaline running buffer. The electric field is created with negative electrode at one end pushing the proteins to the other end where the positive electrode is. The positive electrode also pulls the proteins through the gel to assist the

separation process. When the electric current is applied, proteins will move down the field to the positive electrode while being separated due to the different movement rates.[107]

The most commonly used electrophoresis technique for protein separations are gel electrophoresis. Within the gel electrophoresis, there are sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and native gel electrophoresis. The native gel electrophoresis allows the molecules to be separated in their native higher order structure. Sodium dodecyl sulfate (SDS) as an anionic detergent that helps protein to lose the secondary or tertiary structure. It linearizes the protein for protein separation.[108] Typically, molecular weight markers are included in a gel electrophoresis process and analyzed in the same gel to provide references for protein mass determination.

Isoelectrofocusing (IEF) is another type of electrophoresis that separates molecules by their isoelectric point. It utilizes the relationship between the net charges on the proteins and the surrounding pH to control the movement of the proteins.[109] When the current is applied, a positively charged proteins will migrate through the increased pH gradient while reducing the overall charges. When the protein reaches the pH region that equals to its pI, migration stops. This technique can provide very high resolution of protein separation.

The detection process of gel electrophoresis mainly involves staining.[110] Proteins are often stained with silver stain, Coomassie Brilliant Blue dye, ethidium bromide, and SYBR Green.[111] Photographs can be taken normally or by a gel documentation system that includes an ultraviolet light for protein analysis. Diffusion, cutting, or electroelution can also be used to recover the proteins from the gel for other forms of detection.

Even though gel electrophoresis is one of the most popular protein separation technique, there are several disadvantages. First of all, heat is being created when

current is applied for the separation. Heat, however, can melt the gel and cause protein to denature or proteolyse during analysis. Secondly, significant amount of buffer needs to be used to control the pH fluctuation. Yet, the capacity of the buffer can still be exhausted during runs. Also, it is known that sample preparation can influence migration consistency. Therefore, reproducibility of protein separation in gel electrophoresis is harder to master.

Capillary electrophoresis uses voltage to separate ions based on the electrophoretic mobility. The electrophoretic mobility depends highly upon the molecule charge, viscosity, and the atom's radius. There's a direct relationship between the molecule moving rate and the applied electric filed. In another word, the higher the electric field, the faster the molecules moves. It is important to note that only ions will move with the electric field and the neutral compounds will not be affected. If two compounds with different sizes obtain the same charges, the smaller one will move faster. In the case of two compounds being the same size but different charges, the one with more charges would move faster.[112]

Capillary electrophoresis was first introduced in the late 1800s. However, it was until the 1930s when researchers used electrophoresis to separate proteins. Even then, this technique did not get popular until the 1960s, when capillaries were added into the process. Since then, capillary electrophoresis has proven itself to be a fast and versatile method to separate both large and small molecules while requiring small amount of sample and reagents.[113] Capillary electrophoresis not only outperforms traditional electrophoresis in efficiency of separation but it also eliminated problems such as overheating at high voltages.

A capillary electrophoresis setup involves a high voltage power supply where each side is connected to an electrode in a electrolytic solution. The electrode creates the

electric field to encourage analyte migration from anode to cathode through the capillary tube. The capillary is typically made of silica. A small window is designed on the capillary close to the cathode for UV-Vis absorption. A photomultiplier tube can also be connected to the end of the capillary closer to the cathode for detection.

Capillary electrophoresis includes different types of separations, including capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic capillary chromatography (MEKC), capillary electochromatography (CEC), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP). They can first be segregated into continuous and discontinuous systems. While a continuous system has the electrolyte throughout the capillary at all time, a discontinuous system separates the sample into different zones based on the presence of different electrolytes. Out of all the separation methods mentioned above, only CITP falls under the discontinuous system. The others can be further segregated into kinetic process and steady-state process within the continuous system. The kinetic process (CEC, CZE, MEKC, and CGE) offers constant electrolyte composition while the steady-state process (CIEF) provides changing electrolyte composition.[114]

Out of the six separation method mentioned above, it is worth mentioning CGE and CIEF that are particularly useful for protein separation. Similar to the gel electrophoresis, capillary gel electrophoresis separates using compound migration through gel and the separation is based on mainly on size. The gel minimizes the diffusion process and prevents the analytes being absorbed into the capillary. The capillary elevate this process by requiring very small amount of sample. Capillary isoelectric focusing separates proteins based on the isoelectric point of the proteins and the pH of the surrounding environment. Therefore, for this type of separation, a pH gradient can be useful. In capillary isoelectric focusing separation, the sample is inside of

the capillary at first. Cathode electrode is in a high pH environment where anode is in a low pH environment. When voltage is being applied, proteins will migrate to a region where the net charge became zero and will not move further for being neutral. Using this method, proteins with the same isoelectric point will be forced together in the same region for detection.[114] Capillary electrophoresis can be used with various detectors including UV-Vis and MS. It has many advantages including simple setup and operation while providing high peak capacity and narrow peak shapes. However, it is limited in terms of selection of stationary phases and column dimensions and might not provide enough selectivity in some cases.

For the past few decades, evolution in both column and packing designs for large protein separation has increased greatly, especially in miniaturizing the process and reducing analyte losses.[115] Different protein separation techniques separate protein based on different interactions. Each technique also carries their own advantages and disadvantages. Various established separation methods can be found to fit the specific application or the detector of choice. However, each is likely to require further optimization for different systems and matrices.

Conclusion

In this chapter, we reviewed the most widely used protein detection and separation methods. The classical protein detection techniques have been rapidly replaced by the informative mass spectrometry based techniques. To develop a mass spectrometry based protein quantitation method, various mass analyzers can be chosen from for their distinctive characteristics, advantages, and disadvantages. The high mass resolution mass spectrometers support top-down protein analysis while low mass resolution mass spectrometer such as the triple quadrupole mass spectrometer is widely used for bottom-up protein quantitation. With the increasing number of mass

spectrometry based protein quantitation applications, separation techniques that can be coupled with mass spectrometry continue to be advanced.

Chapter 3

Multiple Reaction Monitoring for Direct Quantitation of Intact Proteins using a Triple Quadrupole Mass Spectrometer

3.1 Abstract

Methods that can efficiently and effectively quantify proteins are needed to support increasing demand in many bioanalytical fields. Triple quadrupole mass spectrometry (QqQ-MS) is sensitive and specific, and it is routinely used to quantify small molecules. However, low resolution fragmentation-dependent MS detection can pose inherent difficulties for intact proteins. In this research, we investigated variables that affect protein and fragment ion signals to enable protein quantitation using QqQ-MS. Collision induced dissociation gas pressure and collision energy were found to be the most crucial variables for optimization. Multiple reaction monitoring (MRM) transitions for seven standard proteins including lysozyme, ubiquitin, cytochrome c from both equine and bovine, lactalbumin, myoglobin, and prostate-specific antigen (PSA) were determined. Assuming the eventual goal of applying such methodology is to analyze protein in biological fluids, a liquid chromatography method was developed. Calibration curves of six standard proteins (excluding PSA) were obtained to show the feasibility of intact protein quantification using QqQ-MS. Linearity (2-3 orders), limits of detection (0.5-50 µg/mL), accuracy (<5% error), and precision (1-12 %CV) were determined for each model protein. Sensitivities for different proteins varied considerably. Biological fluids including human urine, equine plasma, and bovine plasma were used to demonstrate the specificity of the approach. The purpose of this model study was to identify, study, and demonstrate the advantages and challenges for QqQ-MS-based intact protein quantitation, a largely underutilized approach to date.

3.2 Introduction

The demand for protein quantitation has been increasing in fields including, but not limited to, protein markers in biological fluids analysis, protein therapeutics, and drug development and response control. This rapidly increasing trend demands that analytical tools and methods be developed to facilitate these and other relevant applications.[8] In biological fluids, the flux of protein expression is a crucial marker for numerous diseases. By studying easily accessible biological fluids such as urine, one can perform non-invasive clinical diagnostics.[116] Even though trace amounts of protein in urine is common, significant amounts often indicate bladder tumors, kidney disease, preeclampsia, and other maladies. Proteins can also be potential biomarkers for cancer screening, from samples such as plasma or serum.[117]

For the past few decades, increasing numbers of proteins and peptides have been approved for clinical use by the US Food and Drug Administration.[118] However, since the process of making protein therapeutics using DNA expression techniques can generate unintended protein byproducts and compromises product activity and stability, it is crucial to have established qualitative and quantitative methods for quality control of protein therapeutics.[119] With the rapid development and the increasing usage of protein therapeutics, the need for methods which can accurately and precisely monitor the protein levels throughout the entire process of production, characterization (e.g. pharmacodynamics and pharmacokinetics), and clinical treatment are particularly necessary.[120]

Quantitative analysis of small molecules is routinely performed using a triple quadrupole mass spectrometer (QqQ-MS), which is known for its outstanding sensitivity and specificity.[121] A triple quadrupole provides several operational modes that can accomplish different means of discovery and quantification. Selected reaction

monitoring (SRM) involves the determination of an analyte of interest by monitoring a unique fragment ion generated by collision induced dissociation (CID). Specificity is significantly improved and background noise and interferences are reduced by monitoring a defined precursor and product ion pair.[122] The enhancement of signal-to-noise ratio allows reliable determination of low abundant compounds in a sample. Multiple reaction monitoring (MRM) mode uses the same concept as SRM, except that multiple fragmentation events for potentially multiple analytes are followed during an analytical run.[123] Two or more product ions can be captured for a given analyte to achieve both quantification and verification.

Although methods that quantify small molecules, such as hormones, drug metabolites, and protein degradation products have been established and commonly used, methods that directly quantify intact proteins using a triple quadrupole mass spectrometer have not yet been significantly developed and utilized.[124] In quantifying proteins, selected ion monitoring (SIM) has been used to select and monitor a single charge state of a multiply-charged protein to directly quantify r2u-globulin in rat urine and kidneys.[125] A method including solid-phase extraction, liquid chromatography, and SRM was developed to quantify angiogenesis inhibitor rK5 protein in plasma.[122, 126] In both papers, Ji et al. expressed the lack of intact protein quantitation methods on QqQ-MS by claiming no reports were found that described detection of proteins over 10000 Da using SRM. Indeed, examples of such an approach in the literature are extremely limited.[127]

The lack of activities of intact protein quantitation using QqQ-MS might be caused by challenges in optimizing intact protein fragmentation. When developing a SRM method for a high molecular weight protein, abundant low mass fragments with irreproducible fragment patterns are often observed.[128] This could be attributed to multiple interdependent parameters. The possible explanations of these occurrences can

be categorized from two main sources: Low resolution instrumentation and analytes with large molecular size and complexity.

A typical triple quadrupole is a low resolution instrument that cannot fully distinguish $\Delta m/z < 0.5$ in a mass spectrum (i.e. unit resolution). Proceeding with MRM of a multiply-charged protein ion formed from electrospray, precursor ion isolation will likely include a mixture of multiple isotopes, adduct ions, isoforms, and conformational states. Differ from going through the highly reproducible high energy collision cell,[129] a large protein ion will be susceptible to ion scattering,[130] charge transfer,[131] sequence rearrangement,[132] and cyclization processes[133] in the low energy collisional cell used in QqQ-MS. The precursor ion will have many degrees of freedom, which can lead to heterogeneous distribution of internal energy and the unpredictable formation of product ions possessing a variety of molecular weights and charge states. The selection of a product ion in the low resolution third quadrupole also provides limits for assigning the charge state and sequence of the ion. Some good top-down mass spectrometry software tools exist,[134] but these provide ambiguous assignments without high mass accuracy data. In general, the potential for irreproducible fragment patterns makes it difficult to optimize an MRM channel and reproducibly quantify an intact protein target using a triple quadrupole instrument. Presumably, it is for these reasons that little literature exists on this topic.

In order to utilize triple quadrupole mass spectrometry for protein quantification, prior protein digestion and quantification of peptide products through a bottom-up approach has been the norm.[121a, 135] However, such methods can be time consuming and possible errors in the protein digestion steps can easily propagate, which can compromise precision and accuracy.[136] In fact, protein digestion steps are often incomplete and the percent protein digested is not always consistent making absolute

protein quantitation very difficult.[120] One can purchase a stable isotope-labeled protein to accompany the entire process of target protein analyte digestion as an internal standard.[137] However, this is extremely costly and not widely practical due to limited availability of such standards. As an alternative, a top-down approach can be used, which involves the direct interrogation of intact proteins in the mass spectrometer. This strategy is most often applied on more expensive high resolving power mass spectrometers including those based on time-of-flight (TOF), orbital trap, and Fourier transform ion cyclotron resonance technologies; triple quadrupole mass spectrometers are not commonly used for top-down protein analysis.[122, 126, 136, 138]

The goal of this work was to explore consideration for the development of a top-down MRM-based method for direct intact protein quantification using a triple quadrupole mass spectrometer. Eliminating potentially inconsistent protein digestion steps will save error, money, time, and resources. It can also accomplish absolute protein quantitation while avoiding the extensive use of the stable isotope-labeled proteins. We investigated variables that directly correlate with the production of stable and reproducible product ion fragments for MRM, including mobile phase composition, electrospray ion source conditions, collision energy (CE), and CID gas pressure. A set of model proteins were used to demonstrate method feasibility by developing a reproducible MRM and creating calibration curves. Linearity, accuracy, and precision along with limits of detection and quantitation were determined for each protein. Possible challenges of matrix effects and interferences when directly analyzing intact proteins from biological sample were anticipated. A liquid chromatography method was developed to demonstrate the feasibility of chromatographic resolution of target proteins to minimize matrix effects and other interferences. Biological matrices including human urine, bovine plasma, and equine plasma were used to prove the concept. The success of this work demonstrates

that a top-down MRM approach for protein quantification is indeed feasible; this finding should be attractive to those interested in developing more simplified top-down methods for protein quantification using QqQ-MS.

3.3 Experimental

3.3.1 Material and Reagents

Protein standards lysozyme (Lysz) (14.2 kDa) from chicken egg white, cytochrome c (Cyt c B) (12.2 kDa) from bovine heart, myoglobin (Myo) (16.9 kDa) from equine skeletal muscle, lactalbumin (Lact) (14.2 kDa) from bovine milk, ubiquitin (Ubiq) (8.6 kDa) from bovine erythrocytes, cytochrome c (Cyt c E) (12.3 kDa) from equine heart, bovine serum albumin (BSA) (66.5 kDa), prostate-specific antigen (PSA) (28.4 kDa) from human serum, trifluoroacetic acid (TFA), and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis MO, USA). Protein standards studied ranged in molecular weight from 8.6 to 28.4 kDa. As biological matrices, human urine was donated from a volunteer in our laboratory and plasmas from equine and bovine were purchased from Innovative Research (Novi MI, USA). LCMS-grade water, methanol (MeOH), and acetonitrile (ACN) were purchased from Honeywell Burdick and Jackson (Morristown NJ, USA).

3.3.2 Intact Protein MRM Method Development and Optimization

Sample Preparation and Instrument Parameters

An LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Scientific Instruments, Inc., Columbia MS, USA) was used to study intact protein quantitation. Intact protein standards were prepared in water and further diluted with a mixture of 50% water and 50% MeOH with 0.1 % FA (by volume) to working standard concentrations for different proteins due to their differences in sensitivity. From the multiply-charged ion envelope for each protein acquired from a Q3 scan event (200-2000 *m/z*), the highest

intensity ion signal was selected to be the precursor ion for further MRM method development. The sample was directly injected using an autosampler (SIL-30 AC; Shimadzu) with a mobile phase of 50:50:0.1 (v/v/v) H2O:MeOH:FA. Optimal operation parameters of the electrospray ionization – triple quadrupole mass spectrometer for intact protein quantification were determined to be as follows: Interface temperature at 300 °C; interface voltage at 4 kV (positive ionization mode); desolvation line temperature at 250 °C; Heat block temperature at 400 °C; heat gas flow, nitrogen at 10 L/min; and drying gas flow, nitrogen at 10 L/min.

Collision Energy and Collision Induced Dissociation Gas Pressure

A series of CE and CID gas pressures were used in different combinations to identify reproducible product ion signals generated from the intact protein precursor ions. The LCMS-8050 uses ultra-high purity argon as the collision gas. Once a few stable product ions were observed, automated MRM optimization was initiated using LabSolutions software (version 5.65) to obtain the optimal collision energy in the range of -5 to -180 V. Ubiq, Myo, and Cyt c B were used to study the relationship between CE and CID gas pressure. The MRM optimizations were conducted at low (40 kPa), medium (100 kPa), and high (270 kPa) CID gas pressure to determine optimal CE to generate selected product ions. Replicates (n=8) at different CID gas pressures for the three proteins were collected to study the signal intensity and the reproducibility of the product ions.

Intact Protein Fragmentation Reproducibility

To demonstrate a) the poor reproducibility of intact protein product ions that hinder the discovery of an unique and reproducible MRM transition for quantitation purpose under certain conditions, and b) the good reproducibility and sensitivity obtained under alternate settings, experiments involving Myo and Ubiq with multiple replicate (n=500) product ion scans at different combinations of collision energies and CID gas

pressures were performed. Myo (100 µg/mL) and Ubiq (1 µg/mL) were directly injected (30 µL) and flowed from the autosampler to the mass spectrometer. CID gas pressure were set at low (40 kPa), medium (100 kPa), and high (270 kPa) values with different collision energies for Myo (-27, -40, -45, and -76 V) and Ubiq (-25, -37, -59, and -70 V). Various collision energies were chosen to cover the range of fragmentation behavior that included no fragmentation, limited fragmentation (with significant precursor remaining), and complete fragmentation (with no precursor remaining). It should be noted that the latter strategy (complete fragmentation) is commonly used for optimization of small molecules MRMs to maximize generation of product ions and consequently, sensitivity.

Intact Protein Separation and Quantitation

A CID gas pressure at 270 kPa was chosen as a generic condition to perform a more in-depth analysis of intact protein quantitation capabilities. Lysz [+10], Cyt c B [+14], Myo [+17], Lact [+8], Cyt c E [+16], PSA [+18], and Ubiq [+11] were all carried through MRM optimization using the 270 kPa CID gas pressure. Two stable and reproducible product ions were selected from each protein to be optimized. These preliminary MRM transitions were then used to track the protein elution during chromatography to determine the elution solvent composition. Under different solvent compositions (variations in organic solvent and other modifier concentrations), the distribution of protein charge states can change. This could alter the protein profile so the highest intensity charge state might be different from the previously determined MRM. Therefore, after the chromatography was developed, the protein charge state distribution was re-evaluated at the elution composition and the highest intensity charge state was selected for a second MRM optimization process.

To address future analyses from complex matrix mixtures, a liquid chromatography method was developed to separate the set of model target proteins.

Lysz (1 µg/mL), Myo (0.05 µg/mL), Cyt c B (0.1 µg/mL), Cyt c E (0.1 µg/mL), Lact (1 µg/mL), Ubiq (0.01 µg/mL), and PSA (100 µg/mL) were prepared in a mixture. Separation was performed using a Shimadzu LC system (AC-30; Shimadzu Scientific Instruments, Inc., Columbia MD, USA) on a reverse phase wide pore Viva C4 column (2.1 x 100 mm, 300 Å; 5 µm, Restek Corporation (Bellefonte PA, USA). Flow rate was set to 0.2 mL/min and the mobile phase was composed of A (H2O + 0.1% FA + 0.05% TFA) and B (ACN + 0.1% FA + 0.05% TFA) components. 20 µL of the protein mixture was injected and the solvent composition was held at 25% B for 1.35 min. A mobile phase gradient from 25 to 85% B was then applied to the column from 1.35 to 21.25 min, followed by 1 min washing (85% B) and a 5 min re-equilibration step (25% B). This method was designed for generic protein separation in biological samples, and thus some peak overlap was to be expected. Overlapping MRMs are easily compensated by the fast scan speed of the triple quadrupole instrument.

The preliminary MRM method was used to track elution during chromatography for solvent composition determination. Protein standards were injected (15 µL) onto the Viva C4 column with the chromatography method developed then eluted into the electrospray ionization triple quadrupole mass spectrometer. The new protein profile was obtained and the highest signal charge state was isolated to develop MRM method for Lysz [+11], Cyt c B [+14], Cyt c E [+14], Myo [+18], Lact [+10], and Ubiq [+8] to ensure optimal detection sensitivity. In principle, the second most abundant charge state can also be used if the most abundant signal ion overlaps with the precursor ion of another protein of interest (e.g. within 1 Da) or two reproducible and stable product ions could not be found. Due to the cost and sample limitation of PSA, calibration curves were only generated and validation was only performed for Lysz, Cyt c B, Cyt c E, Myo, Lact, and Ubiq. Five point calibration curves were generated in triplicate for the six selected

proteins. Accuracy and precision were tested at low, medium, and high concentrations within the calibration range, but at concentrations distinct from the concentrations used to create the calibration curves. Calibration concentrations ranged from 0.5 to 2000 µg/mL due to response factor variations among the proteins. Since noise is essentially negligible in MRM mode, the limit of detection (LOD) calculation of three times standard deviation of the signal divided by the slope of the calibration curve was not applicable. In this study, LOD was first estimated using the slope and the standard deviation of the calibration curve. Then, protein standards with concentrations around the estimated LOD were prepared and analyzed to determine the actual detection limit using 7 replicates. The lowest concentration that could be successfully detected was reported as the LOD. For all six proteins, quantifiable data was obtained at concentrations three times higher than the LOD and no signal could be detected at half of the LOD concentration. In a minimal noise condition, the actual LOQ is often lower than the commonly calculated 3.3 times LOD. In some cases, here, LOQ is equal to the LOD. In this study, the limit of quantitation (LOQ) of the six proteins were determined experimentally as those protein concentration that produced signals with a coefficient of variance of less than 10% following triplicate measurements.

3.3.3 Matrix Effects and Interferences

When dealing with complex biological fluid samples, matrix effects and interferences from other proteins would be anticipated. In order to assess the specificity and susceptibility of intact protein quantitation to these possible complications, mixtures of Cyt c E at a constant concentration of 10 µg/mL with increasing Cyt c B (close sequence homology to Cyt c E) and BSA (a high abundance matrix component) concentrations were prepared. Cyt c B and cytochrome c E only differ by 3 amino acids (97% sequence homology) and were chosen to represent other proteins similar to the

protein target of interest. Cyt c B and BSA were spiked in the sample at 0, 1, 10, and 100 µg/mL. This range represented other proteins existing in the same sample with concentrations from 10 times lower to 10 times higher than the protein of interest. All measurements were made in triplicates and the sample were directly injected into the electrospray ionization mass spectrometry (ESI-MS) without any chromatographic separation.

Although BSA and Cyt c B were chosen to represent other proteins in a biological matrix as a model, and the experiments above demonstrated the possible impact when the analyte coelutes with proteins that are somewhat higher in concentration and similar in sequence, the complexity of the biological matrix itself should not be overlooked. Human urine, plasma from equine, and plasma from bovine were used as sample matrices to demonstrate the specificity and the feasibility of the method. All biological fluids were filtered with VWR 25 mm syringe filters with 0.45 µm nylon membrane to remove debris in the samples. The filtered biological matrices were then diluted 5 times with water and 0.1% FA to represent working solutions for analysis. Biological matrices were first injected (15 µL) and analyzed against the previously determined chromatographic and MRM method to observe any interferences. If the protein MRM method was not unique to the proteins, detection of interfering matrix background would be observed. Protein standards Lysz (500 µg/mL), Lact (1 mg/mL), Myo (35 µg/mL), Ubiq (50 µg/mL), Cyt c B (85 µg/mL) and Cyt c E (90 µg/mL) were then spiked in the biological matrices at the middle of the calibration curves concentrations and analyzed by LC-MS. This study was designed to show the specificity of the MRM transitions to the intact proteins of interest and the feasibility of quantifying proteins in biological matrices through this method on QqQ-MS.

