Supercharging and Multiple Reaction Monitoring Optimization of High Molecular Weight Intact Proteins using Triple Quadrupole Mass Spectrometry.

By

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THESIS

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Abstract

Supercharging and Multiple Reaction Monitoring (MRM) Optimization of High Molecular Weight Intact Proteins using Triple Quadrupole Mass Spectrometry

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Various supercharging agents, such as *meta*-nitrobenzyl alcohol, dimethyl sulfoxide, trifluoroethanol (TFE), and sulfolane were tested in different concentrations to study the effects of supercharging agents on the charge distributions of the high molecular weight intact proteins. The data were analyzed by triple quadrupole mass spectrometry. For most of the proteins, m-NBA and TFE worked more effectively than other supercharging agents, both to shift the charge state and increase intensity. The settings of different instrumental parameters were also studied. Both increases in source temperature and flow rate had a negative impact on average charge state observed. The goal of this work was to increase charge acquisition and ionization efficiency for proteins ranging from 66 kDa – 150 kDa, to allow subsequent optimization of multiple reaction monitoring (MRM) mode transitions for potential top-down quantitative analysis. The MRM transitions of six to eight different precursor ions of the proteins were optimized to demonstrate the feasibility of this approach and limits of detection were evaluated.

Keywords: immunoglobulin G antibody; trifluoroethanol; albumin; transferrin; top-down; electrospray ionization

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List of Abbreviation

LC-MS: Liquid chromatography – mass spectrometry

DMSO: Dimethyl sulfoxide

m-NBA: *meta*-Nitrobenzyl alcohol

TFE: Trifluoroethanol

QqQ: Triple quadrupole

QQQ-MS: Triple quadrupole mass spectroscopy

MRM: Multiple reaction monitoring

SRM: Selected reaction monitoring

CID: Collision induced dissociation

ECD: Electron capture dissociation

ETD: Electron transfer dissociation

CE: Collision energy

BSA: Bovine serum albumin

hTfr: Holotransferrin

IgG: Immunoglobulin G

Q_1: First quadrupole

Q_3: Third quadrupole

ESI: Electrospray ionization

ESI-MS: Electrospray ionization mass spectrometry

ESI-MS/MS: Electrospray ionization tandem mass spectrometry

Chapter 1

Background on intact proteins and their identification techniques

Proteomic identification is a recent but widely used global method which increases opportunities of biological understanding [1]. It is useful in development of new biomarkers and protein drug efficacy [2, 3]. There are different types of protein identification techniques in relation to mass spectrometric analysis [4, 5]. Intact protein identification by a top-down approach is a recently used, but proliferating technique that has distinct advantages for characterization of proteins and protein isomers and their post translational modification over its counterpart, the bottom-up approach. The bottom-up approach is different from top-down approach in that proteins are digested first before entering them into analytical measurements which is good for increased sensitivity, since many state-of-the-art mass spectrometry instruments are currently best suited for small molecule (i.e., peptide) detection. But, incomplete digestion of the protein often causes unreliable and irreproducible results. In addition, due to incomplete digestion, all proteins may not be detected by the mass spectrometer. Similarly, the digestion of protein into small peptides and further fragmentation into smaller forms are often accompanied by loss of some important information about protein isoforms and post translational modifications in intact proteins [6 - 9]. Therefore, intact protein identification by top-down approach can be considered as a more specific and reliable technique with respect to providing information about the proteins and their genotypes [10].

The intact protein refers to a highly pure form of protein with its native covalent bonds and its native molecular formula. The top-down method involves direct insertion of intact

proteins for characterization by mass spectrometry techniques, where fragmentation is commonly performed to glean additional information about the protein, as desired. Fragmentation is performed in different ways; it can be induced by collision induced dissociation (CID), electron transfer dissociation (ETD), and electron capture dissociation (ECD), among others [11, 12]. Intact protein analysis provides information about different protein isoforms and post translational modifications, which can further provide phenotypic understanding and can help in early stage detection of certain diseases. By matching the exact proteoform with disease phenotype, a specific method for the early detection of disease and its clinical use can be designed [4]. Since we are introducing the intact proteins directly into the mass spectrometer, there is a chance of lowering sensitivity because the mass spectrometer works better in lower mass-to-charge ratio, for example as in bottom-up approach. There are other approaches that we can use to increase sensitivity. For example, supercharging agents are known for shifting of the electrospray protein charge state envelopes towards higher magnitudes, but they may also help to increase ionization intensity to better enable a more sensitive top-down analysis [13].

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Chapter 1.1

Mass spectrometry based protein quantification

Most research-based protein quantification methods are based on mass spectrometric techniques. Mass spectrometry in combination with different chromatographic techniques is widely used for better separation and analysis of proteins. Some of the widely-used techniques are matrix assisted laser desorption/ionization – time of flight (MALDI-TOF), surface enhanced laser desorption/ionization (SELDI-TOF), liquid chromatography/ mass spectrometry (LC/MS), and liquid chromatography- tandem mass spectrometry (LC/MS/MS). They work differently, and they have their own advantages and disadvantages. Among them, MALDI-TOF and SELDI-TOF are the protein fingerprinting identification based techniques. In matrix assisted laser desorption/ionization (MALDI), the proteins are first mixed with matrix where it undergoes laser desorption for ionization. Usually used matrix for protein analysis are α-cyano,4-hydroxycinnamic acid (CHCA), 3,5-dihydroxy benzoic acid (3,5-DHB) and sinapinic acid. The main function of the matrix is to protonate the sample in the gas phase, after desorption, so that ion formation takes place. The laser is the source for desorption of the ions. Since the ions are usually singly charged (i.e. molecular ion peak only typically observed),less information about protein composition, as well as the different isoforms of the proteins and other biological samples can be gleaned, unless a very high resolution mass spectrometer is available. The ions formed by the MALDI source are typically analyzed using time of flight (TOF) mass spectrometers.

The TOF analyzer separates the ions based upon their masses and time required by each mass to reach the analyzer. In TOF analyzer, the ions formed by the source are accelerated by

the applied potential towards the detector and travel a particular distance. Based upon their masses they acquire the different kinetic energy in the presence of the electric field and are accelerated towards the detector. Generally, the separation of ions takes place based on their different velocities, which are controlled by the masses of the ions. All ions are ideally imparted the same kinetic energy, but the ions with the higher masses will have less velocity, relative to smaller ions; the former will reach the detector after the latter .The flight time (t) required by each mass to reach the detector is recorded in instrument. The m/z value is then calculated from the measurement of t^2 value. Since TOF analyzers have essentially no mass limit (in linear mode), or at least quite a high limit (~30,000 Da in reflectron mode), it is a very useful instrument for the analysis of high molecular weight compounds [14].

Historically, TOF instruments have different modes of operations. In the linear mode, we will get the higher sensitivity, but because of different factors like time distribution, distance of flight and ions with same m/z value having different kinetic energies, linear TOF is generally characterized as having lower resolution. Later on, this problem has been corrected by using different approaches. For example, the delayed pulse extraction, use of longer flight tube, and use of the instrument in reflectron mode help to correct this band broadening problem and increase resolution [15].

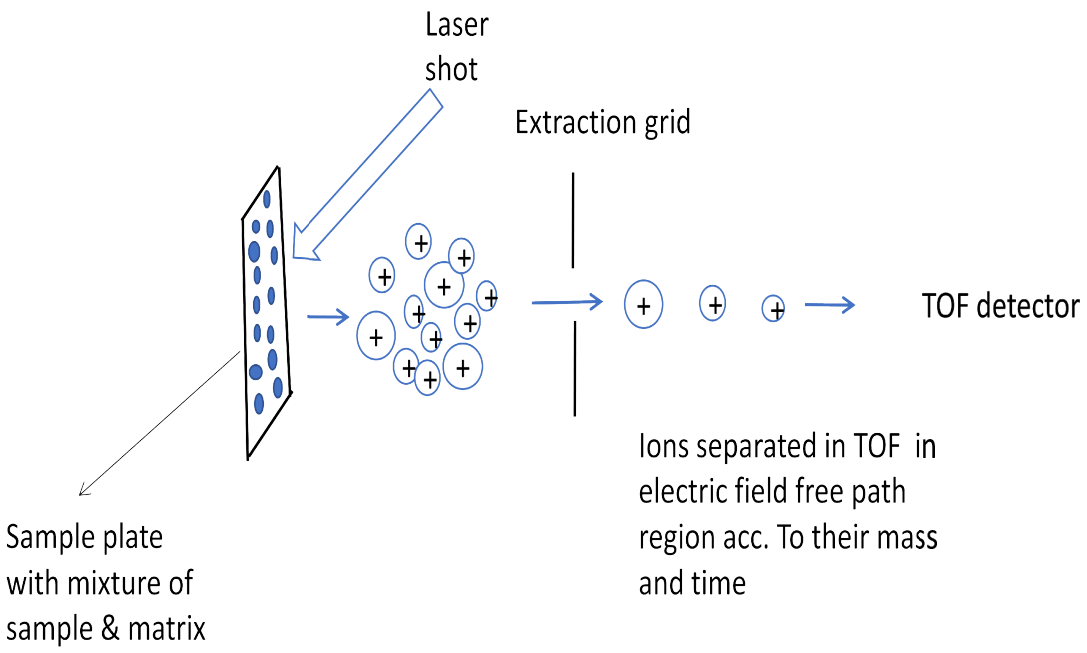

Figure 1: MALDI-TOF diagram

Figure 1 generalizes the instrumental set-up for MALDI-TOF. MALDI-TOF is famous for its use in analyzing polymers and proteins. For proteins, we cannot get the information about different isoforms of intact protein very easily using this technique; capabilities for tandem TOF mass spectrometry are limited. Also, it is not very good for quantification of proteins and biomolecules because of heterogeneity present in the matrix-sample mixture; shot-to-shot and spot-to-spot reproducibility can be hampered by differences in the co-crystallization between matrix and analyte. Therefore the overall scope is less specific and less sensitive, but measurements can be made relatively rapidly compared to LC-MS.

Similarly, SELDI-TOF, which is similar to the MALDI-TOF in that laser desorption ionization is utilized, but rather a sample is spotted on a special surface, which facilitates ionization upon irradiation. There is the specific binding of sample to the surface and over

which matrix is applied for ionization of sample. Matrix can be applied in two different ways, one after applying sample to the surface over which matrix is applied and second it can be applied on the surface first by covalent modification and then sample is applied over it. The use of irradiating laser ionizes and desorbs the protein or peptides from crystal of sample and matrix mixture. The surface used has different properties like cation or anion exchange properties, hydrophilic or hydrophobic interaction, specific metal binding properties, antibody antigen binding etc. Depending upon the specific need of our research we can use different surfaces. SELDI-TOF technique is more useful for the analysis of low molecular weight compounds < 20 kDa mass. It has lower accuracy and sensitivity than the MALDI. Overall, MALDI and SELDI techniques are not preferable for the quantification of biomolecules [16].

ESI-MS and ESI/MS/MS triple quadrupole techniques are different than that of the above techniques. It is the atmospheric pressure ionization techniques in which the ions are first generated at atmospheric pressure, and then they are sent to the mass spectrometer for analysis under high vacuum. The set-up of the electrospray ionization along with the triple quadrupole MS is arranged, so that ions from atmosphere are transferred easily into vaccum medium by using two differently pumped vacuum chambers –a first chamber with low vacuum and second chamber with high vacuum, so that the transmission of ions from atmospheric pressure to the analyzer would be feasible and higher ion transmission efficiency can be attained.

Figure 2 depicts the ESI process, in front of a triple quadrupole mass analyzer (not to scale). On the left is shown the electrospray ionization source and how the ions are formed; and on the right, is the mass analyzer where the quadrupoles are arranged sequentially, one

after another. Presence of the low vacuum system between the two-skimmer cell allows ions generated in the atmospheric pressure to reach to the analyzer part more efficiently than the direct insertion of ions generated in the atmospheric pressure to the high vacuum system.

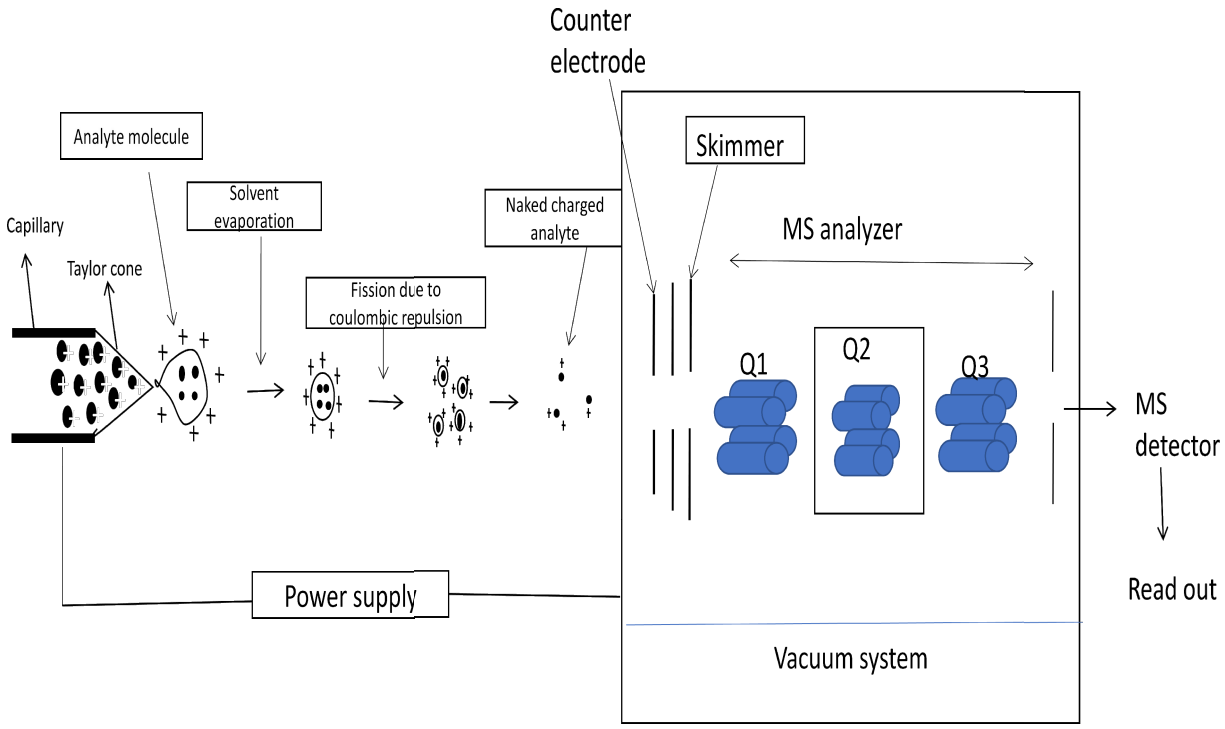

Figure 2: ESI-MS diagram

The triple quadrupole is arranged in the high vacuum so that it can selectively transmit ions of a desired m/z ratio through the application of direct current (DC) and radiofrequency (RF) potentials. The presence of the radiofrequency voltage helps to flow through the central axis and DC voltage helps for the separation of ions following Mathieu's stability equation [17, 18]. In a triple quadrupole, the first quadrupole usually acts as the filter for the precursor ion. Whereas the second quadrupole is maintained in RF-only mode and is used for the fragmentation of selected precursor ions into small product ions in the presence of CID gas

pressure and collision energy without separation. Similarly, the third quadrupole also acts as the filter for the product ions and sends them to the detector. We can also work using only one scan mode usually the Q3 scan mode for the characterization of compounds. In this way, we can characterize and proteins, peptides, and other bio molecules as well as organic compounds using ESI-MS, ESI-MS/MS instruments, with and without fragmentation, as desired. The sensitivity and quantification is very good in triple quadrupole instruments. But lower mass limitation and low resolution is the challenging part of triple quadrupole instrument.