3.3.4 Data Evaluation

Triple quadrupole mass spectrometer data including qualitative and quantitative results were analyzed by Shimadzu LabSolutions Software (v. 5.65). The accurate mass of Lysz, Myo, Cyt c B, Ubiq, and Lact precursor and product ions were obtained using a separate high mass accuracy ion trap - time of flight (IT-TOF) mass spectrometer (Shimadzu) and a Synapt G2 Q-TOF mass spectrometer (Waters). An attempt to assign product ions sequence and charge states was performed using the free web-based top-down mass spectrometry software ProSight Lite.[139]

3.4 Results and Discussion

To develop the capability for direct intact protein quantitation using a triple quadrupole mass spectrometer, to eventually enable absolute protein quantitation, a series of experiments were conducted. In order to quantify target analytes on a triple quadrupole mass spectrometer, one must first find unique and reproducible product ions that represent the protein of interest to create a MRM transition. One of the greatest hurdles of intact protein fragmentation using a triple quadrupole was the irreproducible product ions generated by a complete precursor CID fragmentation process.[128] Figure 1A shows the intact Myo protein charge state distribution generated from the ESI source and measured in scan mode on the QqQ-MS. The highest intensity ion (998.4 [+17]) was selected to be the precursor ion for further fragmentation and eventual MRM method development and optimization. Figure 1B is a full ion scan mass spectrum on the product ions generated from precursor myoglobin ion (998.4 [+17]) at CE = -45 V. At this setting, no consistent or reproducible product ions were obtained. To further demonstrate the fragmentation inconsistency at this setting, the result from 500 scans collected at collision energies sufficient to completely convert all Myo precursor ion (998.4 [+17]) to product ions (CE = -40 and -45 V; CID P = 270 kPa) were analyzed and these are displayed in

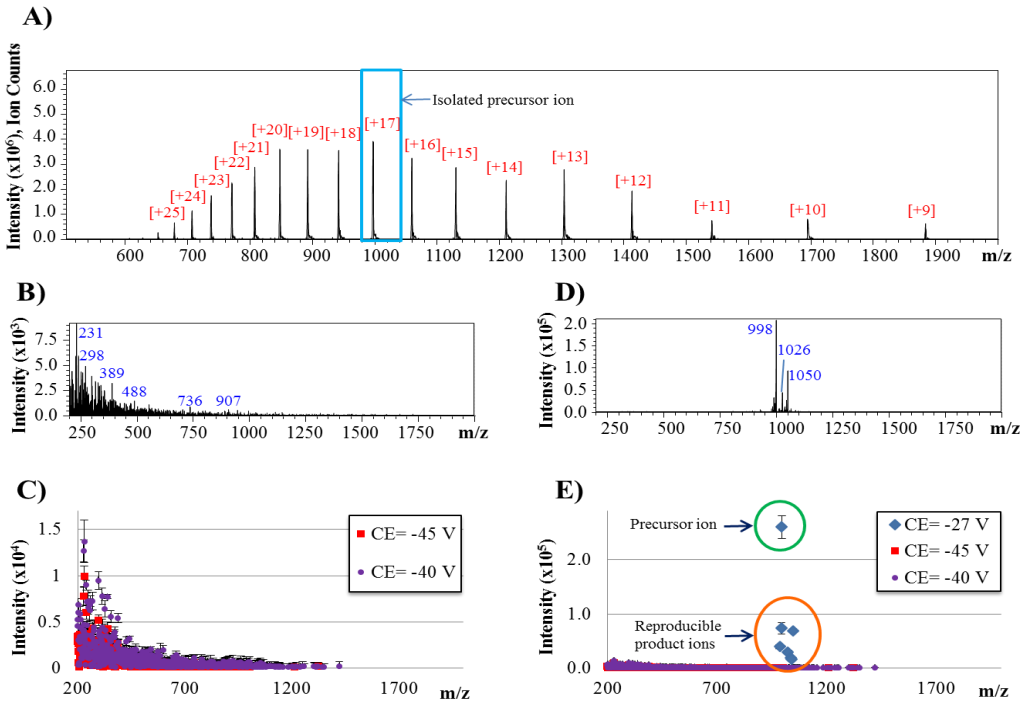


Figure 3-1 Figure 1. A) Myoglobin intact protein charge state distribution generated by electrospray ionization source and obtained by full scan mode. The highest intensity charge state [+17] were chosen to be the precursor ion. B) Myoglobin product ion scan at CID gas pressure= 270 kPa and CE= -45 V. C) Data from 500 product ion scans at CE= -40 and -45 V. D) Myoglobin product ion scan at CID gas pressure= 270 kPa and CE= -27 V. E) Data from 500 product ion scans at CE= -27 V in contrast to CE= -40 and -45 V. Reproducible and consistent product ions at higher signal abundant were obtained while precursor ion was still present.

Figure 1C. Fragment ions were observed across the entire mass range monitored. Approximately 290 product ions were observed above 500 *m/z* with low average signal

intensities (~320 ion counts) and poor reproducibility (120% RSD) revealing their irreproducible and spurious nature. Product ions below 500 *m/z* (n=285) had higher signal intensities (~1600 ion counts), but still had poor stability (27% RSD). The abundance of product ions under these higher collision energy settings made it impossible to select unique and reproducible precursor – product ion pairs for MRM-based quantitation. A similar situation was observed for Ubiq; those data are shown in the Appendix A Figures S1 and S2 for this article.

In order to find settings appropriate for reproducible intact protein fragment ion generation, MS parameters were altered. Among the instrumental parameters adjusted, CE and CID gas pressure applied in the second quadrupole were the two factors that had the greatest effect. Even though the majority of the product ions had low *m/z* ratios and were generally irreproducible, some product ions generated using a combination of moderate CID gas pressure and lower CE were found to be more stable, reproducible, and unique across the different proteins studied. For example, although Myo product ion signals at CE -40 and -45 V were irreproducible and not unique to the precursor ion, reproducible and unique fragment ions were observed at CE = -27 V (Figure 1D). To demonstrate the reproducibility of these product ions generated, an experiment was performed to collect 500 scans under this condition (Figure 1E). The product ion intensities were much higher than the ones from CE = - 40 and 45 V, and the fragmentation was consistent and reproducible. At this lower collision energy, a significant amount of the precursor ion remains. This is in contrast to common practice for MRM optimization on small molecules. Generally, it is thought that better sensitivity is obtained when all of the precursor ion is converted to product. In the case of intact proteins, reproducible (< 10% RSD) and unique product ions were generated at lower CE where precursor ion was still present. Additionally, under these conditions, the intensity

(~25,000 – 75,000 ion counts) of these product ions significantly exceeded that for fragments generated using high CE. It is likely that a combination of greater randomness of fragmentation sites, charge states, and rearrangements are responsible for the lack of reliable MRM transitions at high CE. At lower CE, presumably more selective fragmentations at the weakest links of the protein create unique and reproducible product fragments that could be used for intact protein MRMs.

Since both CE and CID gas pressure were contributors to optimal fragmentation conditions, the combinations of the two were studied. The optimal collision energies obtained following automated MRM optimization at three different CID gas pressures for three model proteins are shown in Figure S3 of the Appendix A Supplementary Information. Two reproducible transitions for each protein were followed. This study revealed that as CID gas pressure was increased, the optimal CE required to form reproducible and intense product ion fragments decreased. Ubiq (8.6 kDa) showed the highest signal and reproducibility at medium CID gas pressure whereas Cyt c B (12.2 kDa) and Myo (16.9 kDa) preferred higher CID gas pressure. The trend of optimal CID gas pressure increasing with increased protein size was apparent.

Though optimal results for protein ions with different sizes were found using different CID gas pressures, the gas pressure cannot be changed during an analytical run. A compromised CID gas pressure of 270 kPa was chosen as a generic condition to perform a more in-depth analysis of the capability for intact protein quantitation. Lysz[+10], Cyt c B[+14], Cyt c E[+16], Myo[+17], Lact[+8], PSA[+18], and Ubiq[+11] were all carried through MRM optimization and two stable and reproducible product ions were selected from each protein at relatively low CE (Table S1 in Appendix A). One of the MRM transitions was used for quantitation while the other was used for confirmation (termed a qualifier ion). This strategy is in accordance with common practice for MRM-

based quantitation on a triple quadrupole and helps ensure specificity.[140] If an interfering matrix ion has the same *m/z* ratio as the analyte of interest and produced a fragment at the same *m/z* ratio as the first product ion, confirming the absence of the qualifier ion aids the assignment of this signal as an interference. While the first product ion can be used to quantify the analyte, the presence of the second product ion can help confirm the existence of the analyte of interest and help discriminate against any interfering ions. The collection of two MRM transitions for each analyte is particularly useful when dealing with quantifying analytes in a complex matrix.

At this point, the identities of the monitored product ions are unknown. However, the accurate monoisotopic mass of all five proteins and their product ions were obtained using separate high mass accuracy mass spectrometers so the fragment identities could be predicted using the amino acid sequence through software.[139] It should be noted that the fragmentation products in these different instruments may not be the same as those generated using the triple quadrupole instrument due to the potentially different fragmentation channels and intensities in different mass analyzers; the use of these supplemental instruments is simply to demonstrate that such steps could be explored if the product ion identity was needed. The Appendix A includes the prospective sequences of the product ions using the high accuracy *m/z* ratio of the product ions with the known protein sequence. Factoring possible charge states can also increase the accuracy of the prospective sequence prediction. Even though higher *m/z* ions may be obtained through the collision induced dissociation process, the product ion is more likely to have a lower charge state than the precursor ion. Each protein produced unique product ions and thus, the general requirement for designating specific MRM transitions for each protein was achieved regardless of the identities of the product ions.

To address determinations from complex biological matrices, a generic liquid chromatography method was developed to demonstrate protein separation in combination with MRM-based detection. The seven protein mixture including Lysz, Myo, Cyt c B, Cyt c E, Lact, Ubiq, and PSA was separated on the wide pore reversed phase protein separation column using gradient elution (Figure 2). Various concentrations were used for different proteins to compensate the different response factors. The separation method was not optimized for the seven proteins since they were only used as an example to demonstrate proof of principle. While baseline separations were not achieved between all peaks, most of the proteins were separated to show the potential for mitigating matrix effects or other ionization interferences. It should be noted that Cyt c E and Cyt c B were baseline resolved using this method, even though they have high sequence homology.[141] The effect of the presence of an interfering protein, BSA, was evaluated with the method as well. Although an MRM transition of the BSA was not determined, in full scan mode, BSA was observed to elute at 8.1 min. In other words, this model high abundance protein could be chromatographically resolved from the model target proteins.

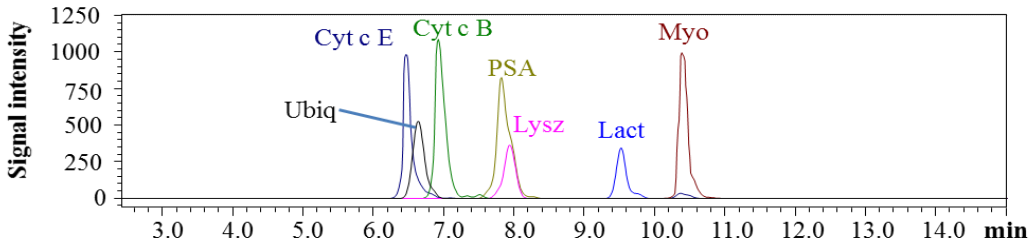


Figure 3-2 Seven Protein Separation Using a Liquid Chromatography Method

A top down intact protein quantitation strategy could mitigate the problems with targeting low abundance proteins experienced with bottom-up approaches, since high

abundance proteins can be confined to individual peaks in a chromatogram during the former. In bottom-up approaches, the peptides generated from high abundance proteins can mask the peptides generated from low abundance proteins – peptides from all digested proteins are spread throughout the chromatographic separation space. In the top-down approach, low abundance target proteins would be single peaks that could be resolved chromatographically from high abundance protein interferences. For specific samples and assays, further optimization can be performed to ensure better separation and increase the accuracy and precision of target protein quantitation.

The previously determined MRM transitions enabled detection of the proteins during chromatographic method development. However, elution of the proteins at different organic solvent compositions or modifier concentrations can cause changes in the protein charge state distribution. Consequently, the highest intensity charge state might be different from the previously isolated precursor ion for MRM determination. The decrease in precursor ion signal intensity can significantly reduce the sensitivity of the method. Therefore, after the chromatography was developed, the protein profiles were obtained again at the elution composition and the highest intensity charge states were re-selected for MRM optimization (Lysz [+11], Cyt c B [+14], Cyt c E [+14], Myo [+18], Lact [+10], and Ubiq [+8] as shown in Table 1). The mass spectra containing the intact protein charge state distribution and product ions are shown in Appendix A Figures S4-S10 and the data for intact protein molecular weights are given in Tables S2-S7.

Table 3-1 Optimized Protein MRM Transitions of Myoglobin, Lysozyme, Ubiquitin, Lactalbumin, Cytochrome c (E), Cytochrome c (B), and Prostate-Specific Antigen (PSA)

Protein	Molecular Weight (Da)	MRM	Q1 Bias (V)	Collision Energy (V)	Q3 Bias (V)
Myo	16942 ± 2	942.8 [+18] > 933.1	-28	-24.0	-26
		942.8 [+18] > 948.4	-28	-8.9	-36
Lysz	14295 ± 1	1301.6 [+11] > 1310.4	-42	-4.8	-50
		1301.6 [+11] > 1315.8	-42	-5.5	-46
Ubiq	8560 ± 2	1072.0 [+8] >1307.0	-30	-37.9	-48
		1072.0 [+8] >1084.6	-30	-10.0	-32
Lact	14168 ± 1	1417.4 [+10] > 1414.9	-42	-43.1	-48
		1417.4 [+10] > 1427.1	-42	-7.9	-44
Cyt c E	12352 ± 2	883.9 [+14] > 824.6	-24	-30.6	-30
		883.9 [+14] > 891.1	-24	-6.7	-24
Cyt c B	12223 ± 1	874.6 [+14] > 881.9	-34	-7.0	-34
		874.6 [+14] > 889.8	-34	-5.5	-36
PSA	28434 ± 2	1580.9 [+18] > 1666.5	-36	-34.6	-32
		1580.9 [+18] > 1564.4	-36	-37.2	-44

Five point calibration curves (Appendix A Figures S11-S16) for each of the six proteins were generated in triplicate. Accuracy, precision, linearity, linear range, limit of detection and limit of quantitation were also determined and are reported in Table 2. Respectable linearities (R^2> 0.99) were obtained in all calibration curves. Calibration linear ranges were different for all proteins due to their different response factors and detection limits. Figure S17 in the Appendix A demonstrates the use of Myo as an example to show a greater dynamic range may be considered if nonlinear responses at higher concentrations are accounted. It is useful to note that the sensitivities for different proteins, as judged by the slope of the calibrations curves, varied by as much as one – three orders of magnitude. The accuracy and precision tests were performed by testing protein standards at low, medium, and high concentrations within the respective calibration ranges for each protein. The errors in accuracy of all three points on all six proteins were under 5%. The data were also characterized by relatively high precisions with low coefficients of variation (1 – 12%). The LOD and LOQ obtained for the six protein standards ranged from 0.5 to 40 µg/mL and 0.5 to 50 µg/mL, respectively. Although the LOD and LOQ using this top-down approach is not lower than typical bottom-up approaches, where detection limits for peptides may be reported in the pg/mL to ng/mL range,[142] the LOD and LOQ of this triple quadrupole mass spectrometer method is on par with the various concentrations expected for proteins in many biological fluid samples.[143] It is very close to levels of the lowest abundance proteins (< 500 ng/mL) that are needed to be detected for some biomarker quantitation applications.[144]

Table 3-2 Large Table 2. Intact protein quantitation using QqQ-MS information including calibration curve linearity, linear range, limit of detection (LOD), and limit of quantification (LOQ) of each proteins. Quantitation precision and accuracy obtained from measuring the protein standard at low, medium, and high concentrations within the linear range of each protein.

Protein	Linearity (R^2)	Linear Range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)	Prepared conc. (µg/mL)	Measured conc. (µg/mL)	Precision (SD/mean)	Accuracy (measured-prepared)/prepared
Myo	0.9996	5-75	1	3	7.5	7.8 ± 0.8	0.1	0.04
					25	25 ± 2	0.06	0.03
					50	52 ± 3	0.07	0.04
Lysz	0.9945	50-1000	40	50	250	255 ± 7	0.03	0.02
					500	520 ± 40	0.07	0.05
					800	770 ± 60	0.08	-0.03
Ubiq	0.9998	1-100	0.5	0.5	5	4.9 ± 0.2	0.05	-0.03
					40	41 ± 2	0.05	0.02
					75	75 ± 2	0.03	0.004
Lact	0.9961	100-2000	30	50	300	301 ± 9	0.07	0.005
					900	890 ± 20	0.02	-0.01
					1600	1600 ± 100	0.03	-0.005
Cyt c E	0.9995	5-175	3	5	40	41 ± 1	0.03	0.02
					75	79 ± 1	0.01	0.05
					125	122 ± 5	0.04	-0.02
Cyt c B	0.9968	20-150	5	10	40	38 ± 5	0.12	-0.04
					75	78 ± 2	0.03	0.04
					140	139 ± 1	0.009	0.008

It is not surprising that the bottom-up approach would have better sensitivity since peptides generally can be separated more efficiently, have high ionization efficiency (their ESI response is only distributed over a few charge states at most), and higher fragmentation efficiency in tandem mass spectrometry than large protein precursors.[145] Yet, the bottom-up approach also quantifies protein based on the assumptions of complete and reproducible protein digestion and that the target peptide is solely derived from the specific protein of interest.[146] These assumptions do not always hold true and the lost connection between protein and its peptides through digestion makes it more difficult to trace back to account for the errors. Even though bottom-up protein quantitation is quite mature, the creation of new standardization procedures to account for these limitations is still an active area of research.[147] Of course, the development of a specific application with the top-down approach on a triple quadrupole mass spectrometer would require comprehensive evaluation of sample preparation, sample enrichment (if necessary), and matrix effects that would be expected to alter quantitative performance from the idealized case presented in this paper.

Matrix effects are a significant consideration when quantifying analytes from a biological sample using ESI-MS.[43a, 148] Not only is the sample complex due to the presence of multiple classes of compounds, other proteins besides the protein of interest often exist in a wide range of abundance. Matrix effects in biological fluids thus come in two main sources: Ion suppression and MRM transition interference. In an effort to study the effect of variable matrix protein concentrations in a sample on the specificity of target protein quantitation, experiments were performed to target Cyt c E as the protein of interest in the presence of Cyt c B and BSA as potential interferences. A general increase of uncertainty was observed as the excess protein matrix concentrations increased (Appendix A Figure S18). Cyt c E peak areas with excess protein at different

concentrations were compared to the result of pure Cyt c E sample in the presence of no additional proteins. In the presence of both BSA and Cyt c B studies, Cyt c E areas were not heavily affected with lower concentrations of excess proteins (at one-tenth the target protein concentration). When excess protein concentrations were raised to be ten times the protein of interest, the peak areas and standard deviations were significantly affected. With high concentration of BSA, the peak area of Cyt c E increased by 60%, but the standard deviation increased 6 fold.

With the increase of BSA as an excess protein, a significant matrix effect was observed. The ion signals generated by the charge state envelope of BSA do not overlap with the precursor ion signal of Cytc E; thus, the matrix effect observed can be attributed to competitive ionization during the ESI process. In the case of excess Cyt c B at high concentration, peak area of Cyt c E doubled and the standard deviation quadrupled. The increase of Cyt c E signal with increasing Cyt c B concentration is likely to be caused by interferences in the mass analyzer. Since the two proteins are very similar (97% sequence homology), there is a better chance of some interferences resulting from similarities in *m/z* for precursor or product ions chosen.

Overall, the result showed that matrix effect from co-elution of a high abundance of excess proteins would likely be a major problem for target protein quantitation. In such cases of targeted protein quantitation using the presented method, it would be important to optimize chromatography and to assess the presence of matrix effects. However, this is no different than standard best practices used for small molecule quantitation. Using the generic chromatographic method developed in this study, Cyt c E (t_R = 6.5 min) was baseline resolved from both Cyt c B (t_R = 7.0 min) and BSA (t_R = 8.1 min). Although the homological sequences of the two Cyt c appeared to pose a problem for discrimination by the mass analyzer, it was not difficult to separate the two using chromatography. In

general, efforts to separate excess interfering proteins using chromatography would certainly be preferred to help mitigate matrix effects in a specific application method.

The BSA and Cyt c B studies demonstrated the impact of coexisting highly abundant protein and protein with similar sequence in the same sample. However, these studies alone cannot approximate the complexity of biological fluids. Therefore, human urine, plasma from equine, and plasma from bovine were used as sample matrices to show the feasibility of the MRM intact protein quantitation method on complex biological fluids. Figure 3A shows the chromatogram of blank urine while the MRM transitions of Lysz, Lact, Myo, Cyt c B, Cyt c E, Ubiq, and PSA were being monitored. Figure 3B is the chromatogram of the urine spiked with Lysz, Lact, Myo, Cyt c B, Cyt c E, and Ubiq. Figures 3C and 3D are the chromatograms of horse plasma without and with protein spikes, respectively; and Figures 3E and 3F are the chromatograms of bovine plasma without and with protein spikes, respectively. As the data revealed, no protein MRM peaks were observed when no protein standards were spiked into the matrices. In other word, no interferences with the specific MRM transitions were detected from the blank matrices. Since no proteins were detected with pure matrices using the MRM method, it

was clear that the MRM transitions were specific to the proteins of interest. With proteins

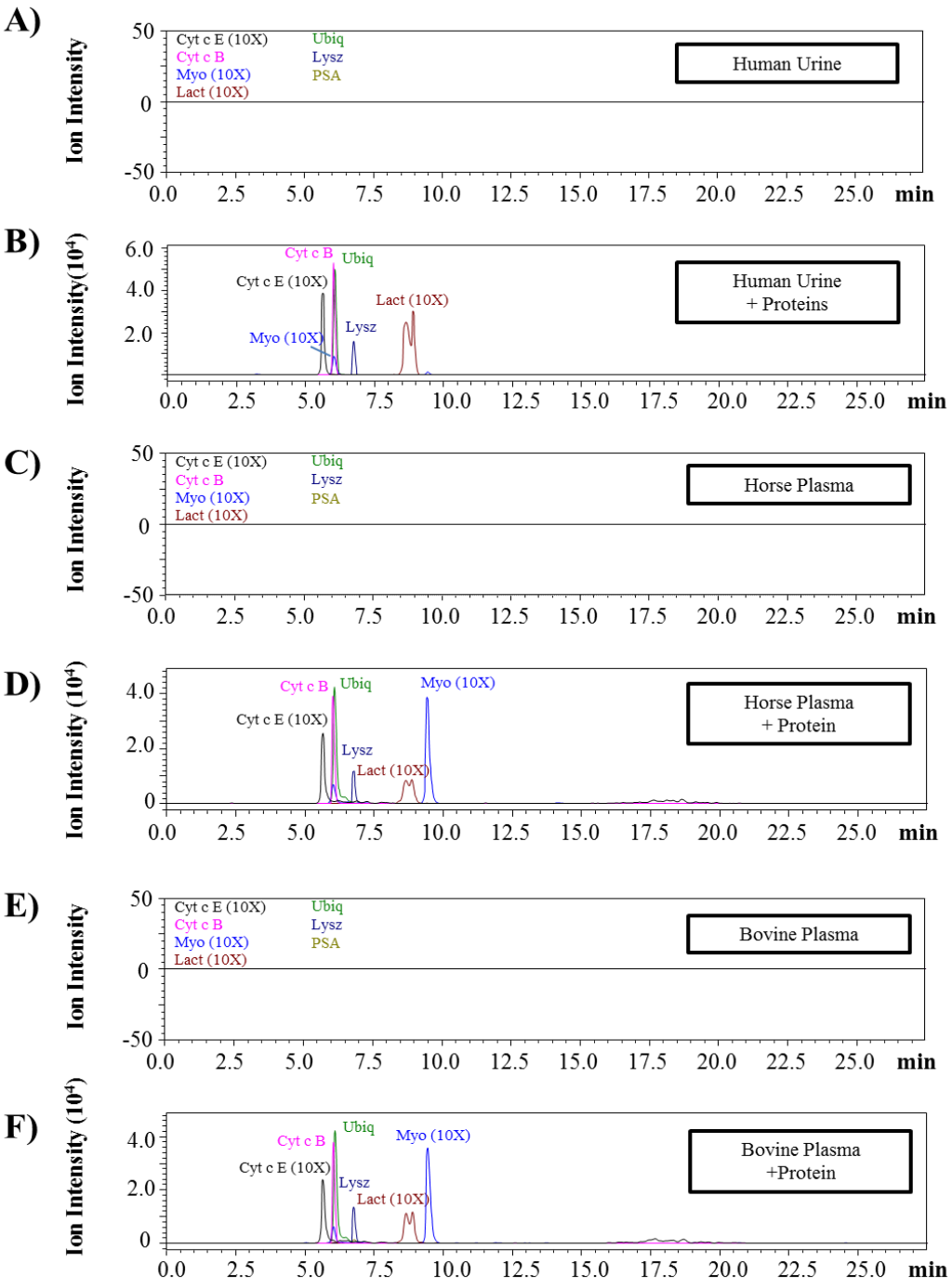


Figure 3. Sample biological matrices including human urine, horse plasma, and bovine plasma were analyzed using a combination of liquid chromatography and QqQ-MS detection. MRM transitions of Lysz, Lact, Myo, Cyt c B, Cyt c E, Ubiq, and PSA were being monitored. Chromatograms of A) human urine, B) human urine with protein spiked in, C) horse plasma, D) horse plasma with protein spiked in, E) bovine plasma, F) bovine plasma with protein spiked in generated by the MRM detection were obtained the demonstrate the specificity and the feasibility of the method.

standards spiked into the matrices, all proteins were successfully detected. Although some matrix effect were seen, especially in the case of Myo and Lact, with some signal suppression and peak splitting compared to the same protein concentration in water. The detection and sensitivity for the other proteins were not significantly effected in the presence of biological matrices. In principle, further variation of chromatography conditions could be made to ameliorate changes in peak quality in the presence of the biological fluid, but this was judged to be beyond the scope of this study.