Chapter 1.2

Background on top-down protein analysis

The top-down approach on LC-MS is the intact protein identification technique in which we directly inject the sample into the mass spectrometer without the use of any digestion enzyme, which can provide information about intact protein, protein isomers, and its combinations of post translational modifications (provided a sufficiently high resolution mass analyzer is available). Intact protein quantitation by a top-down LC-MS approach is a new but growing technique because it removes the need for proteolytic digestion used in a bottom-up approach. But in the case of sensitivity, the bottom-up approach is better because MS works better on small mass-to-charge ions produced from digestion of proteins before entering the MS. If we focus on the second half of this approach, there is a loss of important information about intact proteins due to digestion and further fragmentation in the mass spectrometer. Therefore, top-down approach would be better than bottom-up approach for all of the important information about proteins can be obtained [19- 23].

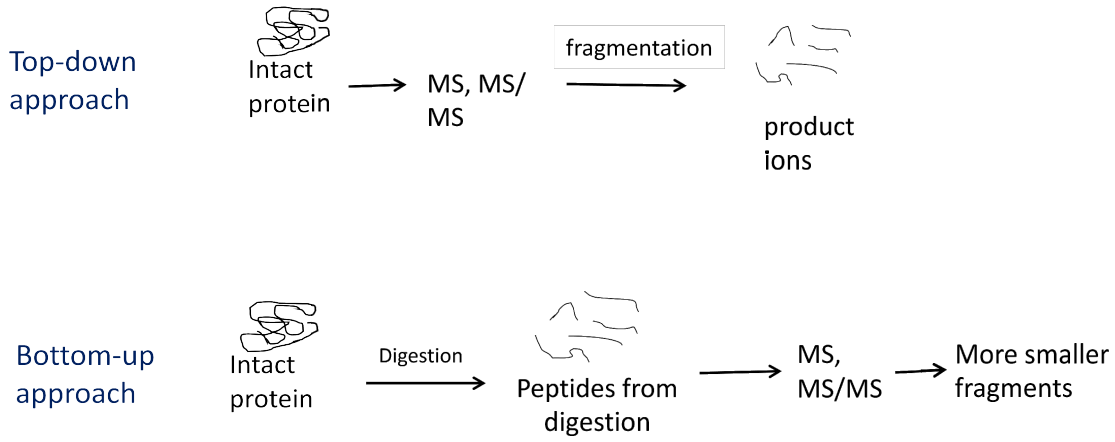

Figure3: Top-down versus bottom-up approach

Figure 3 is a representation of the top-down work-flow versus a bottom-up approach. As in the figure, in top-down approach we insert the intact protein directly into MS/MS system where it undergoes the fragmentation to give product ions, which can provide information about the protein composition and their isoforms. In the bottom-up approach, we first digest the proteins and send the smaller peptides into a MALDI-MS or LC-MS/MS system for the further analysis. Tandem mass spectrometry is still used to provide additional information on the constituent peptides detected. Such information is usually used in conjunction with various bioinformatics software packages to extract information from the sample following analysis [19-23].

Here we have designed the method for the characterization and optimization of large molecular weight intact proteins in QqQ-MS using the top-down method. We have also introduced the use of supercharging agents for the supercharging of intact proteins so that we could see the high molecular proteins more easily using the Shimadzu LCMS-8050 QqQ-MS by multiple reaction monitoring (MRM) operation modes. Protein standards bovine serum albumin (BSA) (66 kDa), holo transferrin (hTrf) (78 kDa), and anti-rabbit immunoglobulin G (IgG) antibody (150 kDa) were target for optimization with and without the addition of supercharging agents. We have also determined the limit of detection of each protein with formic acid only and along with other supercharging agents in MRM mode. Use of the supercharging agents was productive for the higher molecular weight proteins. Here, we have used DMSO, *meta*-nitrobenzyl alcohol (*m*-NBA), trifluoroethanol (TFE) and sulfolane as the supercharging agents. Usually, TFE has been used for investigating conformational changes of proteins but our current work shows that it can also be used as a supercharging agent [24- 26].

Chapter 1.3

Background on supercharging agents

Supercharging agents are chemicals used for unfolding of proteins without breaking down proteins into peptides form. It also offers shifting of charge state towards higher charge state and lower mass charge ratio (m/z) value, which is desirable for MS to work better based upon some advantages like at higher charge state ions are generally induced to fragment with higher efficiency (gives greater sequence coverage) compared to low charge state ions, using techniques like ETD, ECD, and sometimes, CID. Small concentrations of such additives assist in slight unfolding of the protein without affecting the entire protein structure. The mechanism behind supercharging of proteins is that they disrupt the intramolecular interactions, induced by hydrogen bonding, the hydrophobic effect, and vander waals forces of attraction and electrostatic forces of attraction between amino acid sequences and open them to expose the charge states [27 – 30]. Protonation and deprotonation effects of reagents to the protein also lead to unfolding of the protein. Generally, the supercharging agents have neutral pH like water. Therefore, there is no effect of pH in the unfolding mechanism of proteins in the presence of supercharging agents. The mechanism of supercharging agents on the unfolding of proteins is still debated. There are two important mechanisms for protein unfolding by supercharging agents primarily based on volatility, surface tension and the protein-reagent interaction [31 - 33].

1. Mechanism based upon the volatility and surface tension.

The supercharging agents usually have high boiling point. For example, DMSO has a b.p.= 189 ^{0}C, and m-NBA has a b.p = 175 ^{0}C to 180 ^{0}C; thus, their presence in the electrospray droplet

causes differential evaporation. Because of low volatility of supercharging agents as compared to solvent molecules like methanol, acetonitrile, and water, their concentration increases during desolvation which increases the surface tension of droplets. This requires higher degree of surface charging to reach the Rayleigh limit as represented by following equation [33].

$Z_R.e = 8\pi (\varepsilon_0 Y R^3)^{1/2}$

where,

$\mathbf{Z_R}$ is the unit charge limit,

e is the elemental charge,

$\boldsymbol{\varepsilon_0}$ is the permittivity of the surrounding medium,

Y is the surface tension,

and **R** is the radius of droplet

Also, proteins are generally ionized in electrospray ionization according to the charge residue model (CRM), which says that the size of the droplet formed actually determines the average charge state of the protein. Lower small charge state is formed from natively folded proteins when sent to the ESI source. While the unfolded proteins increase their size and form bigger droplets in ESI source due to which it follows longer step in CRM for desolvation and gives higher charge state [31 - 33]. Use of supercharging agent facilitates longer desorption time and undergoes fission at a different rate because of its low volatility than that of the solvents present.

2. Mechanism based upon the protein-reagent interaction

Other hypotheses regarding the mechanism of supercharging simply invoke protein-reagent interactions to explain the role of supercharging agent on proteins. It says that the use

of supercharging agent disrupts the hydrophilic, hydrophobic, and other weak forces of attraction between amino acid sequences by the presence of supercharging reagents that create the unfolding of proteins. The unfolding of proteins exposes the different functional groups and side chains within the protein. This leads to change in charge state of the protein.

Moreover, supercharging agents are also known to increase the signal intensity of proteins and peptides [13]. Increase in signal intensity is also a positive effect of supercharging agents since characterization and optimization of higher molecular weight intact proteins in triple quadrupole is another challenge. Also the higher mass of intact proteins could decrease the sensitivity of instrument. Therefore, the use of supercharging agents facilitate the high molecular weight proteins to be seen in the limited mass range by shifting their charge envelope towards higher charge state and lower mass charge ratio (m/z), as well as increasing the intensity that facilitate the easier optimization of higher molecular weight proteins. [33 - 35].

Chapter 1.4

Aim of Study

The aim of this work was to design a tandem mass spectrometric method capable for the characterization of charge states and optimization of MRM for large molecular weight intact proteins in QqQ-MS using the top-down approach. Besides our desire is to obtain more sensitivity, in order to better compete with bottom-up approach and to overcome the limitation of previous work that has been performed in our lab by Evelyn H. Wang and her co-workers using relatively low molecular weight proteins (9 – 28 kDa) [34]. They found that the limited mass range and low resolution of triple quadrupole instrument, along with that the CID gas pressure and collision energy limitation of some triple quadrupole instruments could be one of the challenges in working with higher molecular weight intact proteins. Therefore, we started working on characterization and MRM optimization of high molecular weight intact proteins along with supercharging agents using Shimadzu LCMS-8050 QqQ-MS instrument. The main purpose of using supercharging agent is that it helps in the shifting of charge state to higher charge state in low m/z range as well as they are supposed to increase the signal intensity [13]. This is desirable because MS works better with higher charge state and lower mass (m/z) value. Here, we have investigated DMSO, *meta*-nitrobenzoylalcohol (*m*-NBA), trifluoroethanol (TFE), and sulfolane as supercharging agents. The top-down approach is not as sensitive as bottom-up approach because we inject intact protein directly into the mass spectrometer. Therefore, use of supercharging agents facilitates the intact proteins to come down to the lower m/z ratio as well the increase the sensitivity of the analysis.

Chapter 2

Multiple Reaction Monitoring (MRM) of intact proteins on QQQ-MS

MRM optimization of bigger proteins is a challenging task but it is very important and it is of high interest in medicinal biology because proteins can be used as biomarkers for identification of different diseases. Characterization and optimization of these proteins with less effort and more reliability is thus desired. Multiple reactions monitoring (MRM) is a technique in mass spectrometry by the use of it, we can identify and quantify the peptides and proteins in better war. The MRM is similar to selected reaction monitoring (SRM) but just the multiple SRM. SRM or MRM approach is good for higher sensitivity and selectivity of our analysis. It is usually done by selecting particular precursor ion for the fragmentation and by the selection of particular product ions obtained from fragmentation of precursor ion. In this way, we will get the information about particular precursor to product ion transition which provides higher sensitivity and selectivity to particular peptide or proteins.

The MRM technique is the targeted analysis technique which needed the series of mass spectrometers one after another. For example as in the triple quadrupole there is the presence of three quadrupoles sequentially in a row. The first quadrupole usually acts as the filter and select the particular precursor ion and send them for fragmentation in second quadrpole which is also called collision cell; in the presence of CID gas pressure and collision energy. Then, all the product ions are sending to the third quadrupole where it filter the most intense product ions and send them to the detector for identification with their collision energy. We can set-up the software for the selection of particular number of the product ions and generally go for the more than one product ions because the most intense product ion is used for quantification

and remaining helps for more specificity of proteins. As in the figure below we have selected the particular precursor ion for fragmentation with their three most intense product ions.

More recently, triple quadrupole (QQQ)–MS was used for MRM-based quantitation of intact proteins in prior studies from Dr. Evelyn H. Wang *et al* [34]. Small proteins were successfully analyzed following MRM optimization. They have optimized and successfully quantified the small molecular weight intact proteins using the same top-down approach and they also found that there is the limitation of work for higher molecular weight intact proteins because of CID gas pressure and Collision Energy (CE). The presence of collision energy has also great effect in fragmentation and reproducibility of MRM optimization of proteins. Additionally, the nature of gas used in collision cell can affect stable and reproducible product ion formation. They showed that the increase in CID gas pressure and high CE simultaneously does not give reproducible product ion. Therefore, they optimized these variables to get reproducible, unique, and stable product ions for each target protein of interest [34].

Limits of detection were determined to be in the low- to mid-nanogram quantity on column, and varied considerably for different proteins. They have also performed an intact protein separations study, and they studied transmission efficiency of proteins using different parameters [36 - 37]. Based upon their work advantages and limitations of triple quadrupole instrument, we started working on the high molecular weight proteins using the same top-down approach using supercharging agent because the optimization of high molecular weight intact proteins in triple quadrupole mass spectrometer is a challenging job and use of supercharging agents facilitate them to see in triple quadrupole instrument Multiple reaction monitoring is like selected reaction monitoring (SRM) technique where we search for at least

two product ions for a selected precursor ion. Among these selected product ions, the more intense product ion is selected for quantification and the other product ions are used to qualitatively confirm the ion assignment. Such an approach increases the specificity and confidence in the analyte targeted for quantification. Additionally, mobile phase composition, source of electrospray ionization, nature of gas used in collision cell, among other variables, can affect stable and reproducible product ion formation. Our study shows that increase in CID gas pressure and high CE simultaneously does not give reproducible product ions. Therefore, it is important to optimize these variables to obtain reproducible and stable product ions.

Here we have designed the method for the characterization and optimization of large molecular weight intact proteins in QqQ-MS using the top-down method. We have also introduced the supercharging agents for the supercharging of intact proteins so that we could see the high molecular proteins more easily using the Shimadzu LCMS-8050 QqQ-MS using the multiple reaction monitoring (MRM) operation modes. The **Figure 4** below is the representation of triple quadrupole in MRM mode.

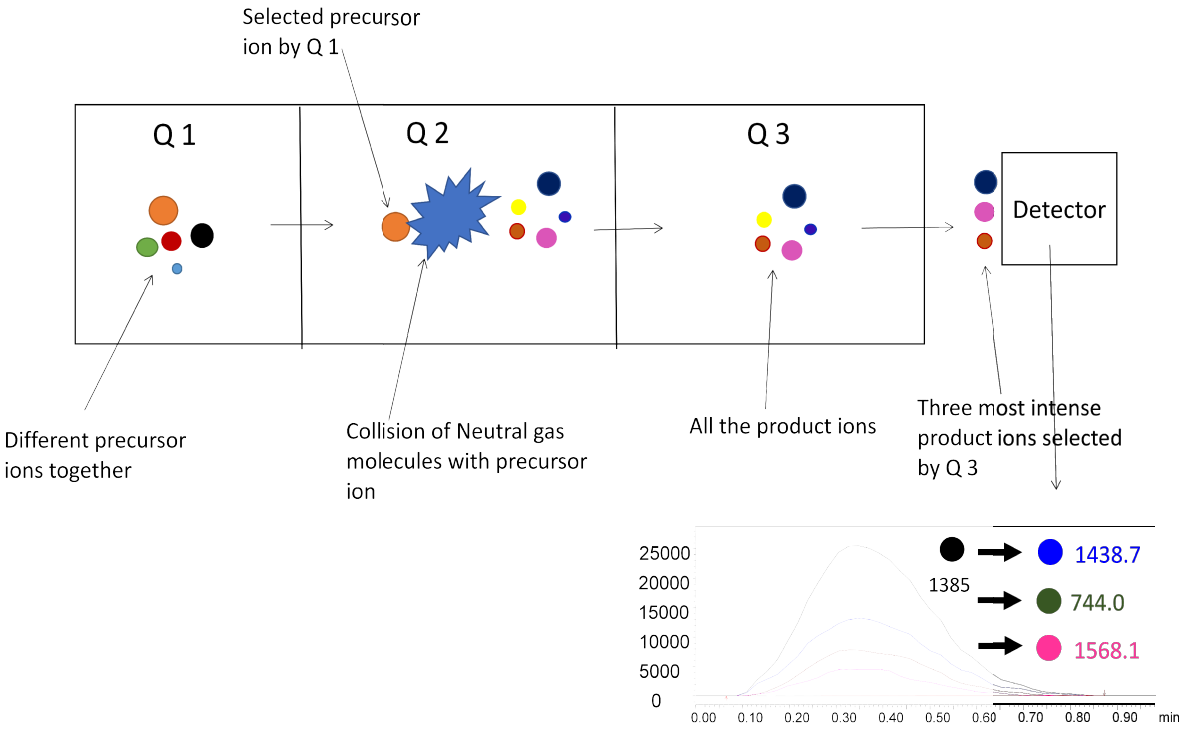

Figure 4: Representation of MRM optimization in triple quadrupole with one of the chromatogram