3.5 Conclusion

In this work, the difficulties associated with generating reproducible MRM transitions in a top-down approach to intact protein quantification on a triple quadrupole mass spectrometer were evaluated and overcome. Although abundant low mass fragments were still observed and much of the recorded fragmentation patterns for each protein were irreproducible, a few unique, stable, and reproducible product ions generated under moderate CID pressure with low CE were found for each model protein. We believe that extensions of this approach to higher mass proteins is feasible, assuming that the multiply-charged protein ion envelope is visible in the limited mass range of the triple quadrupole instrument. Protein supercharging may prove useful in this regard. Not

all commercial triple quadrupole instruments allow variation of CID gas pressure, and this may limit the ability to generate reproducible fragment ions and MRM transitions in some systems. Even so, triple quadrupole instruments are generally more affordable than many high resolution instruments currently used (but, often for different purposes than quantitation) for top-down protein analysis.

To quantify intact proteins, two stable and reproducible protein fragment ions need to be identified and optimized. One of the product ions will be used as the main transition for quantitative analysis and the other as the supportive confirmatory signal for aided specificity. This fast and direct method that bypasses the hassle of protein digestion and related preparation should be beneficial in many fields and applications. Being able to achieve absolute protein quantitation without the tremendous investment in stable-isotope labeled proteins can solve the current drawback in clinical fields where quantitative results cannot be compared from lab to lab, or day to day. Acquisition of appropriate standards for external or internal standard quantitation of many proteins is either expensive or lacking. The attractiveness of this technique could spurn new focus on more affordable production of protein standards, e.g., through large fermentation runs and purification, etc.

While our approach may not be as sensitive as more common methods based on targeted peptides from protein digests,[123] it is clear that much less effort has gone into studying ionization efficiencies and potential for matrix effects and interferences when quantifying intact proteins directly. Our demonstrated ability to target intact proteins directly using MRMs should lead to a more concerted effort to both understand ionization effects associated with intact protein analysis and the optimization of instrumentation for more sensitivity, given the fact that it allows for direct quantitation without the need for digestion and it enables absolute protein quantitation. Even so, a few challenges remain,

and these are currently under further investigation in our laboratory. Considerations should be made for choosing and incorporating appropriate internal standards for intact proteins into specific quantitative applications. Intact protein MRM-based quantification may be less feasible for use in detecting various different proteoforms of a target protein analyte if their mass differences are not within the differential power of the QqQ-MS. The methodology would be most suitable for generation of methods that are designed to detect global changes in a protein level. Yet, with appropriate calibration, absolute determination of the levels of target proteins could be determined, in a similar manner to small molecule protein analysis. Although matrix effects from excess proteins and biological samples were preliminarily evaluated in this study, more work is needed to assess the prevalence of matrix effects, and this is best done in the context of specific applications. In fact, the combination of high efficiency chromatography and specific MRM transitions appear to be provide significant advantages when a method is desired for biological samples that contain a high dynamic range of protein concentrations. Use of appropriate MRM settings would make the mass spectrometer blind to high abundance proteins – only interferences and matrix effects need to be considered, but this is no different than the case for small molecule analysis. Further, dedicated methods will also require the use of appropriate sample preparation and chromatographic techniques, fields that are advancing quickly with the increased attention being placed in the realm of biomarker quantitation and biopharmaceutical development.

Chapter 4

Reversed-Phase Separation Parameter for Intact Proteins using Liquid Chromatography – Triple Quadrupole Mass Spectrometry

4.1 Abstract

The separation of intact proteins is inherently more complex than that for small molecules. In reversed-phase liquid chromatography, chromatographic parameters such as mobile phase, temperature, and stationary phase can affect protein conformation, accessible surface functional groups, and interactions with the stationary phase and underlying solid support. Although protein separations have been studied for many years, direct detection of intact proteins with mass spectrometry requires special considerations of mobile phase additives to achieve efficient separation and sensitive detection. Myoglobin, cytochrome c, lactalbumin, lysozyme, and ubiquitin were used as model analytes to investigate chromatographic method development using a triple quadrupole mass spectrometer and detection by multiple reaction monitoring. Chromatographic parameters including the concentration of trifluoroacetic acid, flow rate, gradient volume, temperature, mobile phase composition, and stationary phase chemistry were evaluated. Protein charge state profiles were also monitored for temperature and modifier effect. An optimized method using 0.2 mL/min flow rate, 15% gradient volume, and 75 ˚C with a combined trifluoroacetic acid and formic acid modified mobile phase was developed.

4.2 Introduction

Proteins are primary components for building and repairing life processes. With new technologies and methodologies, proteomics has gained momentum and supported developments in the fields of biomarker discovery and biotherapeutics. This trend requires not only protein identification and discovery work, but also methods that reliably quantify proteins. Protein quantitation is especially useful for monitoring disease

indicators in biological fluids, as well as quantifying protein levels during protein therapeutic treatments and in the stages of drug development and response control throughout clinical trials. Though the need for protein quantitation is great, the complexity of biological matrices is also high. To address these challenges, methods that enable efficient separation and specific detection of proteins are desirable.

Protein separation methods have been widely used for many years and several modes of separation have been developed. Size exclusion chromatography separates proteins according to their hydrodynamic volume.[149] It is useful for studying protein aggregation, which is a major consideration for protein therapeutic quality control.[150] Ion exchange chromatography separates proteins based on charge. Proteins can differ in their isoelectric points and protein charges can change due to post-translational modifications or processing and handling.[141] Affinity chromatography refers to extremely selective isolation of specific proteins, usually from a complex mixture. However, it requires specific antibodies for proteins of interest that can be costly and of limited availability. Reversed-phase liquid chromatography (RPLC) has also been widely used for protein separation.[151] Separation is typically according to hydrophobicity and protein conformation. Other RPLC benefits include a high degree of selectivity, and if volatile buffers or additives are used, mass spectrometric detection can be considered.

Chromatographic separations of proteins are complicated relative to small molecules due to complex protein structures, which can exhibit multiple conformations and a plethora of functional units. It is important to understand that the chemical forces governing protein conformation (ionic, non-polar, van der Walls, and hydrogen-bonding) are the same forces governing chromatographic interactions. When changing chromatographic parameters, such as mobile phase, temperature, and stationary phase, protein conformations are likely to change as well. Interactions with the mobile and

stationary phases can change the protein structure and thus, alter the functional group accessibility on the protein surface, in turn affecting its chromatographic behavior.[152] RPLC involves the use of aqueous and polar organic (e.g., acetonitrile, methanol, and isopropanol, among others) solvents. While a specific combination of the two may be used for isocratic separation of small molecules, the efficient separation of multiple proteins requires the use of a gradient mobile phase method, where the organic composition is constantly increasing throughout the analysis. This is because proteins exhibit sharp elution isotherms; they essentially remain bound and will not elute until an appropriate organic composition is reached to release the protein from the stationary phase.[153] Different proteins exhibit different elution isotherms. For the same reason, slow shallow gradients are generally believed to improve chromatographic resolution. A steeper gradient may increase sensitivity by sharpening the peaks, but resolution can be compromised due to early elution.[154] Research has also shown that increased retention of proteins is sometimes observed at higher organic concentration.[155] This means that the organic solvent concentration window for protein elution is much smaller than for small molecules. For example, a small molecule's elution window (as defined by capacity factors between e.g., 2 and 10) can be from 10-70% acetonitrile, where as a protein's elution window may only be from 55-60% acetonitrile.

To enhance protein separation, ion-pairing additives are often added to the mobile phase system. Trifluoroacetic acid (TFA) is generally known to improve protein peak shapes in chromatography by masking the silanolid interaction. However, it is widely known as an additive that will suppress electrospray ionization efficiency and mass spectral detection sensitivity.[156] The pros and cons associated with additives in terms of separation and detection should be carefully weighed and necessary compromises often need to be made.

Mobile phase flow rate is another parameter that can have a profound effect on protein chromatography. With use of modern small diameter particles, faster flow rates reduce analysis time but can sacrifice resolution. It is reported that slower flow rates are better for proteins; however, such conditions can allow proteins desorbed from the stationary phase to partially renature while passing through the column. If multiple conformations exist, the result can be extreme peak broadening or peak splitting.[157] Nevertheless, because protein – stationary phase interaction kinetics are slow, slower flow rates may better preserve the integrity of an analyte band on column.

Column temperature is another critical parameter for protein chromatography. Higher temperatures generally aid separation by avoiding the formation of macromolecular secondary structure,[158] stimulating the diffusion process due to reduced mobile phase viscosity, and accelerating adsorption/desorption rates. However, high temperatures can also degrade some proteins, as well as some stationary phases.[154] While the highest operational temperature provided by the column manufacturer should be respected, protein separations should generally be performed at higher than ambient column temperatures to obtain the best results.

The current gold standard for quantifying proteins is through their digestion into constituent peptides and the use of RPLC with electrospray ionization (ESI)-triple quadrupole mass spectrometry (QqQ-MS), termed a bottom-up strategy.[121a, 135] For quantification, QqQ-MS is usually operated in multiple reaction monitoring mode (MRM), where the ions of interest are isolated as precursor ions in the first quadrupole, fragmented in the second quadrupole, and unique fragments, generated from the analytes of interest, are targeted as product ions in the third quadrupole prior to detection. The signal-to-noise ratio is significantly enhanced due to decreased noise in MRM mode. QqQ-MS is both sensitive and specific enough to address the very low

concentrations of analytes, especially when isolated from complex mixtures using appropriate sample preparation and chromatography.

Bottom-up methods can be time consuming and errors associated with the digestion step can easily propagate, which can compromise the ultimate precision and accuracy attainable. Incomplete digestion steps may make absolute protein quantitation very difficult. However, direct quantitation of intact proteins using QqQ-MS in a top-down fashion has not been demonstrated to a significant extent. The lack of development is partially due to abundant irreproducible fragment patterns observed during macromolecule fragmentation in a QqQ-MS at commonly used collision energies, which complicates MRM method development.[128] Others reasons are the combination of using a low resolution instrument and the overall complexity of the protein.

An intact protein MRM method using QqQ-MS was recently developed in our lab and was proven to be feasible for combination with RPLC; model calibration curves of five proteins with respectable linearity and sensitivity were shown, along with specificity for detection of target proteins present in biological fluids.[159] A key was tuning collision energy and collision gas pressure in the second quadrupole to maximize the abundance and reproducibility of a select number of unique fragment ions from a single intact protein charge state. Such an approach may help simplify protein quantitation; eliminating the digestion step saves money, time, and resources while potentially improving quantitation accuracy and enabling absolute protein quantitation so results can be compared across different laboratories.

The aim of tqis study was to evaluate the optimization of RPLC in combination with MRM-based QqQ-MS detection. Although significant work has been performed in protein separation, LC-MS-based intact protein separations have not been widely reported. Consequently, less effort has been placed on evaluating optimal RPLC

chromatography settings for intact protein separation in the context of mass spectrometric detection. While reversed-phase chromatography is widely used for protein separation, common problems that deviate from ideal chromatography include the observation of broad and asymmetrical peaks, multiple peaks from one protein, low recovery, and ghost peaks. In this study, we systematically investigated chromatographic parameters that affect reversed-phase protein separations. We evaluated the effect of chromatographic parameters on protein charge state distributions, since the selection of an appropriate charge state of a parent ion for MRM optimization is a key consideration for sensitivity. Parameters including the addition of different concentrations of TFA, column oven temperature, flow rate, and gradient volume were assessed. The optimal separation conditions were then applied to compare the performance of a set of stationary phase and mobile phase combinations for further selectivity and resolution enhancement.

4.3 Experimental

4.3.1 Reagents, chemicals, and materials

Protein standards lysozyme (Lysz; 1) (14.2 kDa) from chicken egg white, cytochrome c (Cyt c B; 2) (12.2 kDa) from bovine heart, myoglobin (Myo; 3) (16.9 kDa) from equine skeletal muscle, lactalbumin (Lact; 4) (14.2 kDa) from bovine milk, ubiquitin (Ubiq; 5) (8.6 kDa) from bovine erythrocytes, cytochrome c (Cyt c E; 6) (12.3 kDa) from equine heart, trifluoroacetic acid (TFA), and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis MO, USA). LCMS-grade water, methanol (MeOH), and acetonitrile (ACN) were purchased from Honeywell Burdick and Jackson (Morristown NJ, USA).

4.3.2 Sample Preparation and Instrument Parameters

Intact protein standards were prepared in water and then diluted with 0.1% FA to working concentrations. Due to differences in response factors, working solution concentrations varied for different proteins. For each protein standard, a multiply-charged ion envelope was acquired from a Q3 scan event (200-2000 *m/z*). The highest intensity ion signal was selected as the representative precursor ion to proceed through MRM optimization in a QqQ-MS, as described previously.[159] Precursor ions were isolated and passed through the collision cell to produce unique product ions that were used for selective protein detection. The optimized MRM was used for the detection of the proteins during chromatographic method development. The optimized MRMs for each protein are given in the Supplemental Information in Appendix A Table S1.

10 µL of a protein mixture (detailed below) was injected onto the column using an autosampler (SIL-30 AC; Shimadzu Scientific Instruments, Inc., Columbia MD). Separation was performed using a Shimadzu LC system (AC-30) on a reversed-phase wide pore Viva C4 column (2.1 x 100 mm, 300 Å pore size, 5 µm dp; Restek Corporation, Bellefonte PA) for all studies. After optimal separation conditions were determined, mobile phases (ACN and MeOH) and stationary phases were investigated further with additional Viva C8, C18, Biphenyl, and PFPP columns (2.1 x 100 mm, 300 Å pore size, 5 µm dp; Restek).

A LCMS-8050 triple quadrupole mass spectrometer (Shimadzu) was used as the detector. Optimal operation parameters of the electrospray ionization – triple quadrupole mass spectrometer for intact protein quantification were determined to be as follows: Interface temperature at 300 °C; interface voltage at 4 kV (positive ionization mode); desolvation line temperature at 250 °C; Heat block temperature at 400 °C; heat gas flow, nitrogen at 10 L/min; and drying gas flow, nitrogen at 10 L/min; and collision induced

dissociation gas pressure, argon at 270 kPa. Data was analyzed using Shimadzu LabSolutions Software (v. 5.65).

4.3.3 Liquid Chromatography Separation parameters

A protein standard mixture (mixture 1) was prepared to contain the following analytes: Myo (100 µg/mL), Cyt c B (100 µg/mL), Ubiq (0.1 µg/mL), Lact (1000 µg/mL), and Lysz (100 µg/mL). Initially, a generic method was developed using a 0.2 mL/min flow rate of mobile phase A (H2O + 0.1 % FA) and B (ACN + 0.1 % FA) on a Viva C4 column. (It is worth noting that these are not the final mobile phase compositions determined to provide the optimum results; the best results were achieved with the addition of 0.1% FA and 0.05% TFA to both the A (H2O) and B (ACN) mobile phase channels, as detailed below.) 25% B was held for 1.35 min followed by a gradient from 25 to 85% B from 1.35 to 21.25 min. A 1 min wash and a 5 min re-equilibration step were added to the end of the gradient.

TFA effect

Generally, the use of TFA in LC-MS analysis is discouraged due to its ionization suppression effect. However, addition of TFA as an ion-pairing reagent for better peak shape has been commonly practiced, especially when chromatographic detection was accomplished by other means, such as UV absorption. [160] To evaluate the effect of TFA on chromatographic quality and mass spectrometric sensitivity, in hopes of finding a practical compromise, 0, 0.01, 0.05, and 0.1% TFA were added to the mobile phase. The additions were made in addition to the presence of 0.1% formic acid. Studies using TFA alone did not result in good performance (data not included).

Gradient volume

Since the protein elution window was relatively small, it was reasonable to assume a shallower gradient would better complement protein separations. However,

peak broadening and impractically long analysis times could be potential drawbacks. The effect of various gradient volumes (5, 10, and 15 %B/mL) on chromatographic separation were evaluated. A second protein mixture (mixture 2) was prepared to contain Lysz (400 µg/mL), Myo (0.04 µg/mL), Cyt c B (0.04 µg/mL), Cyt c E (0.04 µg/mL), Lact (2 mg/mL), and Ubiq (0.012 µg/mL). Cyt c E was added to mixture 2 to study the behavior of proteins with similar sequences. Cyt c E and Cyt c B have 97% sequence homology, differing by only three amino acid residues. Triplicate injections were performed for each gradient volume. Gradients for all gradient volumes were set from 25 to 85% B, thus the length of the gradient duration varied. 5 %B/mL required 60 min, 10 %B/mL required 30 min, and 15 %B/mL required 20 min for the gradient to progress from 25 to 85% B. Flow rate was set to 0.2 mL/min, and the Viva C4 column with mobile phases A (H2O + 0.1 % FA + 0.05% TFA) and B (ACN + 0.1 % FA + 0.05% TFA) were used.

Flow rate

Optimal flow rate determination was performed with the gradient volume set to 15 %B/mL. Mobile phase was composed of A (H2O + 0.1 % FA + 0.05% TFA) and B (ACN + 0.1 % FA + 0.05% TFA), and the Viva C4 column was used. The gradient time of the methods was adjusted to deliver the same amount of solvent at each solvent composition with different flow rates. For example, gradient time for 0.1 mL/min will be double the gradient time for 0.2 mL/min to allow the same volume of the specific solvent composition to pass through. Therefore, the amount of solvent at a specific organic composition can be controlled to be unbiased for protein elution at different flow rates. Flow rates of 0.1, 0.2, 0.3 and 0.6 mL/min were investigated in triplicate with protein mixture 2.

Temperature

To study the effect of temperature on protein separation, column oven settings were varied between 30, 45, 60, and 75 ˚C. These temperatures ranged from

approximately ambient to the limit of highest operational temperature suggested by the column manufacturer. Flow rate on a Viva C4 column was set to 0.2 mL/min, gradient volume was 15 %B/min, and mobile phase was composed from A (H2O + 0.1 % FA + 0.05% TFA) and B (ACN + 0.1 % FA + 0.05% TFA). Protein mixture 2 was injected in triplicate to evaluate each column temperature.

Protein charge state profile monitoring

Changes in acid modifier, temperature, mobile phase, and stationary phase can alter protein conformation, and consequently, the charge state distribution for the protein generated by electrospray ionization. Because MRM transitions are optimized using a single charge state (ideally the most abundant one), it is practical to monitor changes in charge state abundance as chromatographic parameters are varied.

Protein mixture 3 was prepared with higher concentrations of proteins to more easily visualize changes in charge state distributions. Mixture 3 contained Lysz (1 mg/mL), Myo (0.4 µg/mL), Cyt c B (0.4 µg/mL), Cyt c E (0.4 µg/mL), Lact (2 mg/mL), and Ubiq (0.12 µg/mL). The effect of TFA concentration, temperature, mobile phase, and stationary phase on charge state distribution for each protein was monitored in triplicate. Protein profiles were collected in a Q3 scan event (200 – 2000 *m/z*). The effect of TFA was monitored with both 0 and 0.05% TFA present in the mobile phase. Column oven temperature profiles at the extremes of 30 and 75 ˚C were compared.

4.4 Result and Discussion

4.4.1 TFA effect

Trifluoroacetic acid is a common ion-pairing reagent. Its volatile nature allows it to be used in conjunction with electrospray ionization MS detection. Due to its ion-pairing nature, the presence of TFA in the mobile phase can significantly improve chromatographic peak shape for proteins relative to other weak acids, but it can also

reduce ionization efficiency.[161] The compromise with addition of variable amounts of TFA was investigated first.

The susceptibility of column chemistries to extreme pH values was also considered. The pH of aqueous mobile phase mixtures with different TFA concentrations, in the presence of 0.1% formic acid, were tested with a pH meter. Changing TFA concentration from 0 to 0.1% resulted in a variation of pH between 2.80 to 2.07. The changes in the acidity with the minimal addition of TFA were relatively small and remained within the tolerances of the Viva column line tested in this study.

Figure 1 showed the effect of changing TFA concentration on generated chromatograms for the model protein mixture. With formic acid alone (no TFA added), peak shapes were wide and asymmetrical. Some of the proteins did not elute completely until the end of the gradient (Figure 1A). Results obtained with 0% TFA were not reproducible with respect to chromatographic retention and MS response. Even with this simple protein standard mixture, some co-elution was observed. Co-eluting proteins myoglobin and lactalbumin also exhibited MRM detection interferences.

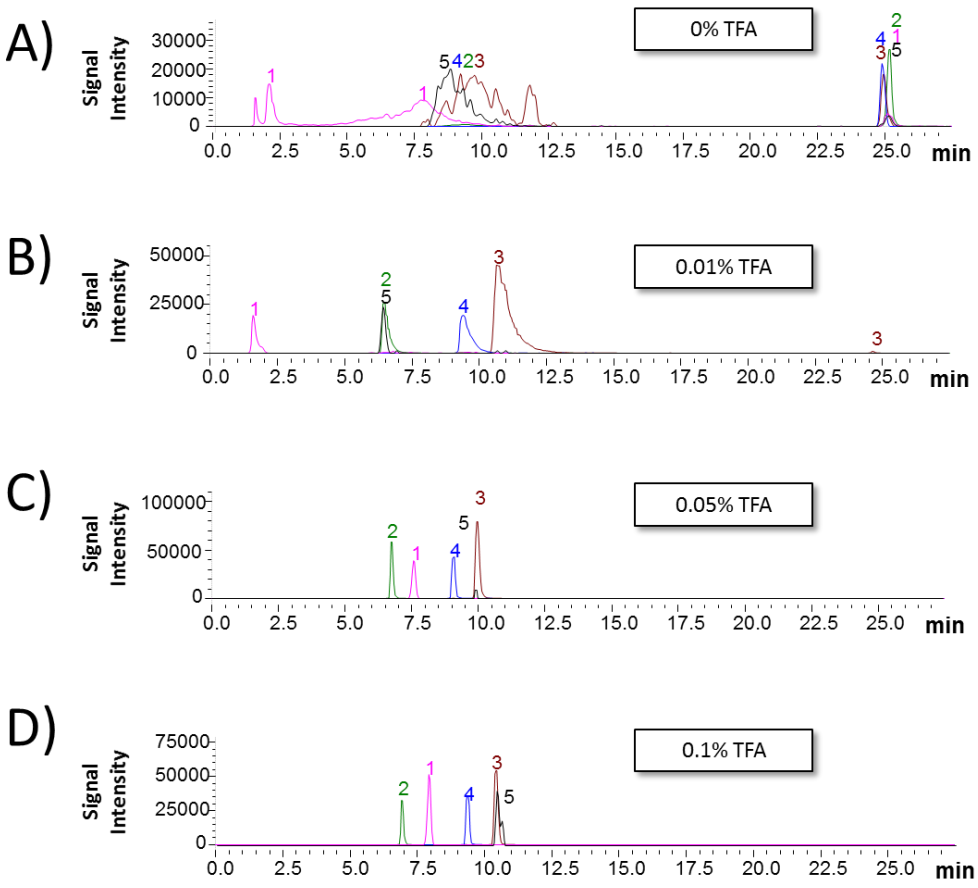
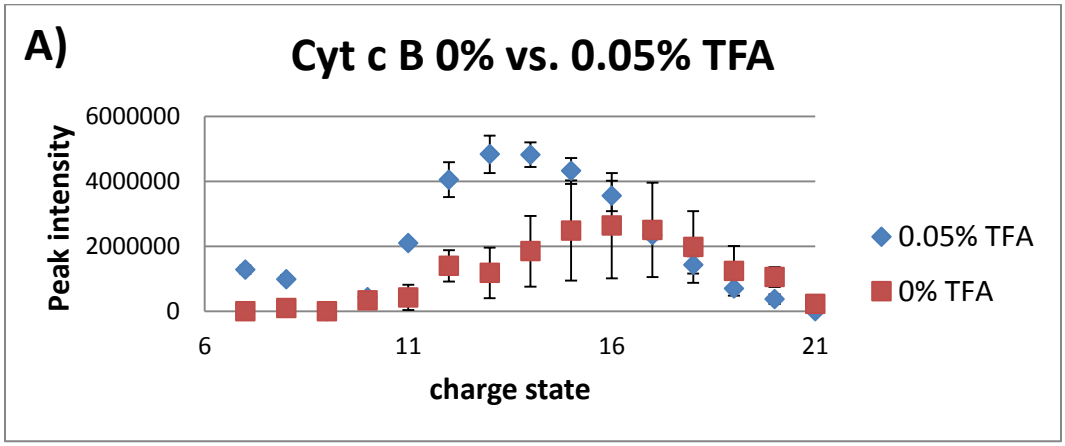


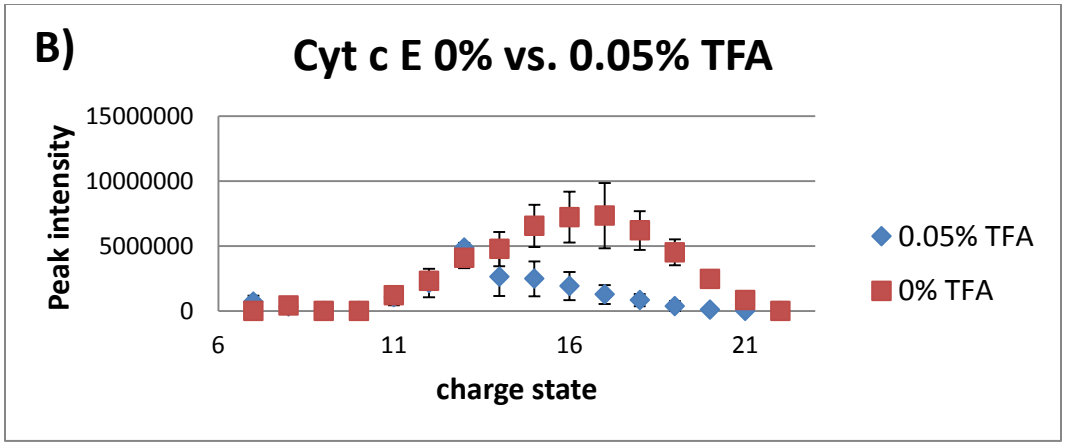
Figure 1. Chromatograms of the standard proteins (1. Lysozyme (3X) 2. Cytochrome c B 3. Myogobin (50X) 4. Lactalbumin (300X) 5. Ubiquitin (25X)) with various TFA concentrations at A) 0%, B) 0.01%, C) 0.05%, and D) 0.1%.