Chapter 3

Experimental Section

The experimental details are based upon the top-down approach on an LC-MS/MS instrument. We have used the Shimadzu LCMS 8040 and Shimadzu LCMS 8050 instrument for the characterization and optimization of the proteins. For the set up for top-down approach in Shimadzu LC-MS 8050 and 8040, the default setting is used keeping everything same of instrument. Using this top-down approach, we are directly injecting the sample by using LC auto sampler which is carried out through the LC to the MS by the help of high pressure pump flow. The flow rate has been maintained usually 0.2 mL/min for 2 to 4 minute for the characterization of sample. There is no separation of sample since we are injecting the pure standard proteins; therefore no use of column. The ionization source is heated ESI source. An important aspect of this work is the optimization of standard high molecular weight intact proteins for quantitative analysis by using multiple reactions monitoring (MRM) technique. To work for the MRM optimization; we have two important variables which plays important roles. They are CID gas pressure and collision energy. Shimadzu LCMS 8040 has the limitation of CID gas pressure up to 230 kPa. Therefore, we are unable to optimize the high molecular weight proteins like BSA, hTrf, and IgG in Shimadzu 8040. Therefore, we worked on the Shimadzu LCMS 8050 for characterization and optimization of all bigger proteins because CID gas pressure can be set in the Shimadzu LCMS 8050up to 400 kPa.

Chapter 3.1

Chemicals and Reagents

Standard proteins BSA) (66.5 kDa) from human, hTrf (78 kDa) , anti-rabbit IgG (whole molecule) peroxidase antibody produced in goat (150 kDa), formic acid (FA) >95%, supercharging agents for proteins like ≥99.5%m-nitro benzyl alcohol (*m*-NBA), 99% , ≥99% 2,2,2-trifluoroethanol (TFE) from Sigma Aldrich (St. Louis, MO, USA) and . dimethyl sulfoxide (DMSO) from 4L bottle from Macron fine chemicals were purchased. LC-MS grade water, acetonitrile (ACN), methanol and isopropanol (IPA) were purchased from Honeywell Burdick and Jackson (Morristown, NJ, US).

Protein Models

Bovine Serum Albumin (BSA): BSA is a type of plasma protein and found almost 50- 60% of total protein concentration present in plasma serum. It has the molecular weight of 66.5 kDa with 583 amino acid sequences. It has a secondary structure with alpha and beta sheets. Since it is stable and non-reactive protein, it can be used as blocker in immune histochemistry [38].

Holo Transferrin: It is a type of glycoprotein.It is also called the iron storage protein and important component of growth medium. Transferrin receptors on cells bind the transferrin for iron transport to and from different cells. Because of the presence of iron on it , it is called holo transferrin. Transferrin without iron is called apo transferrin. The molecular weight of holo transferring is 78 kDa with its secondary structure [39].

Antibody Immunoglobulin G (IgG): IgG is a type of Antibody present in the plasma serum of the human being having usually two binding sites. It has the molecular weight of 150 kDa and

contains the tetramer two with 50 kDa mass and two with 25 kDa mass. It protect the body from the infection by binding with different kinds of pathogens like bacteria, viruses etc [40].

Chapter 3.2

Sample Preparation

For the characterization and optimization of high molecular weight intact proteins in LCMS 8050 triple quadrupole mass spectrometer and LCMS 8040 triple quadrupole mass spectrometer from Shimadzu Scientific Instruments, Inc. (Columbia, MD, USA) were used. Most of the proteins are soluble in water, but some of the proteins have high hydrophobic amino acid sequence therefore use of the both polar and non-polar solvents was found to be effective for sample preparation. Trial started with the 100% water and 100% methanol but ended up with the use of 50/50 acetonitrile/water along with 0.5% formic acid for final solution preparations of the proteins for analysis. Use of the different concentration of supercharging agents needed the higher % of organic phase to dissolve on it, therefore (50/50) polar and non-polar solvent mixture was found to work well. Different concentrations of protein standards were prepared per its sensitivity to the instrument LCMS 8050. The concentration of BSA and hTrf used were 7.5 µM, 15 µM, and 30 µM. Similarly, for anti-rabbit IgG 100µL sample was diluted to make 1 mL using (50/50) water and acetonitrile solvent mixture with 0.5% formic acid and different concentration of supercharging agents. The original solution of IgG in 1 mL bottle solution was 0.67 mg/mL. The dilution was performed by taking 100 µL from that original solution to make it 1000µL (1 mL) that make our sample solution concentration 0.45 µM and flow rate maintained was 0.2 ml/min throughout the injection.

Chapter 3.3

Instrumentation

The instrumentation is based upon the use of top –down method on Shimadzu LCMS 8050 triple quadrupole MS. Use of the triple quadrupole instrument using ESI source is good for the analysis of proteins because we can generate the multiple charge states of them which is helpful for more information about their structural analysis. Triple quaqrupole uses three quadrupole Q1, q2 and Q3. Q1 and Q3 act like the filters and fragmentation takes place only in the middle one at different collision energy. The most advantage of using triple quadrupole is that only the selected mass can enter the detector and other fragments are not detected. When analyte enter into the mass spectrometer in first quadrupole Q1, it select the particular precursor ion for the fragmentation and send them into the second quadrupole (q2) where they undergo further fragmentation into the product ions using high CID gas pressure (usually inert gases like Nitrogen, Argon, Helium) along with higher collision energy and Q3 filters particular fragmented product ions and allowed to be detected by the detector. It is done by using LC/MS/MS, using the MRM mode for the precursor and product ion search which increases its sensitivity and specificity. While, in single quadrupole using QQQ-MS mode, the characterization of proteins and peptides is done to get the charge envelope with different charged precursor ions.

The sample was injected directly using the auto sampler. For the characterization of these proteins first used Q_3-Scan mode from mass m/z ratio 350 to 2000 and observed the multiple charge envelope of the protein. Deconvolution of this different charge state has been performed by using the ESIprot online software. For the MRM of the protein standards, the

most intense peaks (6 to 8) with comparable intensity were selected as the different precursor ions and MRM method for each of them were identified and at last the summation of MRMs was also performed in different ways; one by sequential addition of intensity of different precursor to product ion transition and second summation of 10 MRM of same channel i.e. same precursor to product ion transition Similarly, the characterization of standard proteins was done in different interface temperature in LCMS 8050 to know how the interface temperature effect on the charge state distribution of proteins. Similarly, work done on different flow rate also gave an idea about charge state distribution on different flow rate.

Figure 8: Shimadzu 8050 Triple Quadrupole instrument

Chapter 3.4

Experimental Design and Procedure

For the characterization and optimization of high molecular weight intact protein we have used the Top-Down approach in triple quadrupole instrument. The experimental design is based upon the different variables as below:

Experimental design based on supercharging agent used:

Different supercharging agents like dimethyl sulfoxide (DMSO), *meta*-nitrobenzyl alcohol (*m*-NBA), sulfolane, trifluoroethanol (TFE) are used at different percentage in the solvent system 50/50/0.5 i.e. acetonitrile/water/formic acid, as well as in the sample solution, so that the concentration of supercharging agent does not vary across the flow injection profile.

Experimental Design based on flow rates:

The optimized flow rate used on all proteins was 0.2 ml/min with isocratic flow of 50/50 acetonitrile and water with 0.5% F.A. and different concentration of supercharging agents by using two pumps A and B. To work on the other flow rate for specific purpose, just the flow rate has been changed from 0.02 ml/min to 0.2 ml/min keeping everything constant and obtained the effect of different flow rate on charge distribution of different proteins

Experimental Design based on Temperature:

The interface temperature also influences the charge distribution of proteins. The interface used in the LCMS 8050 is a heated electrospray ionization (ESI) source. For the set up of interface temperature in Shimadzu 8050 triple quadrupole instrument, we can go upto 400 ^{o}C. Therefore, we worked on different interface temperature from room temperature to 400^{0}C temperature and worked at 25 ^{o}C, 100 ^{o}C, 200 ^{o}C, 300 ^{o}C and 400 ^{o}C. To set up the optimum

interface temperature i.e. room temperature we turned off the interface and worked at room temperature.

Experimental design based on solvent properties:

Usually in LCMS the solvents used are acetonitrile, methanol, water, formic acid, , trifluoro acetic acid (TFA) and some bases. Acids are usually used to work in positive mode because they are responsible for the formation of positive ion and bases are used usually to work in negative mode for negative ion formation. Similarly, different mobile phase solvents like water, acetonitrile, methanol have different properties like different viscosity, their polar and non-polar nature, acidity and basicity. These are responsible for size of the droplet formation which is related to charge formation in sample. These are extremely important for the charge formation especially in the proteomics. Therefore, we use the different solvent combination for different proteins depending upon the nature of proteins.

Chapter 3.5

Parameters for MRM Optimization

For the MRM optimization of the high molecular weight proteins LCMS 8050 was used. Important operational parameters were: desolvation temperature 250 ^{o}C, heat block temperature 300 ^{o}C, interface temperature 300 ^{o}C, nebulizing gas flow (Nitrogen) 2L/min, drying gas flow (Nitrogen) 10 L/min,and interface voltage 4 kV. Solvent system was (50:50:0.5) acetonitrile, water and formic acid with the flow rate of 0.2 ml/min flow rate with the 50% isocratic flow for 2 min. Ratio of solvent system changes with the addition of different percentage of supercharging agents. For example, with the addition of 1% DMSO ratio becomes (49.5/49.5/1/0.5 = ACN/water/ DMSO/F.A) with the 5% DMSO, ratio becomes (47.5/47.5/1/0.5 = ACN/water/ DMSO/F.A). The most important variables needed for the MRM optimization are the CID (collision) gas pressure and Collision energy. For the characterization of protein, Q3 scan mode is used with the minimum CID gas pressure 17kPa and the multiple charge envelopes was with different m/z obtained. In the MRM mode, one of the most intense precursor ion from the multiple charge envelope was selected for fragmentation and worked under the CID gas pressure of 400 kPa and collision gas energy from -1V upto -100 V for BSA and Anti-Rabbit IgG while for the hTrf, CID gas used was 400 kPa and CE from -1V upto -70 V for better reproducibility. After getting some reproducible product ions the MRM optimization was done by using the automated Lab Solutions software (ver.5.65) and optimum collision energy required to obtain that product ion was obtained. In this way MRM optimization of all proteins with precursor to the three most intense product ions was performed in triplicate to check the reproducibility and intensity each time. For each of the protein, for each precursor ion at least

three product ions were selected in the MRM mode. Also, the summation of the 10-same product ion for one product ion is done in the same CID gas pressure and collision energy. Similarly, 6 different precursor ions were selected for one protein because they have the similar intensity and MRM optimization was performed for all of them. The mass obtained was de-convoluted by using the software called ESIprot.Online (http://www.bioprocess.org/esiprot/esiprot_form.php) and Microsoft Excel.

Chapter 4

Results and Discussion

Chapter 4.1

Effect of supercharging agents on charge distribution and intensity response of high molecular weight intact proteins

To test the effect of supercharging agents with high molecular weight proteins like BSA, hTrf, and antibody IgG, in a triple quadrupole instrument, we worked with the different concentrations of supercharging agents along with the sample as well as in the solvent system. The composition of solvent mixture changes along with the different composition of supercharging agents used. The run time was fixed for two minutes for each run and flow rate maintained was 0.2 ml/min with 50% isocratic flow of water and acetonitrile solvent mixture. The run was in positive scan mode and mass range selected from m/z 350 -– 2000. The charge envelope for each protein with different charge states with different supercharging agents was obtained using Shimadzu lab solution software for LCMS 8050. The effect of different supercharging agents with different proteins is tabulated below as **Table 1**.

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Table1: Effect of additives on the supercharging and response of high molecular weight proteins

Protein	Additive[a]	No. observed chargestates[b]	z_{base} (*m/z*)	z_{max}[b]	z_{avg}	Molecular weight (kDa)[c]	Response factor/10^3 (ion cts/μM)[d]	Fold change vs. no additive[e]
BSA	none	27	46+ (1445)	60+	45.4+	66.44 ± 0.01	20 ± 4	1 (reference)
	1% DMSO	23	49+ (1357)	56+	46.7+	68.00 ± 0.10	85 ± 4	4.3
	5% DMSO	22	48+ (1390)	55+	46.1+	66.64 ±0.09	60 ± 8	3.0
	10% DMSO	21	46+ (1451)	54+	44.5+	66.67 ± 0.06	53.5± 0.5	2.7
	0.5% m-NBA	21	44+ (1515)	54+	44.0+	66.70 ± 0.10	20.6 ± 1.5	1.0
	1% m-NBA	28	49+ (1357)	61+	48.2+	66.48 ± 0.06	76 ±5	3.8
	1% TFE	29	50+ (1330)	62+	49.2+	66.48 ± 0.06	274± 8	14
	5% TFE	29	50+ (1330)	63+	49.2+	66.45 ± 0.08	288±7	14.5
hTrf	none	22	50+ (1561)	61+	49.6+	78.00 ±0.01	24.0± 4.5	1 (reference)
	1% DMSO	22	52+ (1501)	61+	51.3+	78.2± 0.1	33.4±0.5	1.4
	5% DMSO	20	50+ (1568)	59+	48.8+	78.3± 0.1	29 ±2	1.2
	10% DMSO	20	51+ (1531)	59+	49.3+	78.3± 0.2	43.4±0.8	1.8
	0.5% m-NBA	19	49+ (1599)	58+	49.0+	78.3± 0.7	14± 1	0.6
	1%m-NBA	24	53+ (1473)	63+	52.7+	78.4± 0.1	43 ± 2	1.8
	1% TFE	23	51+ (1531)	62+	51.27	78.1± 0.1	77 ± 5	3.3
	5% TFE	23	52+ (1501)	62+	51.0+	76.5 ± 0.1	75± 3	3.1
IgG	none	20	95+ (1546)	106+	98.0+	150± 5	370± 30	1 (reference)
	1% DMSO	20	97+ (1484)	106+	95.5+	150± 5	1040± 15	2.8
	5% DMSO	20	96+ (1514)	105+	95.5+	149±5	990±60	2.7
	10% DMSO	19	92+ (1668)	106+	94.0+	153± 5	970± 60	2.7
	1%m-NBA	26	101+ (1357)	112+	98.5+	149 ±6	1410± 77	3.9
	1% TFE	26	101+ (1357)	112+	98.5+	151 ± 6	1211± 47	3.3
	5% TFE	26	101+ (1357)	112+	98.5+	151 ±6	1168± 32	3.2

[a] All solutions were 50/50 ACN/Water with 0.5% FA, plus additive.