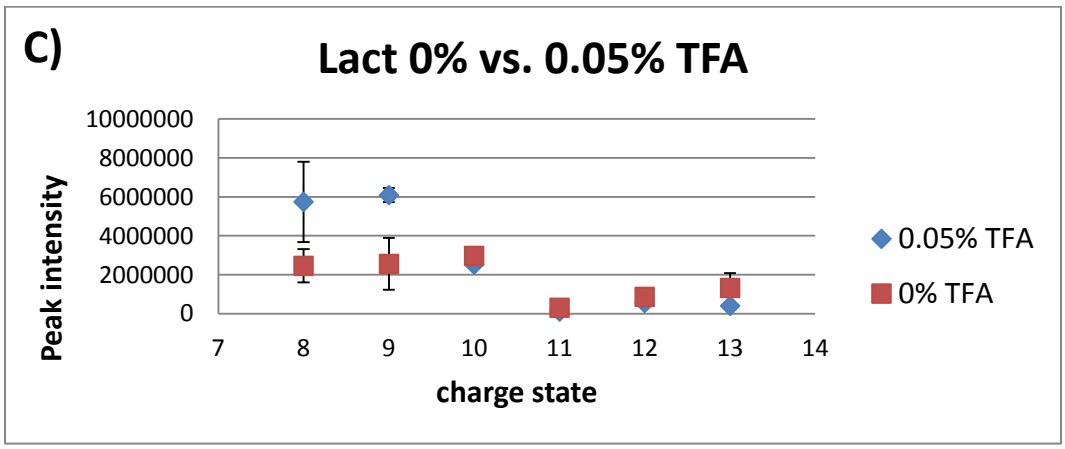
Chromatographic peak shape drastically improved with the addition of TFA, as seen in Figures 1C – 1D. Optimal chromatographic performance was observed with the addition of 0.05% TFA (Figure 1C). Increasing the concentration further to 0.1% did not further improve peak shape, and significant ion suppression began to be observed. Garcia et al. also reported similar findings as higher concentration TFA decreased signal

yield. In that work, a complete disappearance of myoglobin and cytochrome c signals were observed in 0.4% TFA.[162]

Since only one charge state was chosen for MRM-based detection, it was important to observe whether TFA enhanced or suppressed the signal of the specific charge state selected initially. Protein profiles obtained in Q3 scan mode revealed the protein charge distribution was altered by TFA (Figure 2 A-F). In general, protein charge state distributions were shifted to lower charge states. This could be attributed to the ion pairing effect of the trifluoroacetate, which reduced the number of chargeable sites on the protein during the ionization process. Looking more closely at individual proteins, with the addition of 0.05% TFA, Cyt c B, Lact, and Myo experienced signal enhancement for the charge state that was being used for MRM detection (with 0.1% FA only). The originally monitored charge state for Ubiq, Lysz, and Cyt c E, on the other hand, experienced suppression. It is worth noting that Cyt c B and Cyt c E differ only by 3 amino acids in their sequence, yet they exhibited different charge state shifts from the addition of TFA. Although the differences in sequence could appear to be minor, the shape of the proteins could be considerably diverse. Additionally, Cyt c E and Cyt c B were easily resolved by the reversed-phase chromatography method further supports the differences in protein conformations.


A)
Cyt c B 0% vs. 0.05% TFA
Peak intensity
6000000
4000000
2000000
0
6
11
16
21
charge state
0.05% TFA
0% TFA


B)
Cyt c E 0% vs. 0.05% TFA
Peak intensity
15000000
10000000
5000000
0
6
11
16
21
charge state
0.05% TFA
0% TFA


C)
Lact 0% vs. 0.05% TFA
Peak intensity
10000000
8000000
6000000
4000000
2000000
0
7
8
9
10
11
12
13
14
charge state
0.05% TFA
0% TFA

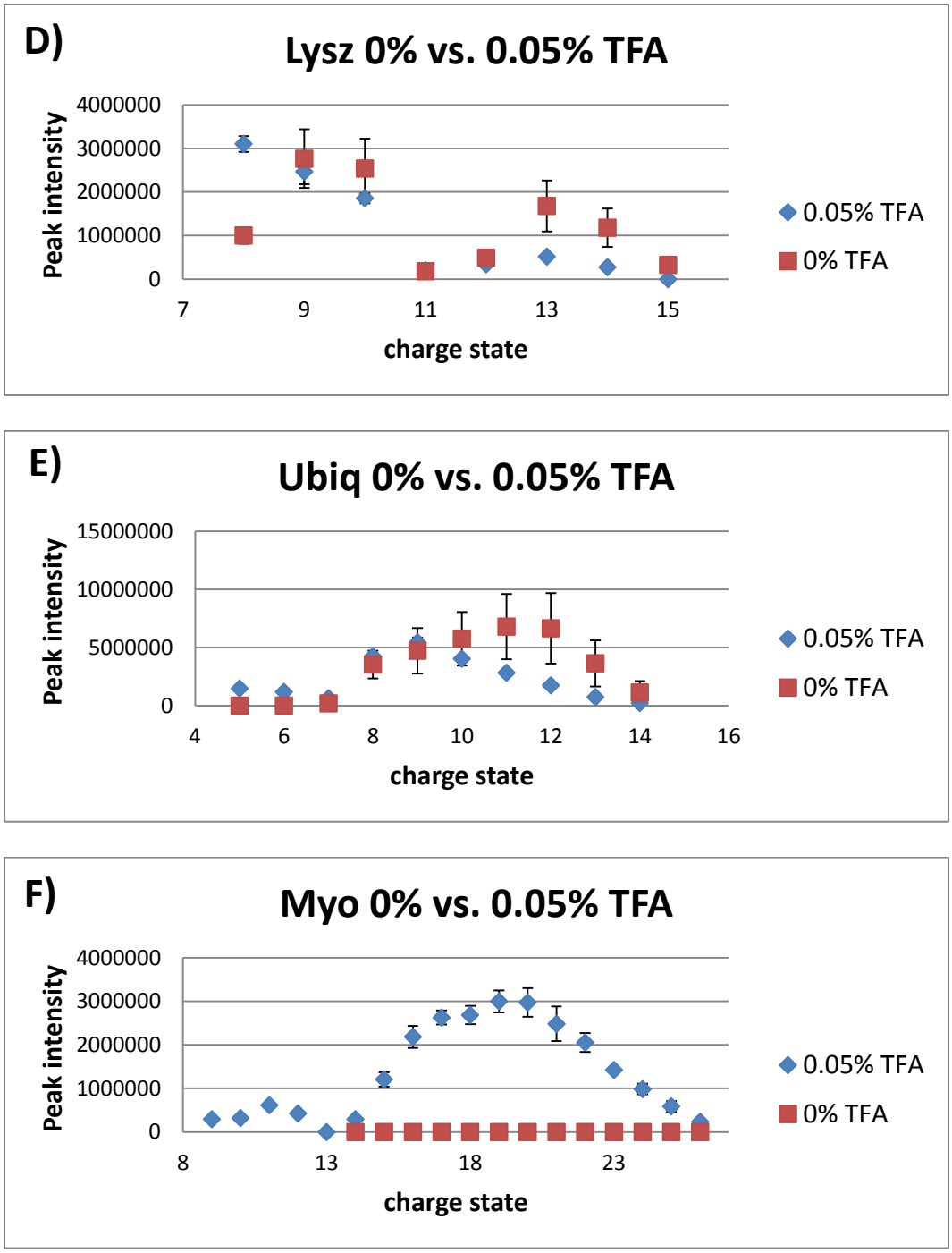


Figure 4-2 Alteration of (a) Cyt c B, (b) Cyt c E, (c) Lact, (d) Lysz, (e) Ubiq, and (f) Myo Charge State Distribution with Additional TFA

Lactalbumin also exhibited a substantial charge state shift and thus, potential conformational changes with the addition of TFA. The drastic peak splitting observed in the absence of TFA could be attributed to the presence of both hydrophilic and hydrophobic conformational states with formic acid alone. With the addition of TFA, the later eluting peak increased substantially in area, and the early eluting peak disappeared, indicating that a more uniform relatively hydrophobic conformation was adopted under optimum chromatographic conditions. Moreover, with the addition of a low concentration TFA, carry-over was reduced to less than 0.3%. Without TFA, carry-over for some proteins we studied approached 12%. In cases where TFA is not suitable for the analysis, addition of stronger solvents such as isopropanol in low percentage to the organic solvent has been reported to reduce protein carry-over and improve quantitation.[163] We did not evaluate the effect of other mobile phase modifiers on carry-over in this study.

4.4.2 Gradient Volume

The small elution window of proteins has encouraged scientists to develop shallow gradient methods that are significantly lengthier than small molecule methods. Ostlund Farrants et al. developed a linear gradient from 35% to 55% for 60 min (0.33 %B/min increase) to separate five GSH transferases.[164] Bobe et al also developed a shallow gradient (mostly <0.5 %B/min increase) with the total run time of 52 min for Bovine milk protein analysis.[165] In this study, we examined the relationship between the protein elution and the steepness of the gradient. To conduct this study, a simple change of the gradient slope could suffice. However, since the gradient slope (%B/min) is flow rate dependent, gradient volume (%B/mL) was a more representative parameter that could be applied to different flow rates. While it is independent from the flow rate, gradient volume still represents the steepness of the gradient. High gradient volume correlates to a steep gradient whereas low gradient volume correlates to a shallow

gradient. As gradient volume was increased from 5 to 15 %B/mL, significant increases in peak area were displayed in Cyt c B (180%), Myo (2400%), and Cyt c E (160%), while those for the other proteins remained unchanged. (Fig. S1 in Appendix B) Shallower gradients retained proteins longer on the column. A gradient volume of 5 %B/mL more than doubled the retention time for proteins relative to that for 15 %B/mL. Although resolution slightly improved with the increased retention at 5 %B/mL, base line resolution still could not be achieved for closely eluted Cyt c E and Ubiq (Rs = 0.2 ± 0.1 at 5 %B/mL and Rs = 0.542 ± 0.004 at 15 %B/mL). For peaks that were resolved at 15 %B/mL, the increased resolution did not bring any additional benefits. A steeper gradient generally sharpened protein peaks, and this was especially noticeable for Cyt c B, Cyt c E. and Lact while Ubi, Myo, and Lys did not show significant improvement. (Figure S2 in Appendix B) Therefore, the optimal gradient volume is likely to be analyte dependent and should be optimized for the specific analyte of interest. Contrary to the understanding that proteins should be separated with shallower gradient due to their sharp elution isotherms, a modest gradient volume of 15 %B/mL was shown here to be more practical and saved considerable time. However, in samples containing multiple co-eluting proteins, lower gradient volumes should be considered to achieve better resolution. Because proteins are inherently complex, some applications may show more significant effects from different gradient volumes than were observed in this study.

4.4.3 Flow rate

After the most practical gradient volume of 15% B/mL was chosen as one of the parameters for this method, a series of flow rates were tested. From a chromatographic stand point, protein desorption is highly dependent on the organic solvent concentration delivered from the gradient volume. However, flow rate still has effect on protein separation efficiency. Based on the Van Deemter equation, low flow rates are preferable

for larger molecules where slow diffusion and mass transfer are observed.[166] For mass spectrometric detection, if no drastic peak broadening effect was observed, lower flow rates would be better for enhanced electrospray ionization efficiency. Consequently, in our experiments, peak broadening effects were observed at lower flow rates for all proteins. The effect was especially pronounced with Myo and Cyt c B. Myo peaks at 0.05 and 0.1 mL/min were broadened to a point where no measurable peaks could be observed. The different degree of peak broadening that different proteins experienced at various flow rate led us to believe that within the general trend, the peak broadening effects are compound dependent. Theoretically, higher flow rates should sharpen the chromatographic peaks and higher peak heights should be obtained up to the ideal flow rate. In our study, we found that peak width did not considerably decrease yet the peak height decreased significantly. The decreased sensitivity at higher flow rates was attributed to decreased electrospray ionization efficiency, which is commonly known.[167] During the flow rate comparison, the lower flow rates experienced peak broadening and the higher flow rates experienced decreased ionization efficiency, therefore, 0.2 mL/min was an ideal mid-point to obtain reasonable data.

4.4.4 Column Temperature

Elevated temperatures can improve efficiency for protein separations by reducing protein refolding, reducing protein-protein interactions, and increasing stationary phase interaction kinetics.[29] Care should be taken to ensure that column chemistries can withstand higher operating temperatures. As expected for the model set of proteins investigated here, increased temperature decreased retention times. (Figure 3) Additionally, peak areas were generally found to increase with increasing temperature without peak widening. The increase in peak areas was especially apparent in the cases of Myo (3 fold increase) and Ubiq (2 fold increase) when comparing separations

performed at 30 ˚C vs. 75 ˚C. In contrast to the effect of modifiers, these changes in sensitivity could not be attributed to changes in the charge state distributions as a function of temperature. Protein profiles obtained from 30 ˚C and 75 ˚C for all of the proteins studied were virtually indistinguishable. Minor discrepancies of signal intensities were observed but the protein profile distribution remained the same.

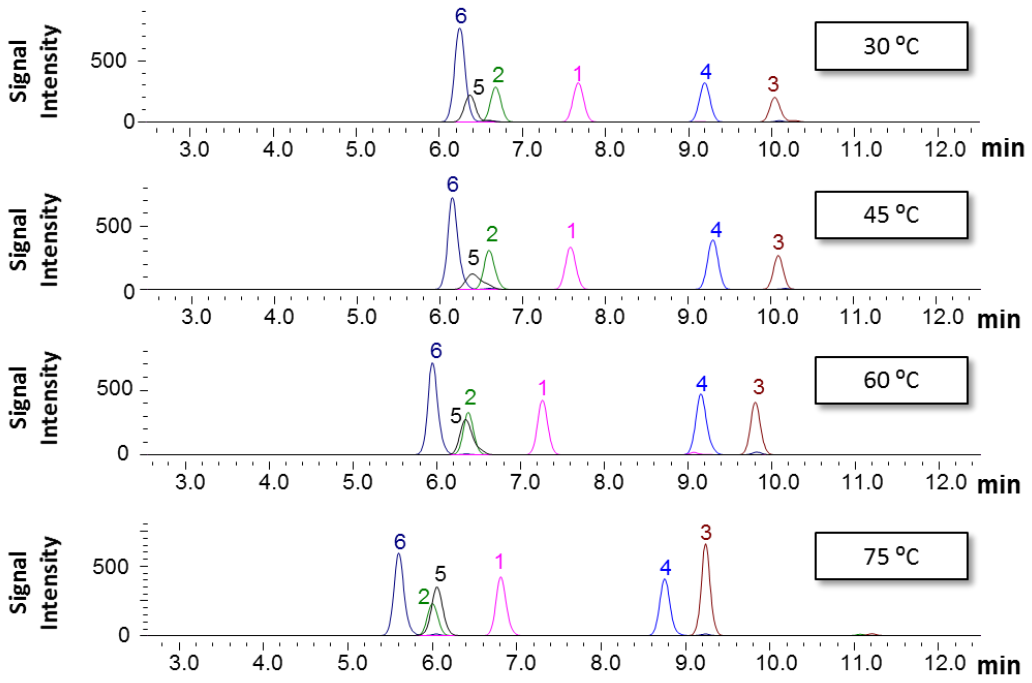


Figure 4-3. Chromatograms of the protein standards (1. Lysozyme 2. Cytochrome c B 3. Myogobin 4. Lactalbumin 5. Ubiquitin 6. Cytochrome c E) obtained at different column oven temperatures, 30˚C, 45˚C, 60˚C, and 75˚C.

According to Geng and Regnier's solvent displacement model for proteins in reversed-phase liquid chromatography, temperature alteration can change either the forces of interaction or the protein conformation.[168] Inferring that a significant change in protein conformation would be signaled by an alteration in charge state distribution,[169] the

effect of temperature on the protein separation in this work appeared to be due solely to changes in the interaction forces. Work by Cohen et al. supports this assertion. They indicated that at high temperature, some proteins favor a more denatured form. The denatured form is usually more hydrophobic, and the retention is thus increased.[157] In this work, retention decreased slightly with increasing temperature. Thus, the proteins studied were likely already fully denatured under the mobile phase conditions used, and the increase in temperature simply decreased the interaction forces in the system and decreased retention time.

4.4.5 Effect of stationary phases

In a typical liquid chromatography method, the chemical nature of the stationary phase has the largest effect on selectivity, which in turn, among efficiency, retention, and selectivity, has the strongest effect on resolution. The stationary phase is an adsorbent material that performs separation by its differential interaction with components in a mixture. In this study, we examined five stationary phases with various active functional groups under reversed-phase chromatography to observe any differences in protein separation. All selected stationary phases were also analyzed with both ACN and MeOH mobile phase systems with 0.1% formic acid and 0.05% TFA.

For the ACN solvent system, resolution and selectivity were found to be very similar for the C4, C8, C18 and biphenyl phases tested (Figure 4 A-D). Cyt c B, Cyt c E, and Ubiq exhibited some minor selectivity changes between the phases. Minor decreases in retention with the increasing alkyl chain length were observed. This was in contrast to results reported by Wang et al.[170]where C18 was shown to provide a nominal increase in separation efficiency and resolution compared to a C4 phase. The results presented by Cooke et al.[171] were more consistent with those that we observed. In that work, a significant increase in retention was not observed for proteins separated on a C8

phase relative to a C3 phase. They still stated that the protein retention on alkyl-bonded silica phases was mainly caused by hydrophobic interactions since the increased coverage of the C8 increased retention of the proteins.

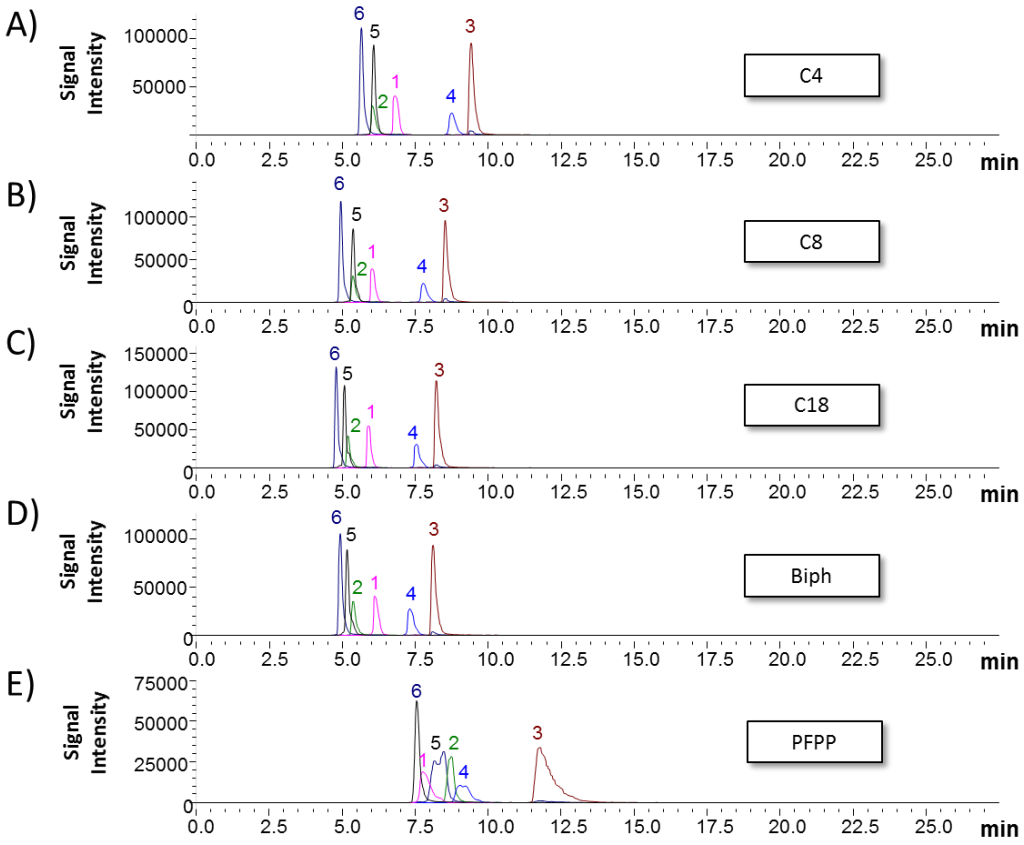


Figure 4-4. Chromatograms of protein standards (1. Lysozyme (10X) 2. Cytochrome c B 3. Myogobin 4. Lactalbumin (10X) 5. Ubiquitin 6. Cytochrome c E) obtained with various stationary phase functional groups A) C4, B) C8, C) C18, D) Biphenyl, and E) PFPP with ACN as the organic mobile phase.

Although the retention mechanism for proteins on alkyl stationary phases appears to be predominately hydrophobic, the fact that the lengthening of the alkyl chain

did not increase protein retention drastically indicates the existence of another interaction. Instead, minor shifts in retention with stationary phase chain length can also be partially attributed to the influence of underlying silica support material and its relative surface coverage. C4 being a shorter chain alkyl would potentially allow proteins to have more chance to access surface silanols. Additionally, bonding density varies with the different phases. The carbon load information (Restek Viva C4 [3.5%], C8 [5%], and C18 [9%]) indicated that the carbon percentages do not scale in proportion to the amount of carbon in the alkyl chain. A C4 column contains more alkyl chains (and thus more bonded silanols) than a C8 or C18 column. Thus, the longer retention in C4 could be explained with additional hydrophobic interaction from the additional alkyl chains. However, this assumption is based on the bulk carbon load and cannot account for the carbon contribution of end-capping groups present in the phases. Therefore, it is ultimately difficult to conclude the relative contributions or the magnitude of effects arising from hydrophobic or silanophilic interactions. Other more complex phenomena further convolute the distinction, including potential for molecular aggregation, steric exclusion, and displacement of solvent from the stationary phase surface and protein surface, among others.[168]

Protein separations using the biphenyl phase was very similar to that using the C18 for Lysz, Lact, and Myo in both ACN and MeOH mobile phase systems (Fig. 4D and 5D). In ACN, Biph behaved similarly to C18 with nominal differences (Fig. 4 C-D). The similar chromatographic behavior of biphenyl and C18 was not a surprise in ACN despite the fact that biphenyl phase containing two aromatic rings. Aromatic stationary phase groups supply electron density which helps retain polarized or electron-deficient analytes. The π character of ACN disrupts the π character induced by the biphenyl on analytes. Biphenyl generally only exhibits major selectivity changes relative to C18 in the presence

of organic solvents without π character (ie. MeOH, EtOH, and IPA).[172] The PFPP phase displayed substantially different selectivity compared to the other stationary phases (Figure 4E). Not only were different retention times observed for the six proteins, different selectivity with different elution order was also observed. The change in selectivity can be attributed to the fact that a C-F bond has a greater dipole character than a C-H bond. This characteristic allows the stationary phase to deliver greater interactions with polar compounds, or in this case, polar sites on a protein.