[b] >5% relative intensity

[c] Average deconvoluted molecular weight and one standard deviation (n=3)

[d] Average ion counts divided by concentration tested and one standard deviation (n=3)

[e] Response factor for additive condition relative to response factor with no additive[a]

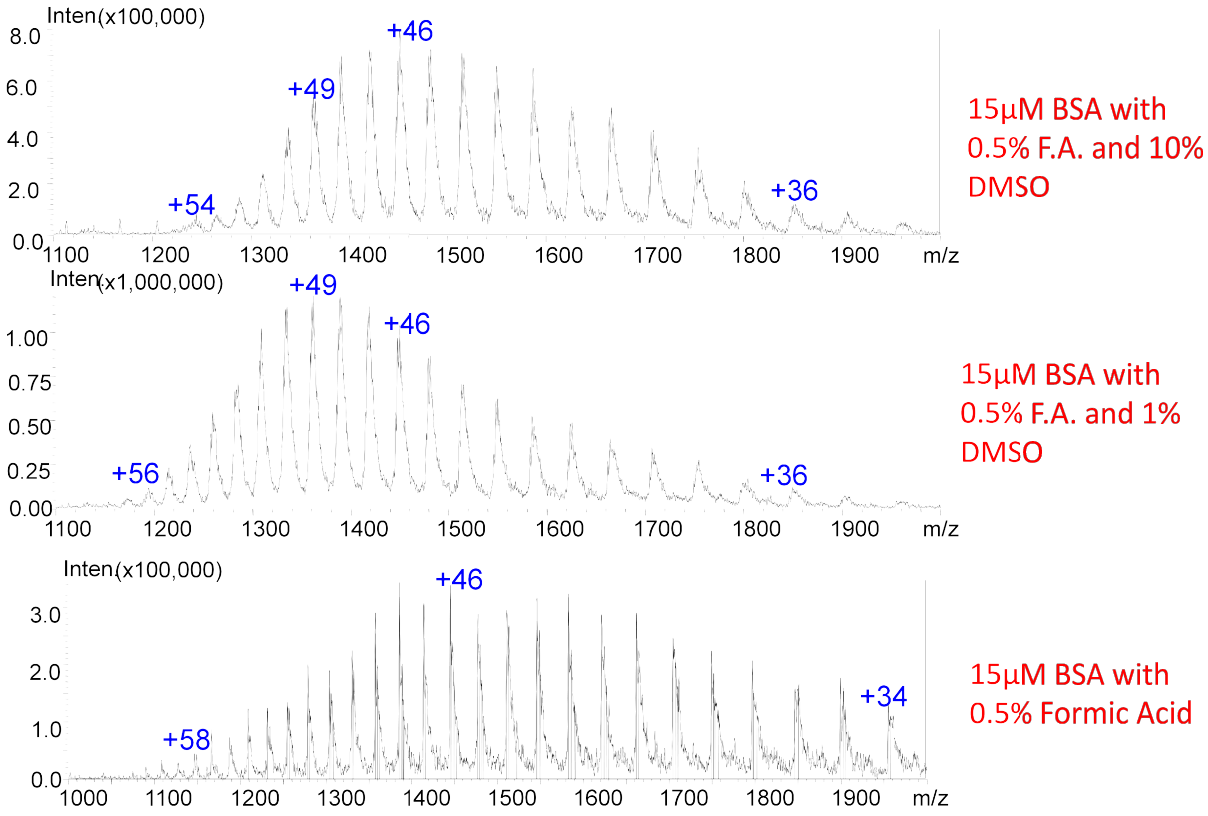

Figure 8: Effect of 0.5% Formic acid and different concentration of DMSO on BSA

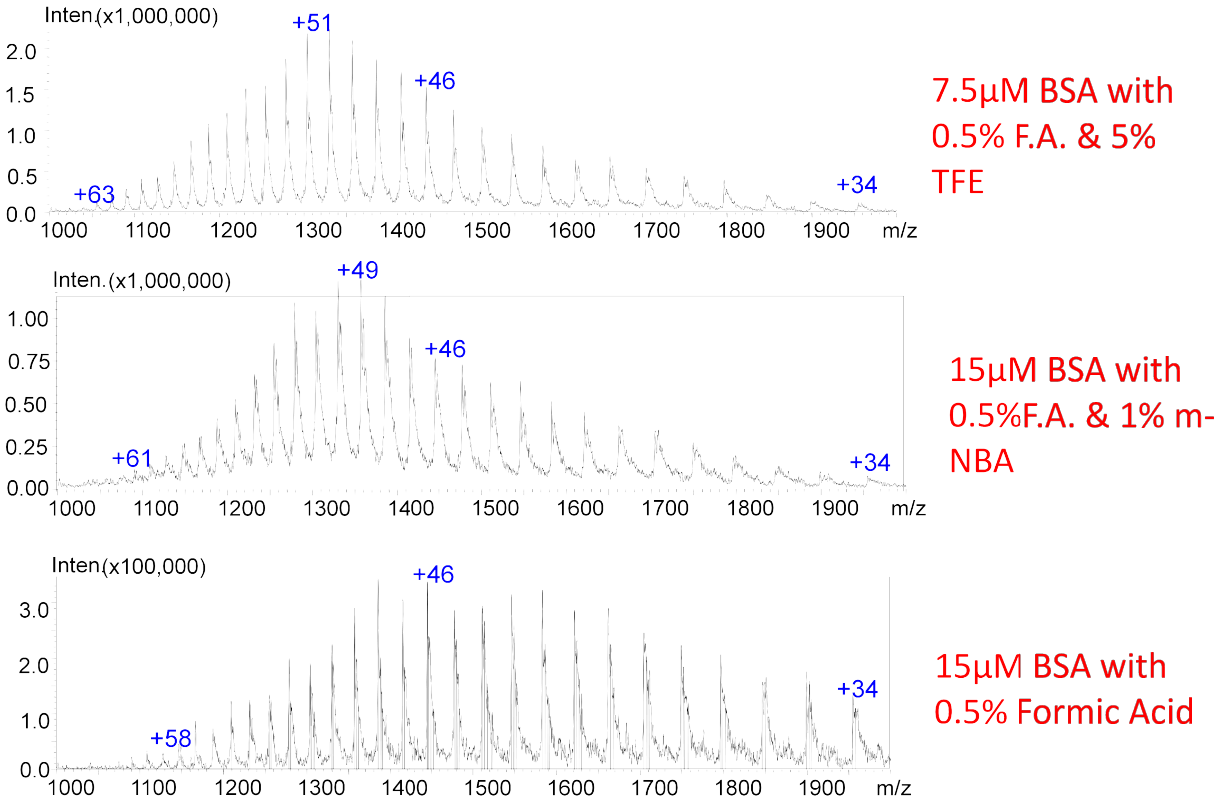

Figure 9: Effect of 0.5% Formic acid along with different supercharging agent on BSA.