4.4.6 Effects of mobile phase

Although stationary phases with various functional groups provide different interactions for protein separation, different mobile phases can enhance or diminish certain interactions that will ultimately affect the chromatographic process. In this study, we compared the results of the five columns using both ACN (Figure 4) and MeOH (Figure 5) under the previously established gradient conditions (which were optimized for ACN). As a polar protic solvent, methanol solvates analytes differently than ACN.[173] Overall, the retention of the six protein standards nearly doubled when switching the mobile phase from ACN to MeOH. This result was to be expected since ACN is credited with approximately three times the eluotropic strength than MeOH in reversed-phase HPLC.[174]

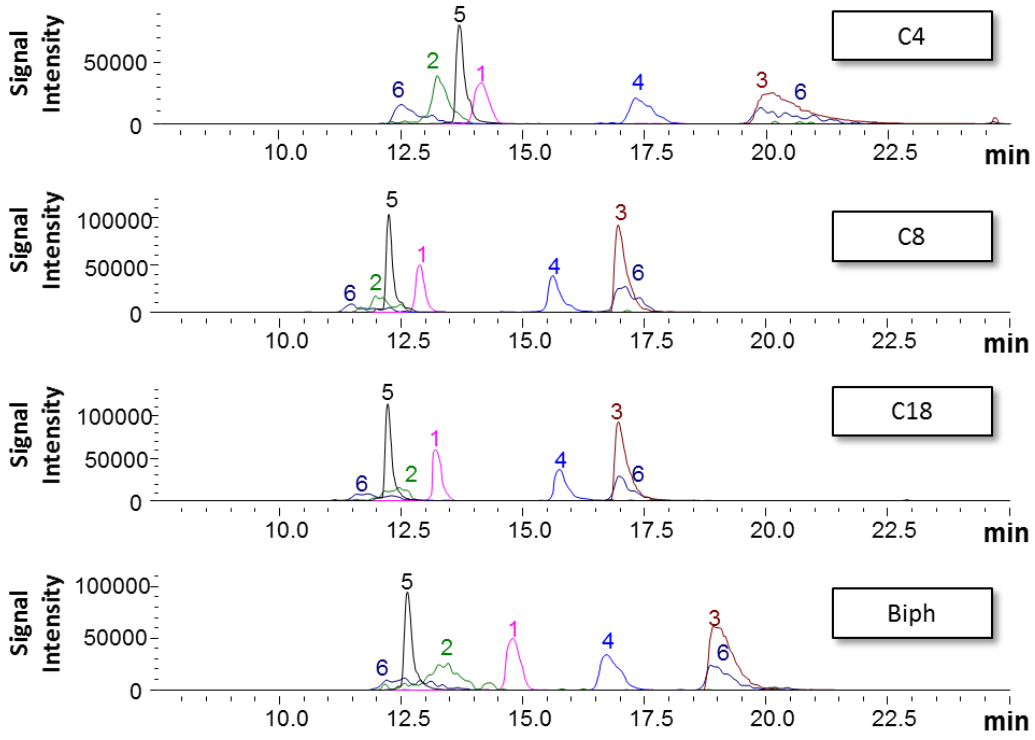


Figure 4-5. Chromatograms of protein standards (1. Lysozyme (10X) 2. Cytochrome c B 3. Myogobin 4. Lactalbumin (10X) 5. Ubiquitin 6. Cytochrome c E) obtained with various stationary phase functional groups A) C4, B) C8, C) C18, D) Biphenyl, and E) PFPP with MeOH as the organic mobile phase.

While the peak shapes of Cyt c B and E were similar to other proteins in the presence of an ACN mobile phase, they substantially deteriorated in the MeOH system. Carry-over peaks of Cyt c E were observed on the C4, C8, C18 and biphenyl phases. Poor and irreproducible peak shapes with significant carry-over in MeOH makes such conditions intractable for Cyt c E and B analysis. However, in MeOH, resolution of Ubiq and Lysz was significantly increased with the increased alkyl chain length (RsC4= 0.7±0.3; RsC18=1.25±0.05), and even considerately greater using the biphenyl phase

(RsBiph=2.27± 0.05). The result showed that Ubiq had the strongest interaction with the C4 phase, whereas Lysz had the strongest interaction with the biphenyl phase. Such changes in resolution on different stationary phases were not observed in the ACN mobile phase system. Although the biphenyl phase behaved similarly to C18 in an ACN mobile phase, the retention of Lysz, Lact, and Myo was more similar to that of C4 in MeOH. Cyt c B and E (despite the significant peak broadening) and Ubiq, on the other hand, were still retained on the biphenyl to a degree similar to C18. PFPP in ACN provided significantly different selectivity than all other phases tested. Nonetheless, PFPP did not work well with the MeOH solvent system. None of the proteins eluted reproducibly and significant ghost peaks were observed from run to run.

The overall protein charge distributions and intensities in the two mobile phase systems were the same. Thus, selectivity differences between C18 and Biph in the MeOH mobile phase could not be attributed to differences in protein conformation. Although the protein signal intensities using the PFPP were lower than for results on the C18 phase in ACN, the protein charge state distributions remained the same and thus, changes in conformation were not likely to be the cause of the unique selectivity. The absence of alterations in the protein charge state distributions with different stationary phases echoes the results reported by Drake et al. where the conformational changes of the α-chymotrypsinogen was studied using circular dichroism with various stationary and mobile phases. Even though it was evident that the protein was modified after interacting with the stationary phases, the conformation was proven to be independent of the alkyl chain length. Similar results were also obtained with a phenyl phase in reversed-phase mode. The authors believed that the conformational changes could be caused by the hydrocarbonaceous layer of the stationary phase material penetrating into the

hydrophobic moieties of the protein, or the reversed-phase material binding to the native protein's hydrophobic moieties.[175]

4.5 Conclusion

In this work, critical parameters that affect reversed-phase chromatographic separation of intact proteins, in the context of direct mass spectrometric detection, were systematically evaluated. The optimal condition described here could be useful as an initial method for protein quantitation on LC-QqQ-MS. Considerations should be made when changing the parameters to accommodate both chromatography and ionization efficiencies for a more accurate and sensitive quantitation method.

Even with all the results obtained in this study, it is rather difficult to formulate a clear understanding of differences in the protein chromatography behavior. Many variables are involved in the process and it is likely that some of them are co-dependent. Chemometric approaches may be able to tease out some of this information, but such an undertaking was beyond the scope of this study. Proceeding empirically, there would be an inherent advantage in time to have multiple columns on a column selector with a generic gradient of different solvent systems to select the most suitable stationary and mobile phases for a specific application.[172] Such a strategy could achieve faster LCMS method optimization than intensively focusing on a single column. Ultimately, to better understand and describe the effect of variables on reversed-phase protein chromatography, one would like to have a much larger set of standard proteins, so that structure – retention relationships could be explored.

For future studies and applications, some reiteration work should be included throughout method development. For example, MRM transitions to detect the proteins of interest should be developed and optimized first, followed by chromatographic method development. Once optimal chromatographic conditions are achieved (including

optimization of additives, mobile phase composition, stationary phase, flow rate, and gradient volume), then MRM should be optimized again. This iterative process will allow one to target the most intense charge state for a given protein under optimal chromatographic elution conditions to generate the highest sensitivity MRM transitions. For peaks that overlap, precursor ions chosen should be substantially different from each other to prevent signal interferences. If the protein profiles of the two overlapping proteins do not share the same *m/z* range, the fast scan speed of the QqQ-MS should be sufficient to compensate multiple simultaneous MRM acquisitions.

Throughout the experiment and method development, a scan event should always be included in a method. At a reasonable concentration, a scan event displays the protein profile that can be particularly helpful in improving MRM and chromatography methods. Although the protein charge state can give indication on protein shape alteration, it is not definitive. Other effects including ion pairing or supercharging can also shift the protein charge states. Circular dichroism spectroscopy can be incorporated to study the protein shape[157] and can be useful to narrow down the source of the problem to improve the LCMS method. Ion mobility mass spectrometry could also be used to observe conformation state of the proteins.

Chapter 5

An Investigation of Ion Transmission for Intact Proteins in a Triple Quadrupole Mass Spectrometer

5.1 Abstract

The demand for protein quantitation is increasing especially for clinical diagnostic and drug development applications. Recently, we demonstrated the feasibility of direct intact protein quantitation using triple quadrupole mass spectrometry (QqQ-MS) and multiple reaction monitoring (MRM) to help meet this demand (J. Amer. Soc. Mass Spectrom. 2016, 27, 886-896). Even though QqQ-MS is known to provide extraordinary detection sensitivity for quantitative analysis, we found that intact proteins exhibited a less than 5% ion transmission from the first quadrupole to the third quadrupole mass analyzer in the presence of zero collision energy (ZCE). With the goal to enhance intact protein quantitation sensitivity to achieve low abundant protein quantitation, a series of experiments were performed to find the source of the 95% lost ion signals. Ion scattering effects, proton-transfer effect, and mass filter width were examined for their contribution to the lost signal. Protein standards myoglobin and ubiquitin along with small molecules reserpine and vancomycin were analyzed together with various collision induced dissociation (CID) gases (N2, He, and Ar) at different gas pressures. The result revealed the low ion transmission has little correlation to the CID gas. Mass resolution settings, on the other hand, played a significant role in blocking ion transmission signal. By narrowing the mass resolution window by 0.35 *m/z* on each side, roughly 75-90% of the ion signal was lost. The multiply charged proteins, however, experienced additional proton-transfer effects, corresponding to ten-fold signal reduction. This study provides additional insight into optimizing the use and sensitivity of QqQ-MS for intact protein quantitation.

5.2 Introduction

The demand for protein quantitation in fields including biomarker discovery, protein therapeutic monitoring, and drug development and response control has increased dramatically in the recent past.[3-4, 5b] Protein quantitation has also been shown to be useful in environmental and agricultural analysis.[1-2] The increasing desire to quantitate proteins in various research fields dictates the needs for more sensitive and efficient methods to meet the demand.

Triple quadrupole mass spectrometry (QqQ-MS) with its excellent sensitivity and specificity is routinely used for quantitative analysis. Most methods that utilize QqQ-MS for protein quantitation involve protein digestion prior to the analysis – a so-called bottom-up approach. Typically, intact proteins in a sample would be digested by an enzyme, such as trypsin, into small peptide fragments. The constituent peptides are then quantified by QqQ-MS to back calculate the intact protein concentration in a sample.[123] However, the digestion step can be variable and is often incomplete, both of which can introduce error into the quantitation method.

As an alternative, a novel top-down method that directly quantitates intact protein using QqQ-MS was recently developed in our lab.[159] Intact proteins were successfully quantified using multiple reaction monitoring (MRM) with respectable linearity ($R^2 > 0.99$). For the six protein standards investigated, 2-3 orders of magnitude linearity with high accuracy (< 5% error) and precision (1-12% CV) were demonstrated. Specificity of the intact protein MRM-based method was demonstrated using spiked standards into plasma and urine. The limits of quantitation (LOQ) of the protein standards were found to range from 0.5 to 50 µg/mL. Although these LOQs were not as low as many bottom-up QqQ-MS quantitation methods (typically with LOQs in the pg/mL to ng/mL range),[142] they were on par with various protein levels of interest in biological fluids.[143] However, if one wishes

to detect and quantify some of the lowest abundance protein biomarkers (concentrations less than 500 ng/mL),[144] some signal enhancement would be needed according to what was reported.

During the development process of the intact protein MRM quantitation method, a less than 5% ion transmission rate from the first quadrupole (Q1) to the third quadrupole (Q3) mass analyzer was revealed. This phenomenon was observed by monitoring the same ion (set as both precursor and product ions) at zero collision energy (ZCE). The Instrumentation specification indicates the transmission of the fragmented product ion of reserpine needs to be more than 5% to be acceptable for normal operation. This specification, however, uses the fragmented product ions to compare to the precursor ion. The low transmission rate is thus reasonable since only a small amount of specific product ions should be expected out of the precursor ions during fragmentation. Nevertheless, when we performed the ion transmission study, the same protein ion was monitored under ZCE. The ion transmission was expected to be higher than 5%, but it was not the case as observed. There are a variety of pathways to explain this effect.

Ion scattering instigated by the collision induced dissociation (CID) gas between Q1 and Q3 is possible.[28] CID gas is generally introduced to the collision cell at a specified pressure to aid the fragmentation process. At ZCE, this gas curtain can partially block the ion path and scatter the ion of interest. It was shown that while 100% transmission could be achieve without the presence of CID gas in the CID chamber, only 50-75% transmission was observed with 2x 10-4 torr of CID gas pressure.[176]

Proton transfer from the protein to the CID gas or other ions can also be a possible source for loss in ion transmission.[177] Inert gases such as argon and nitrogen are used to prevent interactions with the gas phase ion. However, since the intact

proteins are multiply charged, the gas phase ions become increasingly acidic and are more likely to transfer protons to other ions or molecules.[178] Different CID gases are also characterized by different gas phase basicity,[179] and there is precedent for their participation in proton transfer reactions.[131a]

Another possibility is that the mass resolution filter partially excludes the ion of interest. Normally, all MRM analysis are performed using approximately unit resolution (e.g., ± 0.35 *m/z*). That is, if 1000 *m/z* is set to be the ion of interest, the mass filter will allow ions with *m/z* from 999.65 to 1000.35 pass through and be detected. In some QqQ-MS instruments, the mass resolution can be changed from unit to low (± 0.7 *m/z*) or to high (± 0.2 *m/z*) settings. A quadrupole mass analyzer is made of four rods that links to radio frequency (RF) and direct current (DC) voltages. The combination of the RF and DC voltages creates a region that select and/or focuses the ion of interest. The region where ions can pass through the quadrupole with stable trajectory is known as the stable region in a Mathieu stability diagram. The mass resolution is a ratio of DC/RF (the slope of the scan line in the diagram). Increasing mass resolution is done by increasing the slope closer to the apex of the stability region. Lowering mass filter means the slope is being decreased to includes more stable region and allow more ions to transmit. Although the increase in mass filter window might increase ion signal intensity, possible detection of other interfering ions can compromise specificity, and ultimately accuracy. The higher signal intensity can also be voided by the increased noise, which can yield negligible enhances or even negative effects on detection sensitivity, which is defined by the single-to-noise ratio (S/N).

In this article, we performed experiments to investigate contributions from the possible causes for decreased ion transmission for intact protein ions as described above. A variety of CID gases, including argon, nitrogen, and helium were used for the

ion scattering effect study. The CID gas pressures were set to high and low values to observe changes in ion transmission. Proton-transfer effect was also investigated with argon, nitrogen, and helium gas at different CID gas pressure for multiply charge intact proteins (large molecules) and vancomycin (small molecules). Since the multiply charged ions of interest obtain greater gas phase acidity, the chance of transferring proton to other ions or molecules such as CID gas molecules is much higher. The mass resolution setting was altered from unit to low for signal intensity increase observation. Even though the change in the mass window was small, the signal intensity could be affected in a greater scale. The goal of this work was to better understand the sources of loss in ion transmission, to potentially provide for enhancements in sensitivity when performing intact protein quantitation using QqQ-MS and MRM.

5.3 Experimental

5.3.1 Chemicals and Reagents

Protein standards myoglobin (Myo) (16.9 kDa) from equine skeletal muscle and ubiquitin (Ubiq) (8.6 kDa) from bovine erythrocytes, small molecule standards reserpine and vancomycin hydrochloride hydrate, and mobile phase additives trifluoroacetic acid (TFA) and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis MO, USA). LCMS-grade water and acetonitrile (ACN) were purchased from Honeywell Burdick and Jackson (Morristown NJ, USA).

5.3.2 Examining Ion Transmission Parameters

Sample Preparation and Instrument Parameters

A LCMS-8050 triple quadrupole mass spectrometer equipped with a conventional electrospray ionization source (Shimadzu Scientific Instruments, Inc., Columbia MS, USA) was used for ion transmission and related studies. The initial ion transmission observation was performed by comparing the Q3 and Q1 ion signal intensity. Q3 ion

signal intensity was obtained from MRM of the Myo [+18] ion being both the precursor and the product ion selected. Q1 was obtained from Q1SIM mode where Myo [+18] was obtained. Protein standards Myo (100 µg/mL) and Ubiq (10 µg/mL) and small molecules reserpine (0.01 µg/mL) and vancomycin (50 µg/mL) were prepared in water with 0.1 % FA (by volume) to working standard concentrations for further investigations. In some studies, small molecules were included to observe the possible correlation of the effect and the size or charge states of the ions. The concentrations of different standards varies due to their differences in response factor. The sample was directly injected using an autosampler (SIL-30 AC; Shimadzu) with a mobile phase of 50:50:0.1:0.05 (v/v/v/v) H2O:ACN:FA:TFA. The parameters of the electrospray ionization – triple quadrupole mass spectrometer for the following experiments are descried as follows: Interface temperature at 300 °C; interface voltage at 4 kV (positive ionization mode); desolvation line temperature at 250 °C; Heat block temperature at 400 °C; heat gas flow, nitrogen at 10 L/min; and drying gas flow, nitrogen at 10 L/min. Qualitative and quantitative data obtained by triple quadrupole mass spectrometry was analyzed using Shimadzu LabSolutions Software (v. 5.65).

Ion Scattering by Collision Induced Dissociation Gas

Ion scattering effects were monitored using the same method as the initial ion transmission study. Ion intensity of the Q3 was compared to that from Q1 isolation alone. Intact protein standards Myo (943 *m/z* [+18]) and Ubiq (1714 *m/z* [+5]) were used for this study as well as small molecule reserpine (609 *m/z*). Common CID gases including argon, nitrogen, and helium were introduced to the collision cell. The gas pressure was set at normal (250 kPa) or minimal (17 kPa) to observe the CID gas ion scattering effect. The data was collected in triplicates. If CID gas is indeed the major source of the ion transmission loss, the effect would be significantly severe at normal gas pressure in

comparison to minimal gas pressure. 17 kPa was the minimal gas pressure that the instrument software allows to input. Even when CID gas pressure was turned off, a minimal gas pressure around 17 kPa was still maintained in the CID gas chamber. The actual gas pressure, however, could not be easily determined if CID gas is turned off. For the sake of comparing different CID gases and obtaining reproducible results, a CID gas pressure at 17 kPa was set to be the minimal pressure.

Proton Transfer to Collision Induced Dissociation gas

To investigate the potential of proton transfer from multiply charged intact protein ions to the collision gas or other molecule, a separate study was designed. Since only one charge state of the multiply charged protein is selected as the precursor ion for MRM quantitation, the loss (or gain) of a proton will shift the ion signal to another *m/z* and thus decrease method sensitivity and accuracy. Multiply charged proteins myoglobin and ubiquitin were monitored at three different charge states. MRM transmission from Myo [+14], [+18], and [+22] and Ubiq [+6], [+8], and [+11] were used as precursor ions to monitor the signal intensity of neighboring charge states as the product ions under ZCE at various CID gas pressures. Vancomycin (725 *m/z* [+2]) was also used as a doubly charged small molecule to compare the effect. Triplicate data was obtained. The higher the gas pressure, the more gas molecules were present in the CID chamber to potentially undergo proton transfer processes. CID gases with various gas phase basicity (GB) and proton affinity (PA), including argon (GB: 364.3 kJ/mol; PA: 369.2 kJ/mol), helium (GB: 148.5 kJ/mol; PA: 177.8 kJ/mol), and nitrogen (GB: 464.5 kJ/mol; PA: 493.8 kJ/mol) were studied.[179] According to convention, CID gases with higher GB and PA should be more likely to induce proton transfer effects with gas phase acids. Gas phase pressure were set from the minimal 17 kPa to the maximum 450 kPa with 50 kPa increments. Since

nitrogen and helium could only reach 350 kPa before it became unstable, the experiments for these gases were performed up to 350 kPa.

Mass Resolution Affecting Ion Transmission Detection

The mass resolution window of the Shimadzu 8050 QqQ-MS could be set at low (±0.7 *m/z*), unit (±0.35 *m/z*), or high (±0.2 *m/z*) mass resolution. For a typical MRM method, the first quadrupole and the third quadrupole are typically set to unit resolution. Intact protein Myo (943 *m/z* [+18]) and small molecule reserpine (609 *m/z*) were used for the ion transmission detection study. CID gas was held at the minimal level (17 kPa) to eliminate factors such as collision and scattering. Since result should be similar for different gases at minimal pressure, only argon was used as the CID gas for this experiment. As sensitivity enhancement was the goal of this study, the high mass resolution setting, which decreases the ion transmission, was not included as a choice in this study. MRM mass resolution windows were set at low and low, low and unit, unit and low, and unit at unit for Q1 and Q3, respectively to compare the different settings. Q1SIM and Q3 SIM data were also obtained for comparison. Data was collected for all combinations in triplicate.

5.4 Results and Discussion

In triple quadrupole mass spectrometry multiple reaction monitoring, CID gas is introduced to the collision cell (or Q2) to break apart the precursor ion selected in Q1 into unique product ions for quantitation in Q3. The gas molecules in the collision cell not only aid the fragmentation process, but can also scatter the ions of interest. When the instrument was set to monitor the transmission of protein ions without collisional activation, only approximately 5% ion transmission was observed. Such a result was verified to be consistent on a secondary instrument prior to investigating sources of loss more closely.

Ion scattering was examined using intact protein ions from Myo and Ubiq, as well as the small molecule, reserpine. The ion intensity ratio of MRM/Q1 SIM were obtained at minimal and normal gas pressure with common CID gases including argon, nitrogen, and helium. Results are shown in Table 1. Both the proteins and the small molecule yielded low transmission. Less than 10% ion transmission was observed for reserpine and this was only slightly higher than for the proteins. The result also indicated that ion transmission was not heavily affected by increasing CID gas pressure. A decreased ion transmission with increasing CID gas pressure was expected and was observed in most cases. However, the decrease was relatively minor. Ion scattering by the CID gas was apparently not responsible for majority of the transmission loss in the instrument.

Table 5-1 Ion transmission result for ion scattering study. Reserpine, myo, and ubiq were analyzed in minimal (17 kPa) and normal (250 kPa) CID gas pressure with argon, helium, and nitrogen as the gases of choice.

		Argon	**Helium**	**Nitrogen**
Reserpine	17 kPa	8.1% ± 0.3%	6.7% ± 0.1%	7.67% ± 0.07%
	250 kPa	4.5% ± 0.1%	8.2% ± 0.2%	5.08% ± 0.05%
Myoglobin	17 kPa	3.0% ± 0.2%	2.9% ± 0.2%	3.03% ± 0.08%
	250 kPa	0.28% ± 0.03%	2.7% ± 0.2%	0.78% ± 0.08%
Ubiquitin	17 kPa	2.2% ± 0.5%	1.7% ± 0.3%	1.78% ± 0.03%
	250 kPa	1.9% ± 0.6%	2.8% ± 0.2%	2.5% ± 0.3%

Even so, the decrease in ion transmission was more prominent in argon and nitrogen, compared to helium. This is likely caused by the size of the gas molecule. As the molecular diameter of the CID gas can affect the efficiency of the fragmentation

process,[28] it can also affect the efficiency of the ion transmission. The bigger argon and nitrogen could affect the transmission rate by blocking and deflecting the ions while preventing it to exit the CID chamber and increasing the chance of collision and fragmentation. It is worth noting the ten-fold decrease in ion transmission for Myo with increased argon and nitrogen gas pressure. This result that indicated the intact protein was under more effect than small molecule by the increased argon and nitrogen gas pressure was further discuss in the following paragraphs of proton transfer effect.

Proton transfer reactions between the analyte and CID gas or other ions could also be a possible cause of the low ion transmission. Proton transfer is more likely to occur during intact protein analysis since proteins are multiply charged by the electrospray ionization source. More highly charged species will be stronger gas phase acids.[180] To observe proton transfer effects, multiply charged intact proteins, Myo and Ubiq, and small molecule, vancomycin, were used to monitor the generation of different charge state ions at different CID gas pressures with argon, helium, and nitrogen. Myo and Ubi mass spectra generated by electrospray ionization were as shown in Fig. 1. Low, medium, and high charge states were chosen as precursor ions. With ZCE, products ion of the same charge states and neighboring charge states were monitored. If no proton transfer occurred, 100% of the product ions should be the same charge state as the precursor. Product ions of other charge states would be observed if proton transfer occurred.

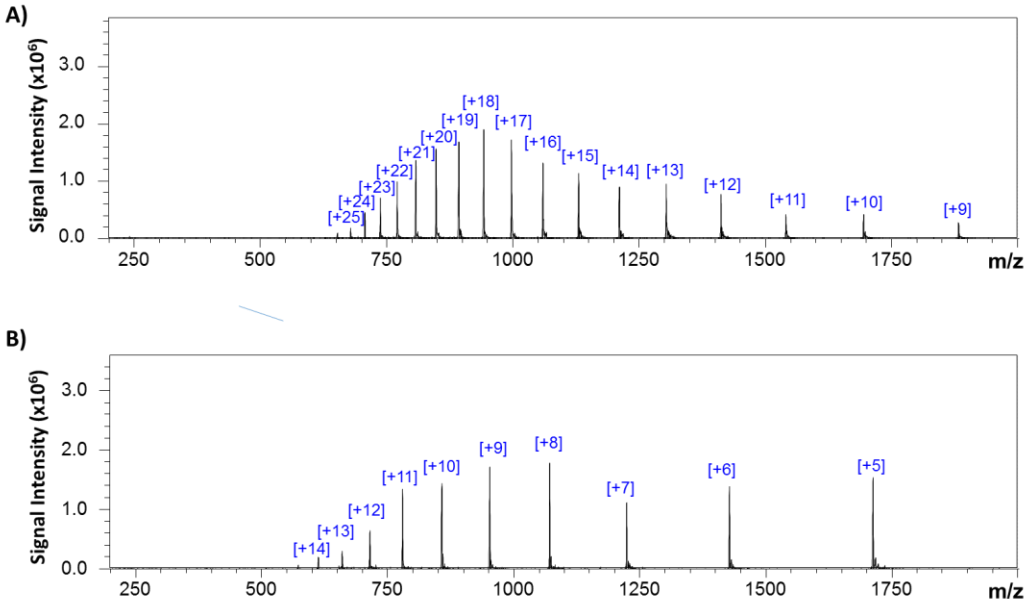


Figure 5-1 Multiply charged intact protein mass distribution of

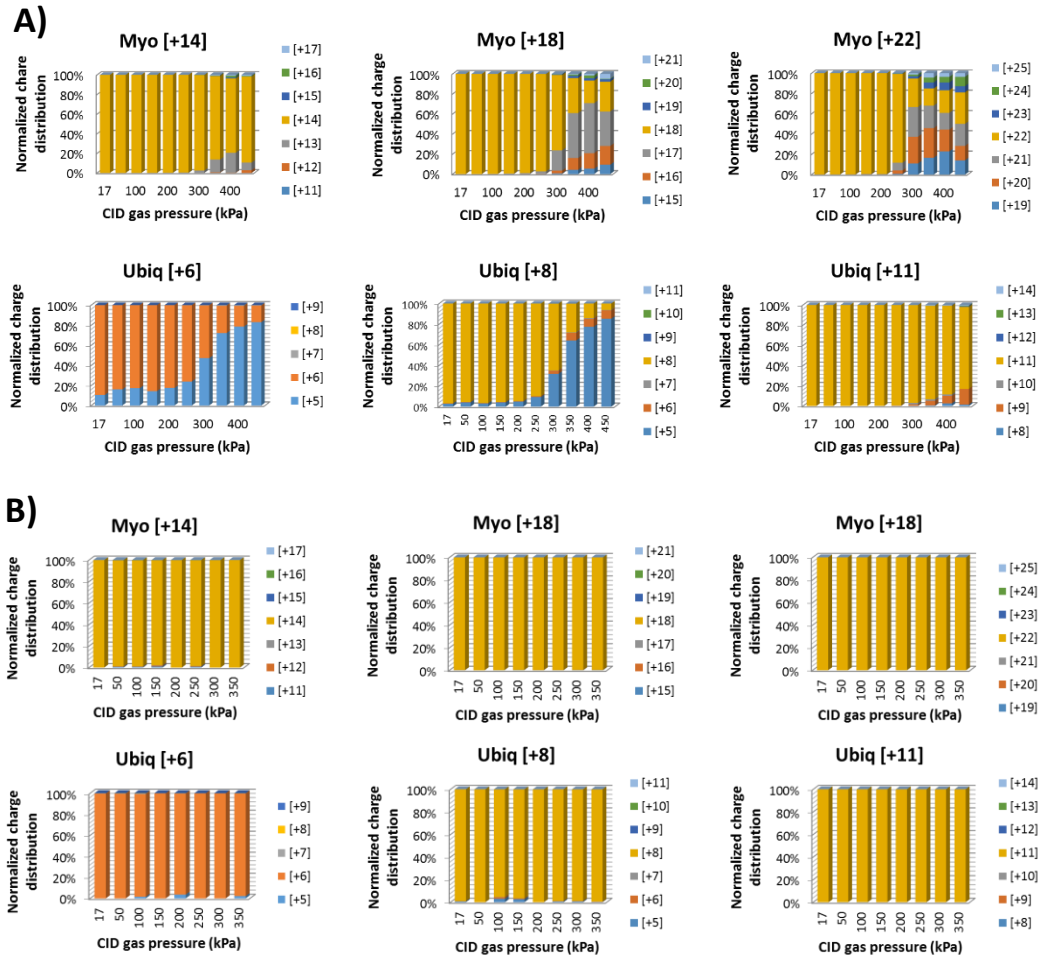
A) myoglobin and B) ubiquitin

The relative product ion charge state distributions of Myo and Ubiq are shown in Figure 3. Argon (Fig. 2A) induced more significant proton transfer for Myo and Ubiq than did helium and nitrogen. As the gas pressure was increased, more proton transfer occurred. After 300 kPa, more than ¾ of the precursor ions underwent proton transfer. This could be an issue as larger molecules such as intact protein with high molecular weight might require higher CID gas pressure to achieve fragmentation. However, the higher CID gas pressure can pose a drastic signal decrease due to proton transfer effect. As expected, the higher charge states of Myo exhibited more proton transfer than the lower charge states. Ions with higher number of protons can be more gas phase acidic and are more likely to transfer protons to ions and molecules that are relatively basic in gas phase. However, Ubiq at [+11] showed less proton transfer than the [+8] and [+6] charge states. This might related to the location of the protons on the surface of the

specific proteins and the accessibility of the proton transfer. The fact that the majority of the Ubiq [+8] signal went to [+5] instead of [+6] and [+7] might be also caused by the same proton accessibility reason. As seen in Figure 2, two charge state distributions were obtained in the mass spectrum of ubiquitin representing different conformation of the protein in the gas phase. The more unfolded form had higher charge states. The folded form had lower charge state. With the proton transfer from [+8], conformation change might happen to stabilize the charge state at the most distinct charge state at [+5]. This phenomenon could lead to discrepancies in trends predicted by gas phase acidity scales.

Helium and nitrogen collision gases induced much less proton transfer than argon (Fig 2B and 2C). Nitrogen actually has a higher gas phase basicity and proton affinity (GB: 464.5 kJ/mol; PA: 493.8 kJ/mol) than argon (GB: 364.3 kJ/mol; PA: 369.2 kJ/mol), so this result was surprising.[179] However, the GB and PA values published by Hunter et al. were derived from data obtained by other studies. The derived value were often a general average and have been reported as a relative scale. Argon and helium values were derived from very limited measurements and a wide range of values were reported. In the paper, authors admitted that the scale of gas phase basicity was not well established in the low basicity region below water and hydrogen sulfide. All gases that were included in this study had gas phase basicity below this region. Therefore, the gas phase basicity and proton affinity values may not be the best standard to predict gas phase proton affinity behavior in this case. Koppel et al. also discussed the inconsistent data that was obtained in ion cyclotron resonance mass spectrometer experiments.[181] Hence, different characters of gas in different system could be expected. Doubly charged vancomycin was also evaluated for proton transfer effects to provide a small molecule comparison. No proton transfer was shown for any of the gases tested in this

experiment. Clearly, the higher charging of protein ions makes them more prone to charge transfer effects, but further studies remain to fully understand the mass-to-charge nature of this effect. The *m/z* ratio of the [+11] ion of ubiquitin is similar to the [+2] ion of vancomycin, yet the former underwent significant proton transfer, whereas the latter did not. Overall, proton transfer effects seem to factor in significantly as part of the explanation for decreased ion transmission for multiply-charged protein ions, especially when argon is used as the collision gas.



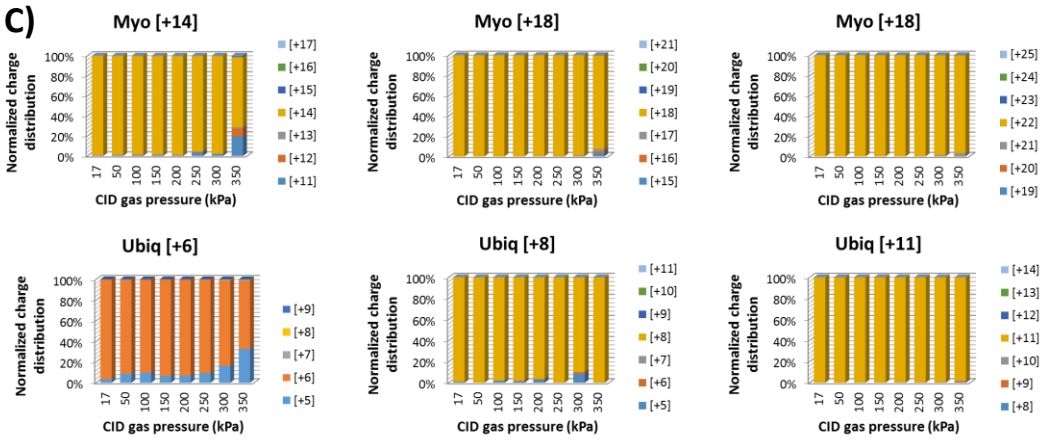


Figure 5-2 Proton Transfer of Myo and Ubi in A) argon, B) helium and C) nitrogen gas

Although proton transfer can explain some of the signal loss of intact protein, the major loss in ion transmission for both small and large molecule was still unclear. Further investigation was performed on the effect of the mass resolution filter setting. As the default, all analysis is performed in unit resolution where the mass-to-charge ratio for isolation is ±0.35 of the input *m/z*. If mass resolution setting is changed from unit to low, the resolution window would change from ±0.35 to ±0.7. Figure 3 is the comparison of MRM ion transmission for reserpine and Myo with the first quadrupole and the third quadrupole being set at low and low (LL), low and unit (LU), unit and low (UL), and unit and unit (UU). As expected, LL had the highest and UU had the lowest ion transmission since the mass resolution windows for both quadrupole was at the widest and the narrowest, respectively. Although UL and LU both had one unit and one low, the order actually made a difference. Typically, a higher transmission in LU than UL could be expected. This was indeed the case for the reserpine transmission study. This might be because LU allowed more ions to pass the first triple quadrupole and be focused by the post rod and pre rod of the quadrupoles. Therefore, ions of interest with a more focused ion path could be detected with higher intensity. UL allowed less ion of interest to pass

through the first quadrupole, thus less ions could be detected even after ion focusing. However, in the case of intact protein Myo, the UL setting returned higher transmission than the LU setting. A possible explanation could be that the multiply charged intact protein obtained many adduct and isotope peaks close to the *m/z* of the ion of interest. In LU setting, adduct and isotope peaks were also allowed to pass through Q1. Ion collision or interactions could happen in the path to Q3 and lowering the chance of the ion of interest to reach the detector. In UL setting, adducts and isotope ions would have less chance to fit in the narrower window. With the ion focusing by the post rod and pre rod, more intact protein ions could be analyzed.

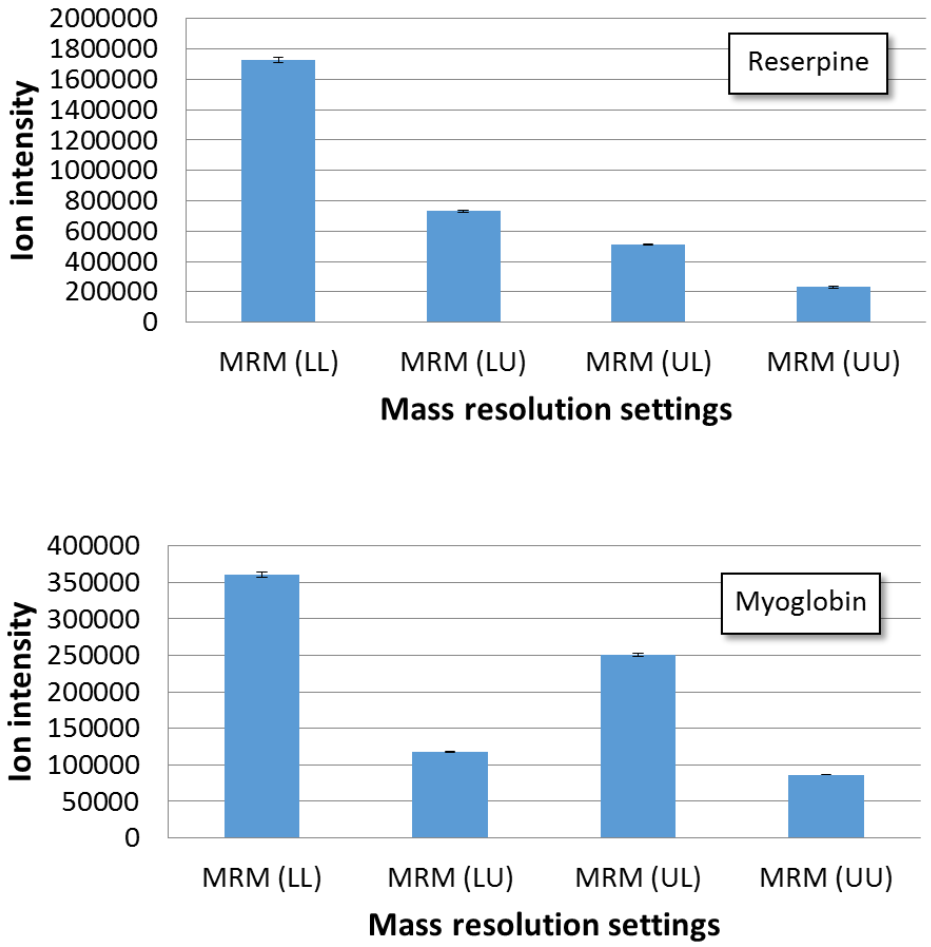


Figure 5-3 Ion throughput of reserpine and Myo with various mass resolution filter at low and low, low and unit, unit and low, and unit and unit for the first quadrupole and third quadrupole respectively.

The preliminary ion transmission study was performed by comparing the Q3 and Q1 signal by using MRM and Q1 SIM. MRM data was obtained using the UU setting and Q1 SIM data was obtained with unit in Q1 and no mass filter in Q3 (rf-only mode). It is

reported that transmission efficiency is around 60% for RF only mode and 10% of the mass filter mode.[28] If the mass resolution filter was the predominant factor controlling ion transmission, the lack of mass filter on Q3 for Q1 SIM could explain the low transmission ratio. In the ion transmission with various mass resolution filter window experiment, a 87% loss of signal for reserpine and a 76% loss of signal for Myo was observed when the mass resolution window was changed from LL to UU. Widening the mass isolation window from ±0.35 to ±0.7 *m/z* increased ion intensities by five- to ten-fold. Thus, the mass resolution filter was likely to be the major cause of the low ion transmission that was observed. The increase in abundance of ion was expected since the decreased slope included more area of the stable ion region and allowed more ions to pass through. Yang et al.[121b] also showed around 2/3 of the signal lost when changing from unit (1 *m/z* full width at half maximum) to enhanced (<0.1 *m/z* full width at half maximum). Nonetheless, they used polypropylene glycol (PPG) to demonstrate the ability to exclude interfering ion signals in the enhanced resolution mode. Although ion intensity could be drastically enhanced by widening the mass resolution window, it is crucial to understand the possibility of including interfering signals in detection. The interfering ions signals could result in noise thus decrease the signal-to-noise ratio, also known as the sensitivity, of the detection. False positives could also be obtained from the less accurate and specific method.

5.5 Conclusion

In this work, possible causes that contribute to the low ion transmission on a QqQ-MS, especially for multiply-charged protein ions targeted in MRM mode, were studied. Mass resolution filter was found to be the major cause of the low ion transmission and could be altered to achieve better sensitivity. Even though widening the mass resolution window can obtain higher ion signal, it compromises the accuracy and

sensitivity of the method. However, in applications where sensitivity is extremely important, widening the mass filter could allow detection and quantitation to proteins that only exist in trace amount. Argon as a CID gas was shown to encourage proton transfer. For a quantitative analysis, proton-transfer sacrifices method sensitivity and might compromise reproducibility and accuracy. By preserving the ion signal at a particular charge state, a higher sensitivity could be achieve by using helium or nitrogen as the CID gas. Nitrogen is cheaper than helium and could be used as an alternative CID gas for multiply charged ion analysis.

As shown in the previous study, the same unique and reproducible product ion for QqQ-MS quantitation could be obtained at different combination of CID gas pressure and collision energy. It was suggested to optimize CID gas pressure for the analyte of interest.[159] Through this study, we understood the possible disadvantages high CID gas pressure could introduce. If it is possible to obtain the product ion at a lower CID pressure, it could be better for overall sensitivity. To increase ion signal at a particular charge state, supercharging reagent such as benzyl alcohol, m-NBA, and sulfolane can also be used.[182] Tuning the instrument around the analyte of interest could be beneficial for quantitation accuracy. A closer interaction with the manufacture may provide more insight to change specific parameters to allow more ion transmission for different QqQ-MS. For future quantitation study, a MRM driven by proton transfer effect performed under ZCE using a charge state (n)+ as the precursor ion and another charge state (n-1)+ as the product ion could be developed. Compare to the low percentage of specific product ion generated by CID, proton transfer MRM might provide higher quantitation sensitivity.

Chapter 6

Summary and Future Work

Through the development and advancement of mass spectrometry, protein analysis has advanced significantly. Although triple quadrupole mass spectrometry has been the gold standard in quantifying proteins, most methods require protein digestion prior to the analysis.[22b, 123] The analysis of the digested peptide is also known as the bottom-up approach. If one desires to study intact proteins by top-down methods, then these are usually performed using high resolution mass spectrometers. Even though high resolution mass spectrometers have shown to be accurate for many applications,[183] the high cost of the instrument limits the technology to be widely spread, and most quantitative analysis is still performed using a bottom-up approach.

Bottom-up quantitation methods do not require high resolution mass spectrometry and are often performed using triple quadrupole mass spectrometry with unit resolution. However, the digestion step does not always reach completion and the result is often inconsistent from day to day and batch to batch. Relative quantitation is usually reported (e.g., healthy vs. diseased, treated vs. untreated, etc.). To eliminate the error, resource, and time involved in the protein digestion step, a method was created to detect and quantify intact proteins directly by triple quadrupole mass spectrometry using multiple reaction monitoring.[159] Even though inconsistent fragmentations were observed initially, a relatively soft collision condition was found to be beneficial to obtain unique and reproducible product ions. Calibration curves of the standard proteins including myoglobin, ubiquitin, lactalbumin, lysozyme, cytochrome c from equine heart, and cytochrome c from bovine heart were obtained. Acceptable linearity, limit of detection and quantitation, accuracy, and precision were obtained through this method. Biological fluids

including urine and plasma were used to test the specificity of this approach and the feasibility for future clinical applications.

Although this top-down quantitative approach has shown to be a possible and attractive technique, the limits of detection and quantitation could be improved to cover target proteins that are present in low abundance. The limit of detection, however, is analyte dependent due to the different response factors. Since intact proteins are multiply charged, it might be possible to use the summation of multiple MRMs from different charges to increase sensitivity. Potential matrix effects can also be addressed by different sample preparation steps or online sample cleanups but should be adjusted for the specific application; sample preparation could also be used to pre-concentrate target analytes. In the future, this method may be used to develop a biomarker quantitation method for targets such as the cancer-related protein AGR2 in urine.[117] The triple quadrupole mass spectrometry method should also be tested for its capability of differentiate and quantify proteins that have undergone post translational modification. Of course, the modification would have to cause a significant enough change in the mass of the protein to be resolved by the low resolution mass spectrometer.

Not only were the detection and the quantitation of intact proteins challenging, the separation of intact proteins is also significantly more complex than the typical small molecules. Reversed-phase liquid chromatography was investigated to pair with the intact protein quantitative analysis by triple quadrupole mass spectrometry. The general compatibility of the solvent and additive system with mass spectrometry, as well as the various commercially available functional groups on the stationary phase providing a wide range of selectivity are two of the greatest advantages for reversed-phase liquid chromatography to be used in this setting. Intact protein separation is often complex. The chemical forces that controls protein conformation are the same forces that controls the

chromatographic interaction. Therefore, changing chromatographic parameters can alter protein conformation that.[152] To study the effect of each chromatographic parameter and create a generic intact protein separation method as a base for future applications, protein standards myoglobin, cytochrome c, lactalbumin, lysozyme, and ubiquitin were used. Chromatographic parameters such as additive, flow rate, gradient slope, temperature, mobile phase, and stationary phase were investigated. As the result, an optimized method with 0.2 mL/min flow rate, 15% gradient volume, at 75 ˚C in a 0.05% trifluoroacetic acid and 0.1% formic acid modified acetonitrile mobile phase system was developed on a C4 wide-pore column.

To obtain better data quality with higher sensitivity, some re-iteration work on MRM optimization should be included. Since proteins can change shape, and consequently the accessibility of surface charged groups, with chromatographic parameters, it is better to re-define the highest intensity charge state as the precursor ion and re-optimize the MRM once the optimal chromatographic conditions have been set. Other ion pairing reagents can be investigated for better protein separation and less ionization suppression. A column selector can also be included to screen multiple stationary phases for the specific application using the generic method to achieve optimal separation with the least amount of chromatographic parameter alteration. Other mass spectrometry compatible separation technique such as hydrophilic interaction chromatography could also be explored.[184]

Although triple quadrupole mass spectrometry based quantitation is known to have high sensitivity and specificity, a less than 5% ion transmission of an intact protein ion signal was found from the first quadrupole to the third quadrupole under zero collision energy. In view of increasing intact protein detection sensitivity for the proteins that exist in lower abundance, experiments were performed to find and characterize the sources of

ion loss. Mass resolution filter width was found to be the greatest contributor as more than ¾ of the signal were lost when narrowing the mass resolution window by 0.7 *m/z*. Although ion intensity could be increased by widening the mass resolution window, the specificity and the sensitivity of the method could possibly be compromised. Since intact proteins are typically multiply charged by the electrospray ionization, proton transfer effect was also observed as the CID gas pressure was increased. Helium and nitrogen exhibited less proton transfer effect and could be considered as an alternative when performing quantitative analysis for multiply charged analyte; however, helium may not provide efficient fragmentation. It remains to be investigated how alternate collision gases affect MRM transitions for multiply-charged ion of intact proteins. For future applications, especially considering the potential to target larger proteins (e.g., > 30 kDa), supercharging reagents can be used to bring the charge state distribution maximum into the range of operation of the triple quadrupole instrument or to increase ion signal at a particular charge state to enhance sensitivity. Tuning the instrument using standards that are close to the analyte of interest can increase the mass resolution which increases method accuracy and sensitivity. Performing MRM by monitoring alternate charge states as the product ions from a specific charge state under zero collision energy (ZCE) could also be a possible method to achieve intact protein quantitation on QqQ-MS.

This work on intact protein detection, separation, and quantitation using reversed-phase chromatography and triple quadrupole mass spectrometer provided a more straightforward method to directly analyze intact protein with minimal sample preparation. The mass spectrometry based method provides quantitative information while confirming the identity of the proteins. Although the triple quadrupole mass spectrometer can only provide unit resolution, it is shown to be sufficient for intact protein quantitation. Availability of lower cost triple quadrupole mass spectrometer is more wide

spread and such systems could be adopted to establish intact protein MRM quantitation methods in a straightforward fashion. Such an approach could be applied for biomarker discovery, protein therapeutic monitoring, and drug response control. The establishment of this technique can greatly facilitate advancement in the clinical world.

Appendix A

Supplementary Information for Multiple Reaction Monitoring for Direct Quantitation of Intact Proteins using a Triple Quadrupole Mass Spectrometer

This supplementary information includes the figures generated from ubiquitin product ion scans at different collision energy, optimal collision energy at different collision gas pressure, preliminary intact protein MRM transitions, intact protein molecular weight determination, the protein fragment ion predictions through accurate mass, protein charge state profile and product ions mass spectra, calibration curves of the six protein standards, and data from studying the matrix effect using BSA and Cyt c B. Ubiquitin product ions produced from different collision energies displayed similar trend as myoglobin figures included in the paper where higher collision energies generated irreproducible fragments and lower collision energy produced reproducible product ions for quantitative analysis. Ubiquitin, myoglobin, and Cytochrome c B were used to study the relationship between the collision energy and gas pressure. Preliminary MRM transitions with the optimal condition is included. The reproducible fragment ion patterns from myoglobin, ubiquitin, cytochrome c (bovine), lactalbumin, and lysozyme were predicted through accurate mass and ProSight lite software. Mass spectra of the intact protein and product ions along with the five point calibration curves of the proteins are presented here.

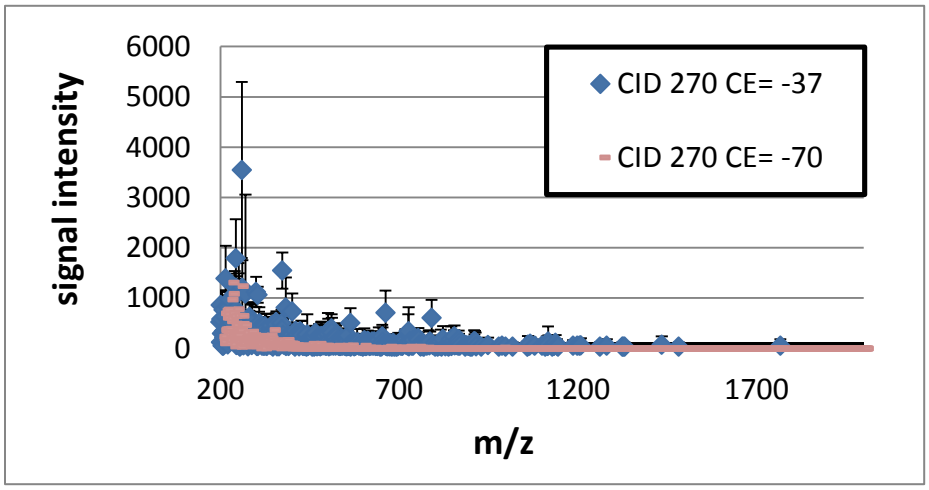


Figure S1. Ubiquitin product ion scans (n= 500) at CE= -37 and -70 V produced irreproducible product ions.

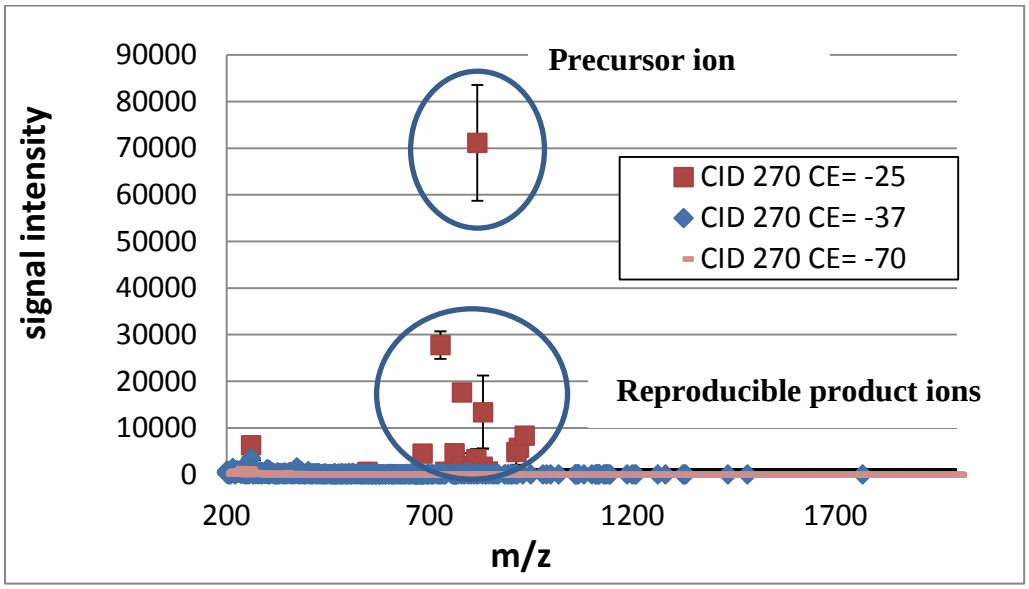


Figure S2. Reproducible product ions of Ubiquitin were found at CE= -25 V while precursor ion was present. The reproducible product ions found at CE= -25 V were significantly higher in signal intensity compared to irreproducible product ions generated at higher collision energy (-37 and -70 V).

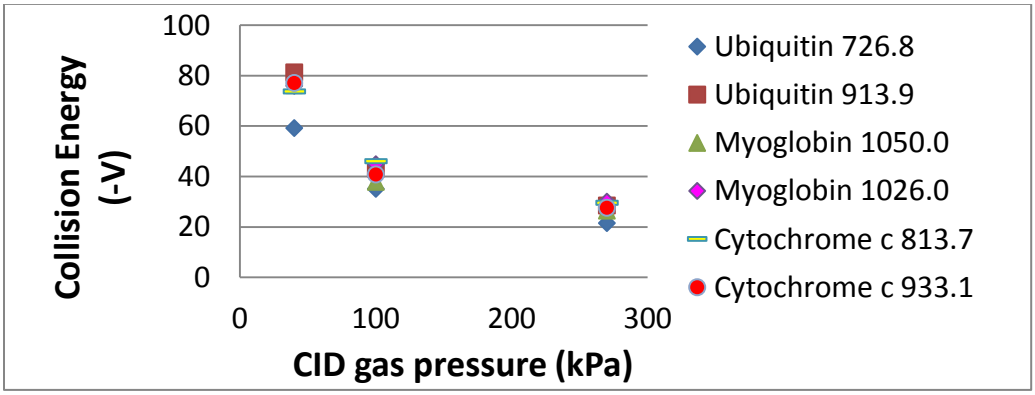


Figure S3**.** Optimum collision induced dissociation energy for product ions of ubiquitin, myoglobin, and cytochrome c at low (40 kPa), medium (100 kPa), and high (270 kPa). As the CID gas pressure increase, the optimal collision energy for producing reproducible and intense product fragment decreases.

Table S1. Preliminary MRM transitions of the protein standards

Protein	Molecular Weight (Da)	MRM	Q1 Bias (V)	Collision Energy (V)	Q3 Bias (V)
Myo	16942±2	998.4 [+17]> 1050.0	-22	-26.4	-30
		998.4 [+17]> 1026.0	-22	-27.1	-30
Lysz	14295±1	1431.7 [+10] > 1427.2	-50	-49.9	-36
		1431.7 [+10] > 1418.5	-50	-49.2	-36
Ubiq	8560±2	779.7 [+11]> 913.9	-22	-28.5	-26
		779.7 [+11]> 726.8	-22	-23.1	-34
Lact	14168±1	1773.2 [+8] > 243.2	-38	-103.4	-11
		1773.2 [+8] > 1770.9	-38	-54.0	-44
Cyt c E	12352±2	773.6 [+16] > 817.9	-40	-21.5	-22
		773.6 [+16] > 838.5	-40	-21.6	-32
Cyt c B	12223±1	874.7 [+14] > 813.7	-40	-29.5	-28
		874.7 [+14]> 933.1	-40	-27.6	-26

Protein fragment prediction through accurate mass of the product ions

The following product ions are measured in Shimadzu IT-TOF or Waters Synapt G2 Q-TOF mass spectrometer with direct injection of the protein standards. ProSight lite was then used to predict possible fragmentation patterns according to the accurate mass of the product ion obtained.

Protein standard concentration: 100 µg/mL

Injection volume: 5-25 µL

Solvent: 50: 50: 0.1 MeOH: H_2O: Formic Acid

Total flow: 0.3 mL/min

Scan range: ±150 *m/z* of the expected ion *m/z*

Scan speed: 393 msec/scan

CDL temperature: 200°C

Heat block temperature: 200°C

Cytochrome C [Fragment 813.4420]

Name	Ion Type	Theoretical Mass	Mass Difference (Da)	charge state	AA sequence
B81	B	8943.56648	-6.79248	11	G1-I81
Y29	Y	3248.795435	0.940565	4	P76-E104
Y42	Y	4871.633235	2.970765	6	T63-E104
Y72	Y	8119.213085	5.126915	10	H33-E104

Cytochrome C [Fragment 933.0529]

Name	Ion Type	Theoretical Mass	Mass Difference (Da)	charge state	AA sequence
B85	B	9331.77752	-11.32852	10	G1-I85
Y40	Y	4657.501495	2.723005	5	M65-E104
Y66	Y	7451.857675	4.501525	8	K39-E104
Y48	Y	5586.950925	5.318475	6	I57-E104
B17	B	1861.95423	2.13557	2	G1-C17

Lactalbumin [Fragment 243.7499]

Name	Ion Type	Theoretical Mass	Mass Difference (Da)	charge state	AA sequence
B18	B	2180.12994	4.54716	9	E1-Y18
Y14	Y	1706.837145	-7.643845	7	L110-L123

Lactalbumin [Fragment 1769.9238]

Name	Ion Type	Theoretical Mass	Mass Difference (Da)	charge state	AA sequence
Y92	Y	10620.03532	-6.540515	6	H32-L123
B93	B	10619.91493	-6.42013	6	E1-K93
Y45	Y	5308.704755	-1.957355	3	K79-L123

Lysozyme [Fragment 1414.9834]

Name	Ion Type	Theoretical Mass	Mass Difference (Da)	charge state	AA sequence
B90	B	9941.71537	-8.88757	7	K1-A90
B25	B	2836.4377	1.5131	2	L1-L25
Y128	Y	14175.78231	13.971695	10	V2-L129

Lysozyme [Fragment 1426.5145]

Name	Ion Type	Theoretical Mass	Mass Difference (Da)	charge state	AA sequence
Y103	Y	11410.41811	-6.366105	8	N27-L129
B76	B	8556.02932	-2.99032	6	K1-C76
B116	B	12830.12622	-0.56772	9	K1-K116
Y38	Y	4275.129865	1.389635	3	V92-L129
Y66	Y	7121.440795	6.091705	5	C64-L129

The following product ions are measured in Waters SYNAPT G2 QTOF mass spectrometer with direct flow injection of the protein standards. ProSight lite was then used to predict possible fragmentation patterns according to the accurate mass of the product ion obtained.

Protein standard concentration: 10 µg/mL

Solvent: 50: 50: 0.1 MeOH: H_2O: Formic Acid

Sample injection flow: 5 µL/min

Scan range: 50-2000 *m/z*

Scan speed: 1s/scan

Capillary voltage: 2.7 kV

Ubiquitin [Fragment 726.5147]

Name	Ion Type	Theoretical Mass	Mass Difference (Da)	charge state	AA sequence
Y58	Y	6527.488745	2.071555	9	P19-G76
Y13	Y	1449.841565	1.171835	2	E64-G76

Ubiquitin [Fragment 913.6602]

Name	Ion Type	Theoretical Mass	Mass Difference (Da)	charge state	AA sequence
B49	B	5476.96427	-1.05107	6	M1-Q49
B41	B	4563.41452	-0.15352	5	M1-Q41
Y40	Y	4561.451665	1.809335	5	P37-G76
Y48	Y	5472.959275	2.953925	6	K29-G76

Myoglobin [Fragment 1025.1360]

Name	Ion Type	Theoretical Mass	Mass Difference (Da)	charge state	AA sequence
B83	B	9217.87182	-0.71982	9	G1-E83
B119	B	13312.16629	1.49771	13	G1-H119
Y147	Y	16382.73609	3.311915	16	W7-G153
Y103	Y	11260.00645	5.401555	11	T51-G153

Myoglobin [Fragment 1049.4140]

Name	Ion Type	Theoretical Mass	Mass Difference (Da)	charge state	AA sequence
Y18	Y	2098.084695	-1.272695	2	E136-G153
B142	B	15730.33003	-4.24003	15	G1-I142
Y142	Y	15728.38715	-2.297145	15	N12-I142
Y151	Y	16770.85911	3.636895	16	S3-G153
B19	B	2096.03266	0.77934	2	G1-A19
B65	B	7335.75662	3.08538	7	G1-G65

Protein Molecular weight determination

The molecular weights reported in the article were calculated from full scan mass spectra obtained. All charge state ions across the protein profile were used to calculate the original molecular weight of the intact protein using the following equation:

$$M.W. = \frac{m}{z} \times \ isolated\ charge\ state - (charge\ state\ \times M.W. of\ Hydrogen)$$

The calculated molecular weight from all charge states were averaged to attain protein molecular weight and the error of seven proteins were all within 2 Da.

Table S2**.** Molecular weight determination of Cyt c B

Charge states	*m/z*	Predicted M.W.
18	680.2	12225.456
17	720.2	12225.414
16	765.0	12223.872
15	816.0	12224.88
14	874.2	12223.988
13	941.3	12223.796
12	1019.6	12222.504
11	1112.1	12222.012
10	1223.3	12222.92
9	1359.0	12221.478
8	1528.7	12221.536
7	1747.0	12221.594

Avg. M. W.	**12223.2875**
SD	**1.490490371**

Table S3. Molecular weight determination of Cyt c E

Charge states	*m/z*	Predicted M.W.
18	687.4	12354.156
17	727.8	12354.614
16	773.2	12354.272
15	824.6	12353.88
14	883.4	12352.788
13	951.2	12351.846
12	1030.4	12352.104
11	1124.0	12352.362
10	1236.2	12351.42
9	1373.2	12349.728
8	1544.9	12351.136
7	1765.3	12349.694

Avg. M. W.	**12352.33333**
SD	**1.68397308**

Table S4. Molecular weight determination of Lysz

Charge states	*m/z*	Predicted M.W.
13	1100.8	14296.646
12	1192.3	14294.904
11	1300.6	14295.512
10	1430.4	14293.92
9	1589.3	14294.178
8	1787.9	14294.736

Avg. M. W.	**14294.98267**
SD	**0.989004887**

Table S5. Molecular weight determination of Ubiq

Charge states	*m/z*	Predicted M.W.
14	612.6	8561.588
13	659.7	8562.346
12	714.5	8561.304
11	779.4	8562.312
10	857.0	8560.42
9	952.2	8560.278
8	1071	8559.936
7	1223.8	8559.194
6	1427.4	8558.052
5	1712.7	8558.21

Avg. M. W.	**8560.364**
SD	**1.55107418**

Table S6. Molecular weight determination of Lact

Charge states	*m/z*	Predicted M.W.
12	1181.7	14167.704
11	1289.2	14169.562
10	1417.7	14166.92
9	1575.3	14168.628
8	1771.9	14167.136

Avg. M. W.	**14167.99**
SD	**1.09934526**

Table S7. Molecular weight determination of Myo

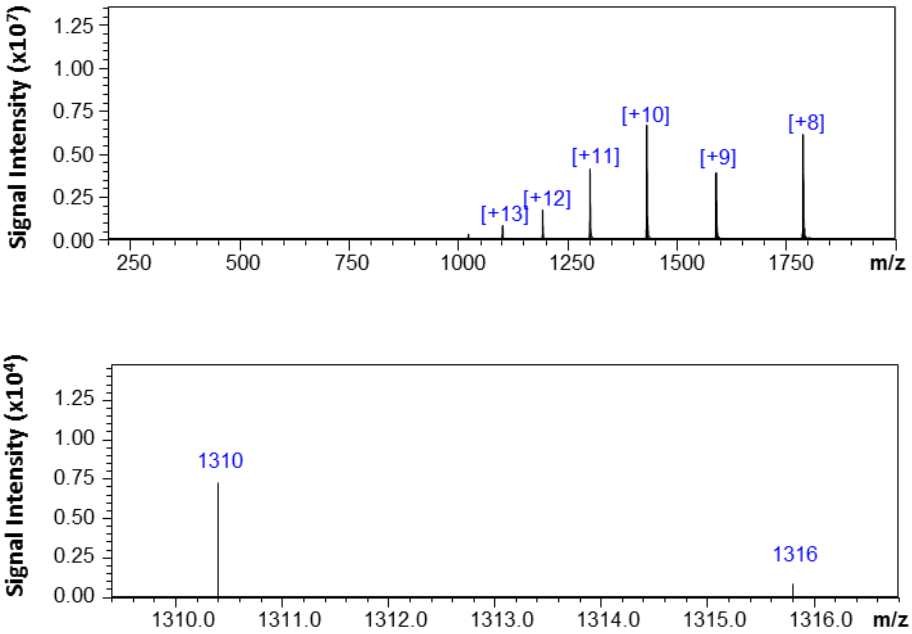
Charge states	*m/z*	Predicted M.W.
25	678.9	16946.05
24	706.9	16941.408
23	737.8	16945.066
22	771.3	16945.324
21	807.9	16943.682
20	848.2	16942.84
19	892.8	16943.098
18	942.3	16942.356
17	997.6	16942.064
16	1060.0	16943.072
15	1130.5	16941.63
14	1211.1	16941.288
13	1304.2	16940.846
12	1412.8	16941.504
11	1540.9	16938.812
10	1695.2	16941.42
9	1883.4	16941.078

Avg. M. W.	**16942.44341**
SD	**1.827765133**

Intact protein charge state distribution profile and product ions fragmented

Protein profiles constituted with the multiple charge states of the proteins were obtained again after the chromatography elution solvent composition were known. The highest signal intensity charge state (or the second highest if no reproducible product ion could be found from the highest) was isolated as the precursor ion for further MRM development and optimization. Product ions selected for MRM transitions are also included as follow.

Figure S4. Intact protein profile and product ions of Lysz



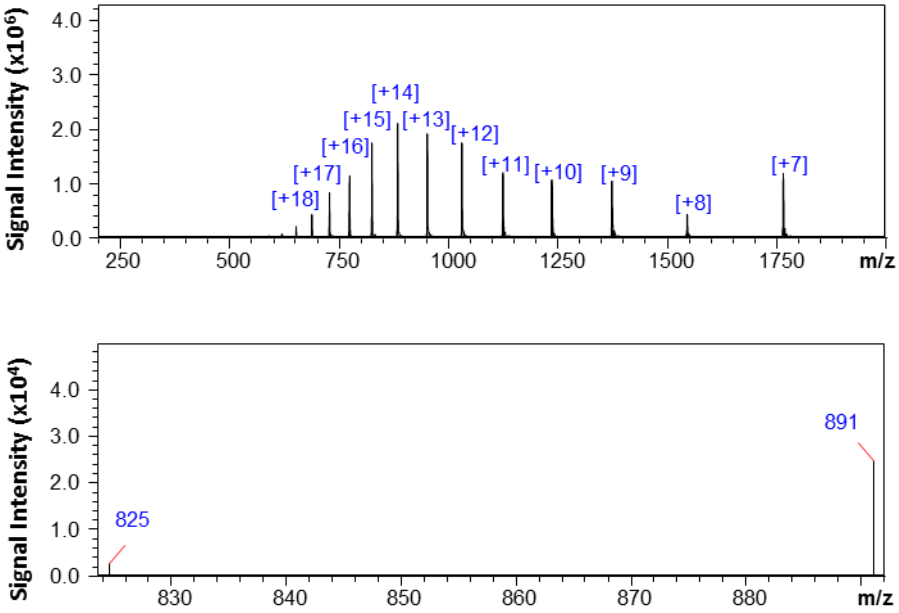


Figure S5. Intact protein profile and product ions of Cyt c E

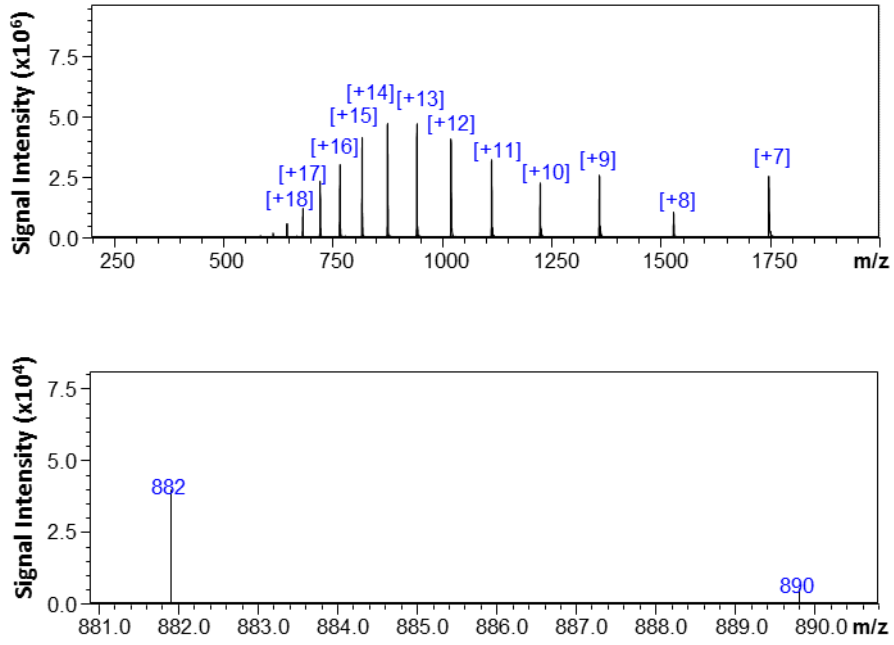


Figure S6. Intact protein profile and product ions of Cyt c B

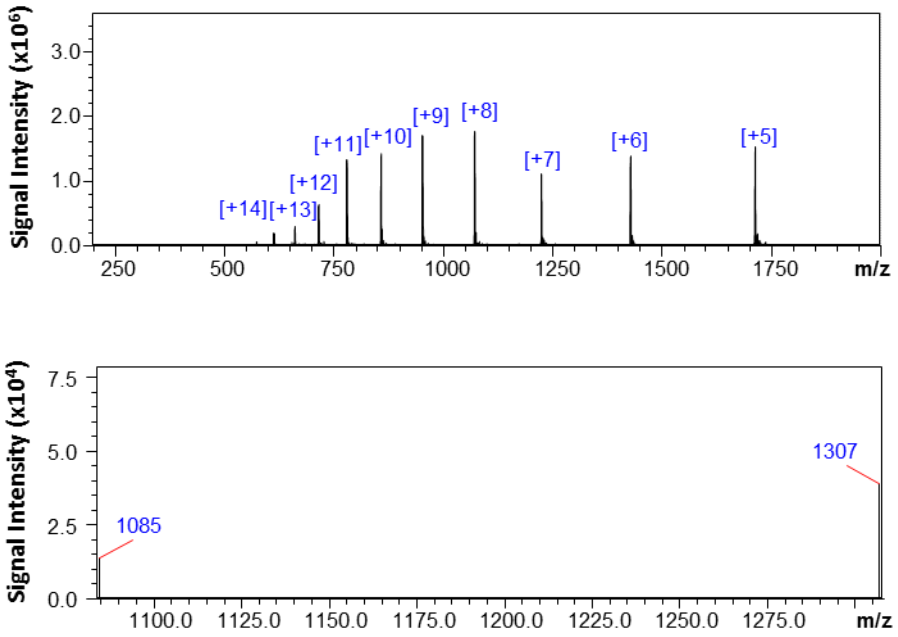


Figure S7. Intact protein profile and product ions of Ubiq

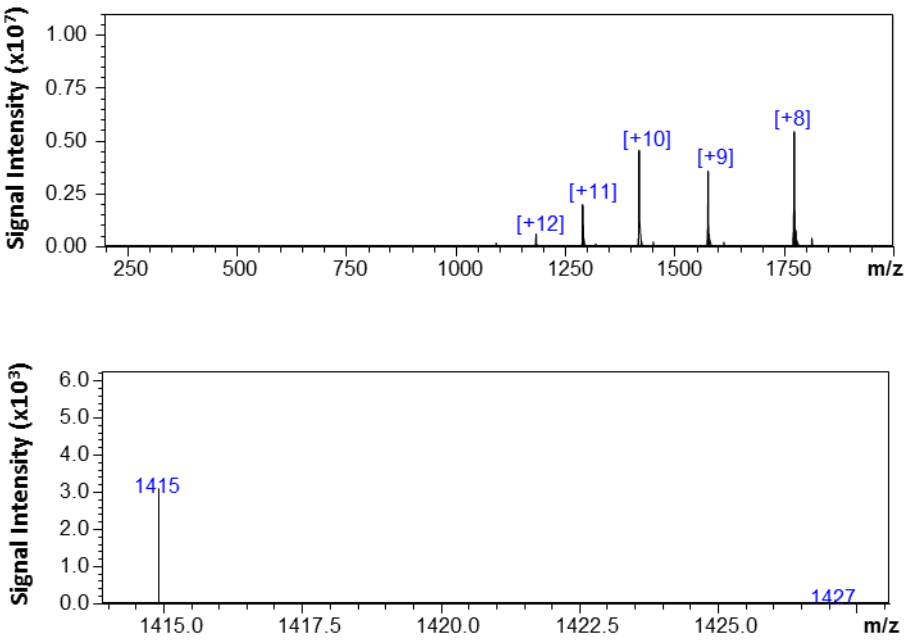


Figure S8. Intact protein profile and product ions of Lact

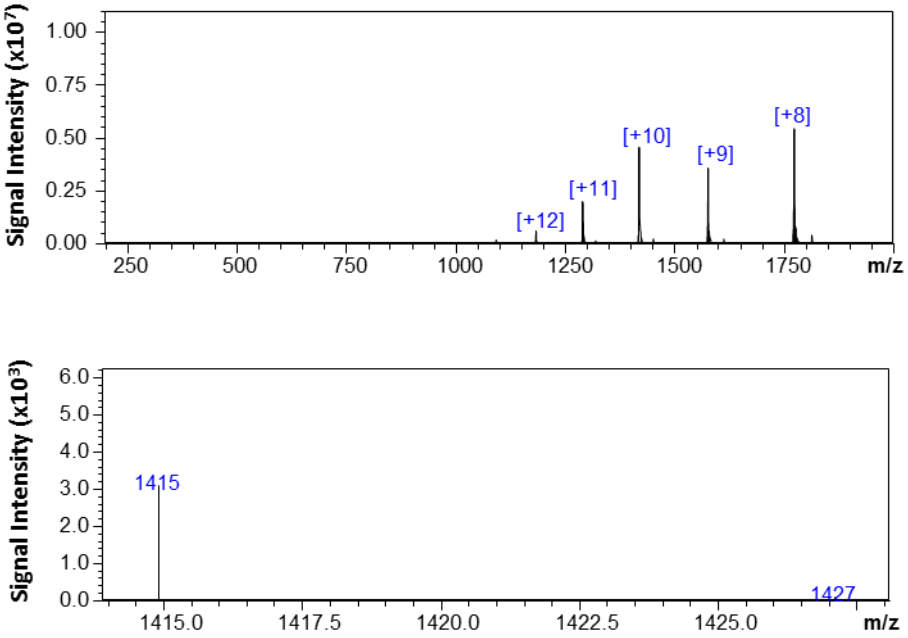


Figure S9. Intact protein profile and product ions of Lact

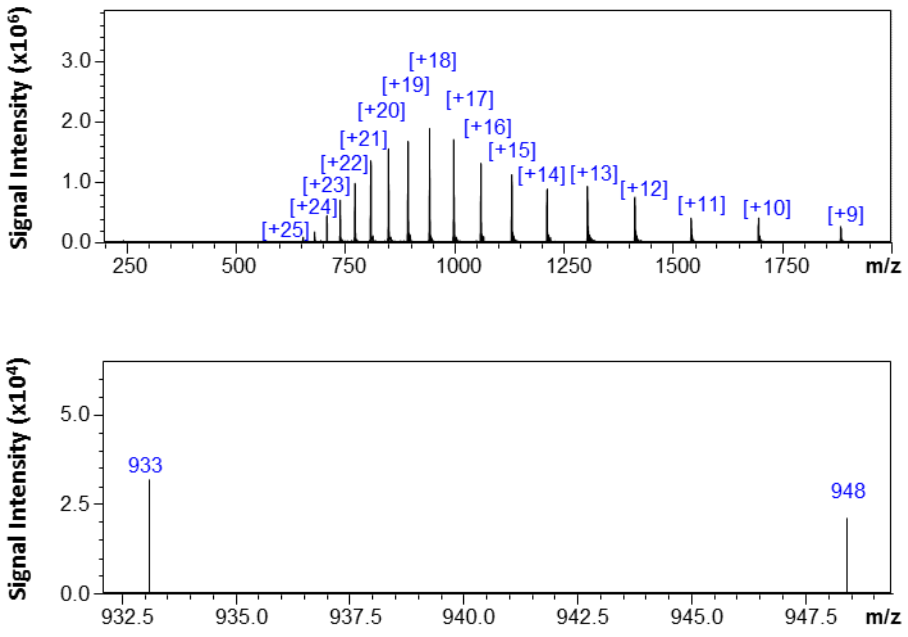


Figure S10. Intact protein profile and product ions of Myo

Calibration curves of intact proteins

Five points calibration curves of the six proteins were generated in triplicates using quantifier product ion MRM transitions. Error bars are included in all points.

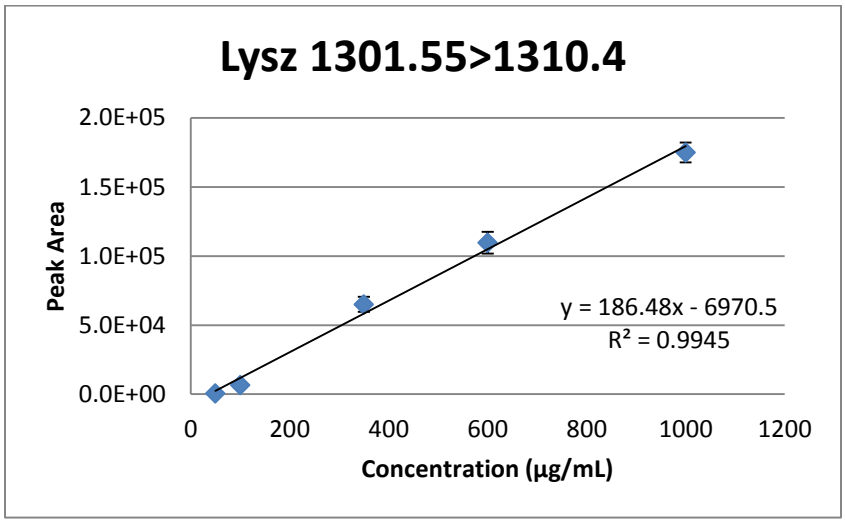


Figure S11. Calibration curve of Lysz

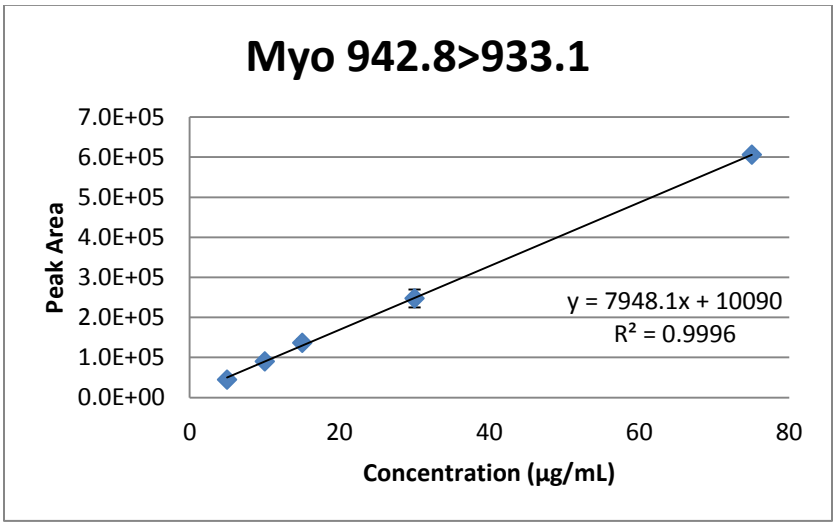


Figure S12. Calibration curve of Myo

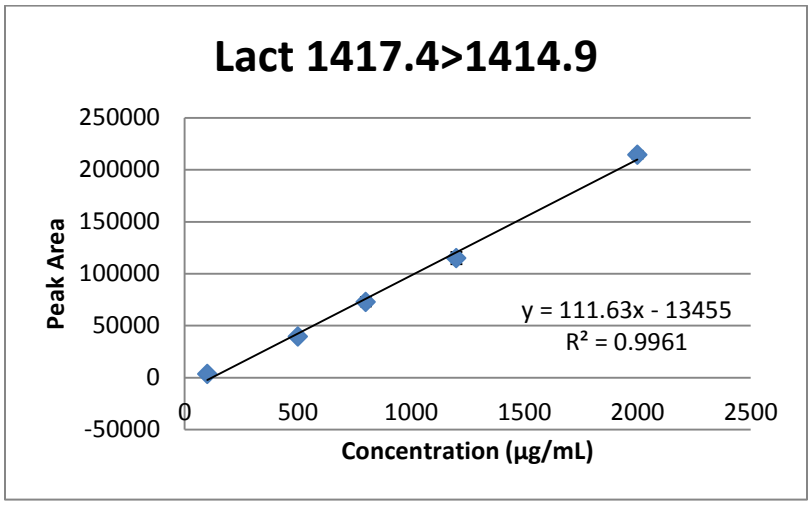


Figure S13. Calibration curve of Lact

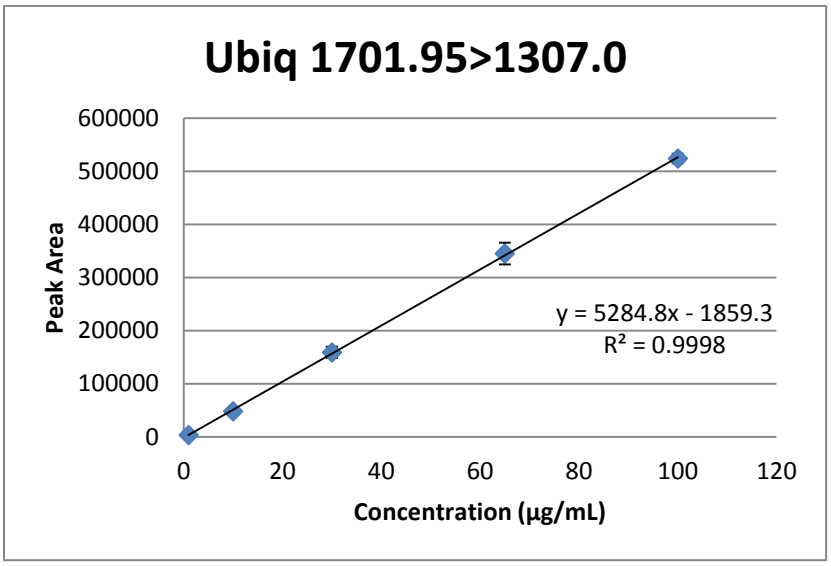


Figure S14. Calibration curve of Lact

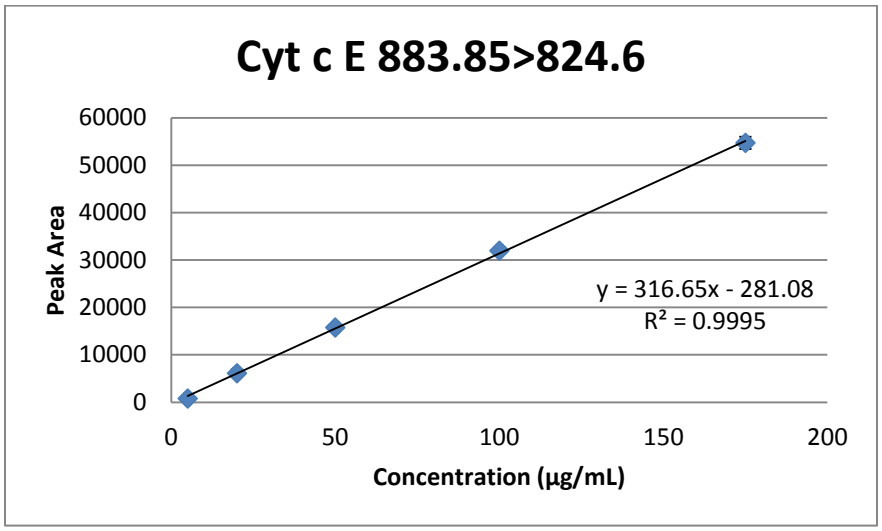


Figure S15**.** Calibration curve of Cyt c E

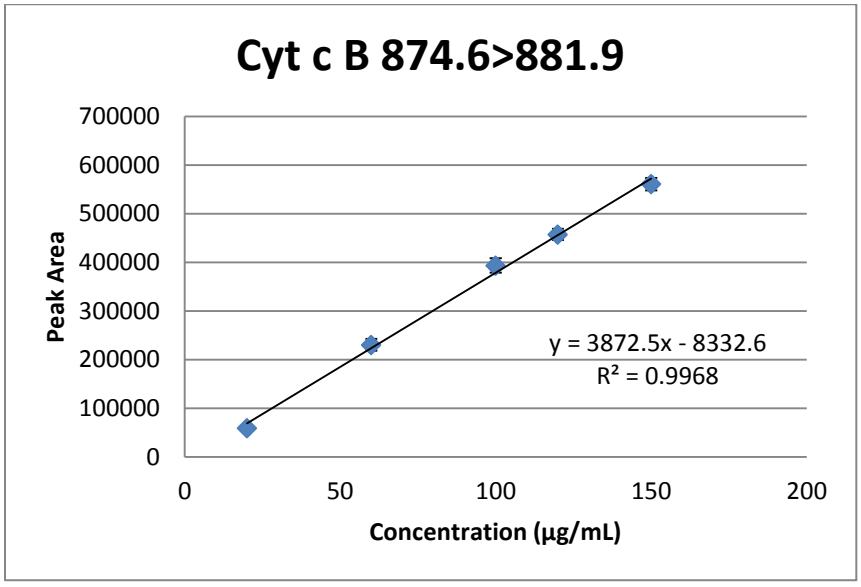


Figure S16. Calibration curve of Cyt c B

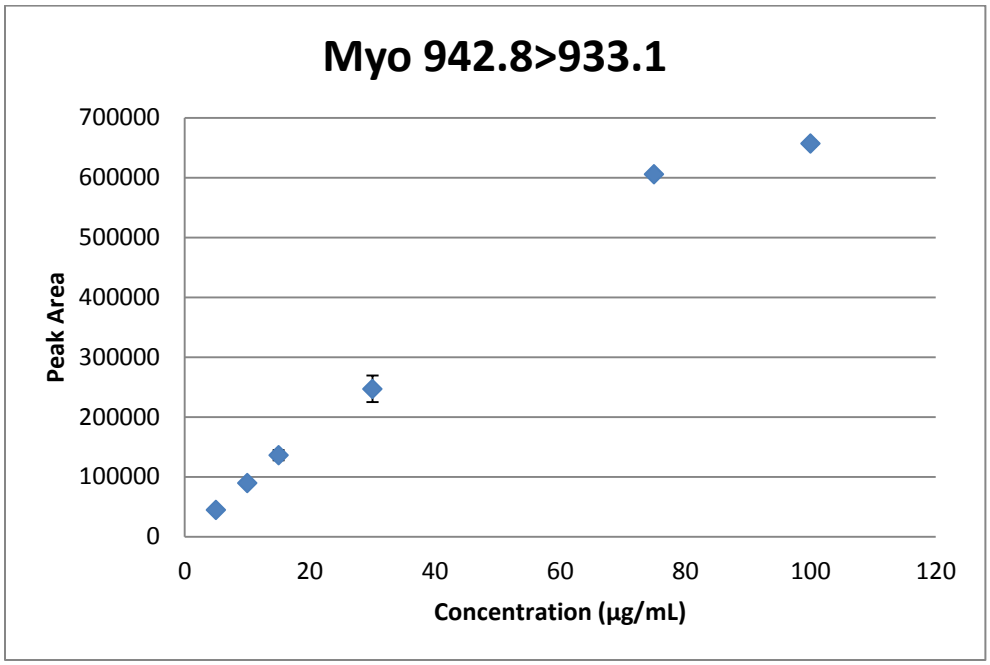


Figure S17. Myo calibration curve extending the linear range.

The myoglobin calibration curve included a point at 100 µg/mL, which was outside of the linear range. This figure demonstrated that a greater dynamic range may be considered if nonlinear response at higher concentrations are accounted.

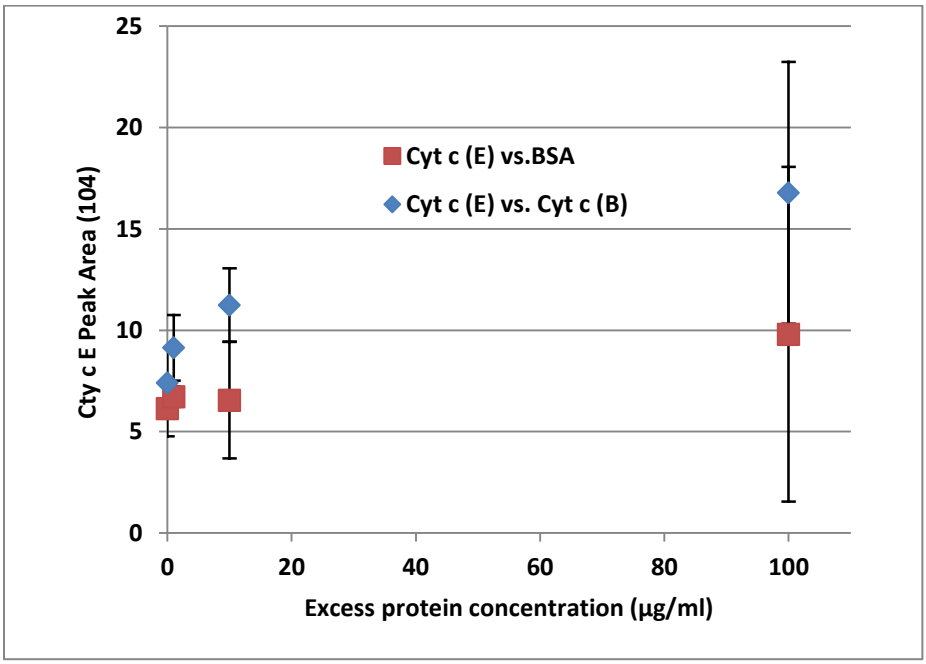


Figure S18. Cyt c E peak area at different excess protein, BSA and cytochrome c B, concentrations are shown to demonstrate matrix effect in a multiple proteins sample.

Excess protein concentrations of Cyt c B and BSA were used to demonstrate the specificity and susceptibility of intact protein quantitation when matrix effect exist. Cyt c B represents protein with sequence homology to the protein of interest and BSA represents a high abundance matrix component.

Appendix B

Supplementary Information for Reversed-Phase Separation Parameter for Intact Proteins using Liquid Chromatography – Triple Quadrupole Mass Spectrometry

This supplementary information includes the protein peak area comparison obtained from methods with various gradient volumes. It also contains the Protein peak half-width comparison with various gradient volumes. Protein standards ubiquitin (Ubi), lactalbumin (Lac), lysozyme (Lys), myoglobin (Myo), Cytochrome c from bovine heart (Cyt (B)), and cytochrome c from equine heart (Cyt (E)) were used to evaluate the effect.

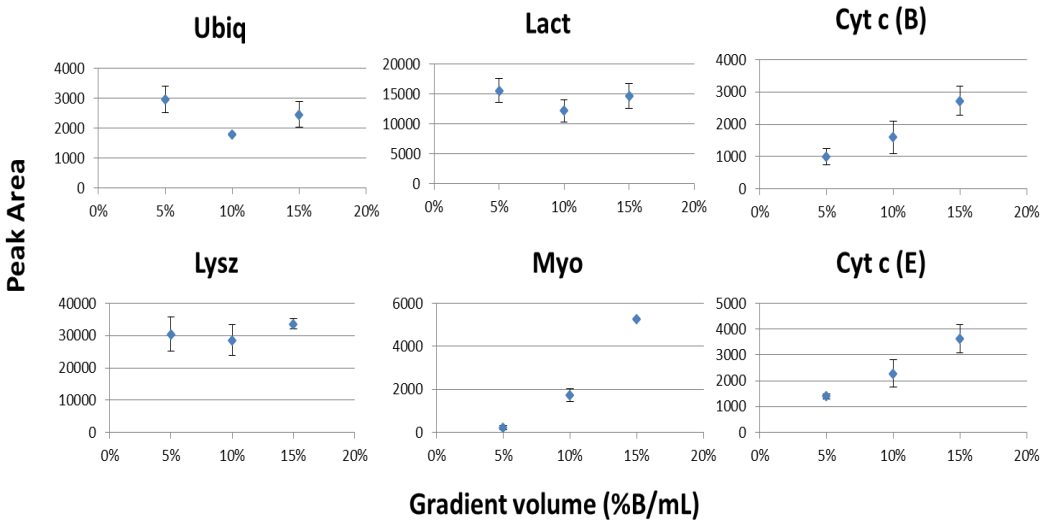


Figure S1. Protein peak area obtained with gradient methods at various gradient volumes (5%, 10%, and 15%)

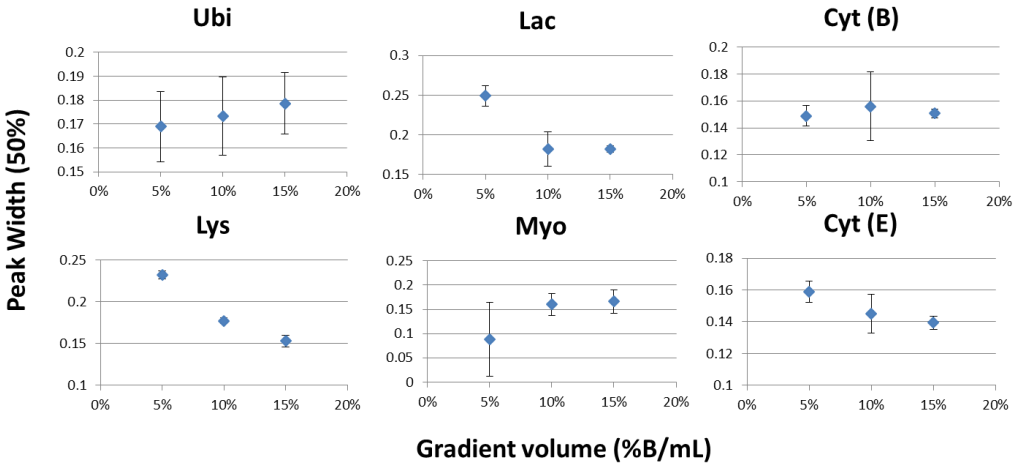


Figure S2. Protein peak widths (50%) obtained with gradient methods at various gradient volumes (5%, 10%, and 15%)

Appendix C

Protocal for Intact Protein MRMs Optimization

The purpose of this protocol is to establish a standard procedure in optimizing intact protein MRM on Shimadzu 8050 triple quadrupole mass spectrometer for quantitative analysis. Although most methods using triple quadrupole mass spectrometer calls for prior digestion, this method bypasses all digestion procedures and can be used to directly quantify intact proteins. This protocol contains the sample preparation, instrument setup, and the step by step direction in optimizing intact protein MRM. A minimum of 25 µg of intact protein standards is required to perform MRM optimization.

Procedure

1. Weigh intact protein standard and make 1 mg/mL stock solution (This concentration can be altered if the amount of the standard is limited) in LCMS grade water. A minimum volume of 200 µL is required to successfully dissolve and mix the solution.
2. Dilute the stock sample solution into a series of working concentrations from 0.1 µg/mL to 1 mg/mL with 0.1% FA (by volume). A minimum volume of 100 µL is required to fill the insert in the vial to be drawn by the autosampler syringe
3. Open autosampler SIL-30 AC and place the vials in the sample rack.
4. Check the lines to make sure the line coming out from the autosampler is linked directly to the ionization source prove of the mass spectrometer.
5. Open LabSolution software on the computer.
6. Under Method in the toolbar, click on Instrument Parameters tab.
7. On the Instrument Parameters view window, go to MS tab to click on Scan (+) and choose Q3 Scan. Set Acquisition Time as 2 min.
8. In the same window, set Start *m/z* at 200 and End *m/z* at 2000. Leave the rest as default.
9. Go to Data Acquisition tab to change the LC Stop time at 2 min and uncheck the PDA (the PDA lamp can be turned off, if desired, in the PDA tab).

10. In Pump tab, set the Mode as Binary gradient and the total flow at 0.1 mL/min with pump B conc. being 50% organic.
11. Uncheck both column ovens under Column Oven tab.
12. Leave the rest of the tab and selection as default.
13. Click Start Single run and input the Sample Name, Data File name, Vial #, Tray number, and Injection volume in µL.
14. Click Start button to start a Q3 Scan.
15. Check the result in Postrun program. Averaging the peak to obtain the mass spectrum of the intact proteins. A protein profile with a distribution of multiply charged ions should be observed.
16. Pick ion with the highest intensity to be the precursor ion for MRM optimization.
17. On the left side of the window, click on Data Acquisition, then Optimization for Method.
18. A Condition Settings for Optimize Method window will pop out providing the choices between three purposes of the study.
19. Check Optimize MRM event for product ion search.
20. Check the boxes for a) Check for Precursor ion, b) Adjust Precursor m/z, and c) Optimize voltage for the initial product investigation steps.
21. Go into the Advanced Setting and check the boxes to optimize Q1 Pre Bias and CE. Uncheck the Q3 Pre Bias and Optimize detail CE. Since no product ion is selected, detailed optimization is not necessary.
22. For CE, change the Lower Limit to -1 and Upper Limit to -70 and leave the Step Width at 5.
23. Return back to the Condition Settings for Optimize Method window and click on Auto Selection Condition.

24. The Auto Selection Condition Settings window will pop up. Uncheck Relative to precursor ion box and set Min Product ions *m/z* at 200 and Max Product ion *m/z* at 2000.
25. Click Okay button to return back to the Condition Settings window and input Compound Name, Precursor *m/z* as previously determined from Q3 Scan, Vial #, Tray, and Inj. Vol.
26. Click on Start to initiate the initial intact protein MRM product ion search.
27. Open the data file with _CE_Select.lcd at the end and average across the chromatographic peak to obtain mass spectra from all collision energy. Look for lowest collision energy where the precursor ion is showing fragmented product.
28. Repeat step 18 to 27. However, as for step 23, change the Lower Limit and Upper Limit to 10 V lower and higher than the lowest collision energy where the product ions are found. Change the Step Width to 0.6.
29. Open the data file with _CE_Select.lcd at the end and average across the chromatographic peak to obtain mass spectra from all collision energies. Compare the mass spectra to find a product ion that appears on multiple collision energies as potential product ions for the MRM.
30. Make a MRM method according to step 6-13. In step 7, choose MRM (+) instead of Scan (+) and input Precursor m/z and Product m/z. Save as a new method.
31. Select the Optimize voltage Optimization for Method.
32. The MRM method should be auto-filled in the window. Check the boxes for Adjust Precursor m/z, Optimize Voltage, and Adjust Product m/z.
33. In Advanced Settings, check to optimize Q1 Pre Bias, CE, and Q3 Pre Bias. Set the Lower and Upper Limit to the setting on step 29 with 0.6 Step Width.

34. Check the box to optimize CE with details. Set Lower and Upper Limits with -0.6 and 0.6 with Step Width at 0.1.
35. Insert Vial #, Tray, and Inj Vol. information then Start the voltage optimization process.
36. The optimized MRM parameters will be saved into the MRM method file previously created.
37. Repeat the process from step 31 two more times to fine tune the optimization

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Biographical Information

Evelyn Wang obtained her Bachelor of Science degree in Biochemistry with a Biology minor from the University of Texas at Arlington in December of 2009. While looking for volunteering hours, she stumbled into Dr. Carl Lovely's Organic Chemistry lab and started doing research on synthesizing organic compounds to make natural product for pharmaceutical use. She later joined Dr. Kevin Schug's Analytical Chemistry lab to work on identifying new antibacterial agents from natural products and synthetic libraries by direct affinity HPLC-ESI-MS.

She enjoyed research and her project so much to the point that she decided to get her Ph.D. degree under Dr. Kevin Schug. During her graduate school years, she developed a synthetic polymeric mesh screen material through collaboration with marine biology, organic chemistry, and physical chemistry research groups to rapidly extract new chemical entities that obtain characteristic of potential antibiotic drugs. This approach focuses on compounds from natural products that target the tripeptite *L*-Lysine-*D*-Alanine-*D*-Alanine. Potential drug compounds were analyzed through a new ambient ionization mass spectrometry technique: Transmission Mode-Desorption Electrospray Ionization (TM-DESI). One of her major contribution was developing a method to directly quantify intact protein in biological fluids including urine and plasma in QqQ-MS. This method bypass the lengthy and error-prone protein digestion step and can possibly achieve absolute protein quantitation.

She graduates with her Ph.D. degree in Analytical Chemistry from the University of Texas at Arlington in Augest 2016. She plans to start a career related to pharmaceutical and clinical field.