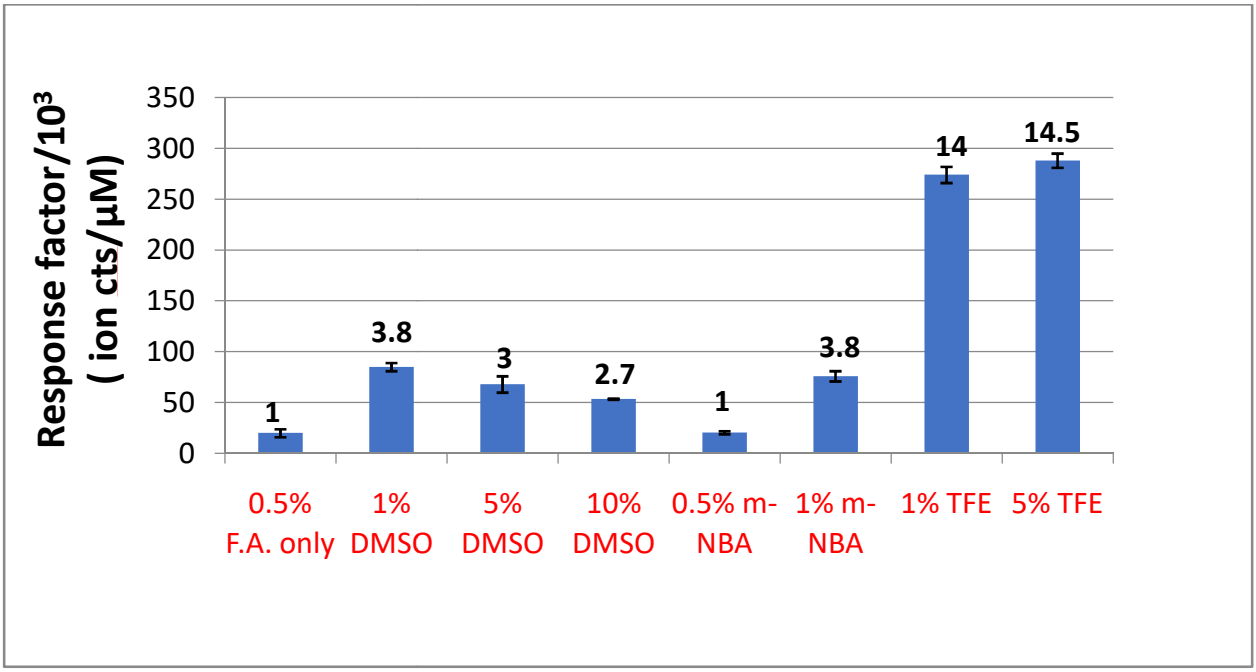

Figure 10: Effect of different supercharging on intensity of BSA

Table 1 and **Figures 8, 9, and 10** represent results obtained by study on the effect of supercharging agents on charge state distribution as well as on the measured intensity for different proteins. First, we worked with 0.5% formic acid only to see charge state distribution for all proteins as the reference. For BSA, different concentration of DMSO from 1% to 10 % were used as supercharging agent along with 0.5% formic and the result obtain shows that with 1% DMSO, there is the shift of most intense precursor ion in higher charge state to +49 (m/z 1357), since +46 (m/z 1445) was the most intense precursor ion with 0.5% formic acid only but there is the narrowing of charge state distribution in the presence of increasing concentration of DMSO. The intensity increased with 1% DMSO is 4.3 fold than that with 0.5% F.A. only. Similarly, with 5% DMSO we can see precursor ion as m/z 1390 (+48) with maximum charge state is +54 and the intensity increased was 3-fold higher than that of the formic acid only. With 10% DMSO, the most intense precursor ion obtained was m/z 1445 (+46) with further decrease in intensity than that of the 1% and 5% DMSO but still 2.7 fold increased than that of 0.5% formic acid only. This result obtained by the use of different concentration of DMSO shows that there is the ion suppression effect with the increasing concentration of DMSO. Similarly, with 0.5% m-NBA, there is the shift of charge state towards the lower charge and precursor ion was found to be m/z 1515 (+44) but intensity obtained is lower with respect to 0.5% F.A. only but with 1% *m*-NBA precursor ion was found to be m/z 1357 (+49) with the maximum charge state of +60 and intensity has been increased 3.82 fold with same concentration and same volume of injection as compared to 0.5% formic acid. Similarly, with 5% TFE the precursor ion was found to be m/z 1330 (+50) and maximum charge state of +63. The intensity profile shows that there

was 14.5-fold increase in intensity of the BSA with 5% TFE even lesser injection volume with respect to 0.5% Formic acid only and it is working most effectively among the entire supercharging agent. Therefore the use of 5% TFE acts as the very good supercharging agent in shifting the charge state as well as increasing the intensity. From, above results in **Table 1**, obtained by the use of different supercharging agents on BSA, the TFE is working as best in shifting charge state towards higher charge state and increasing the intensity. The 1% m-NBA is also found as good in supercharging and increasing the intensity but not in same amount as TFE is working for increasing intensity.

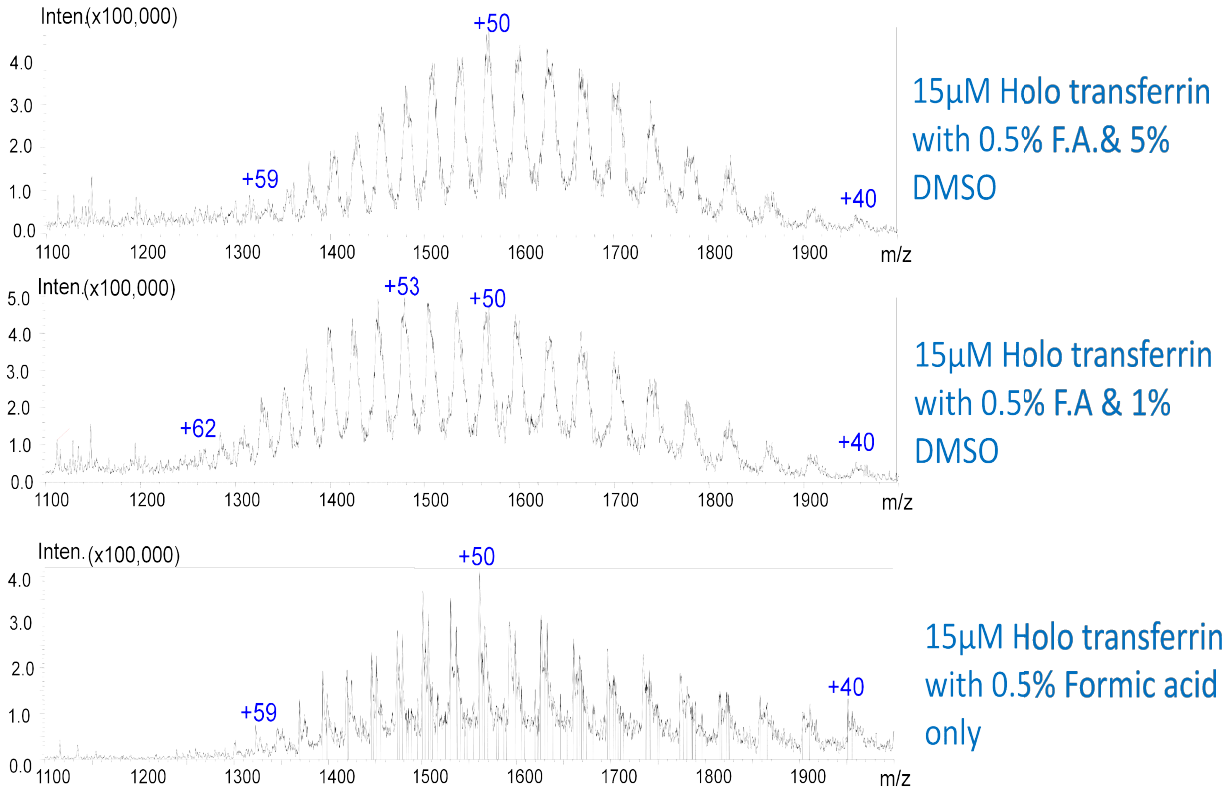

Figure 11: Effect of 0.5% formic acid with different concentration of DMSO on hTrf

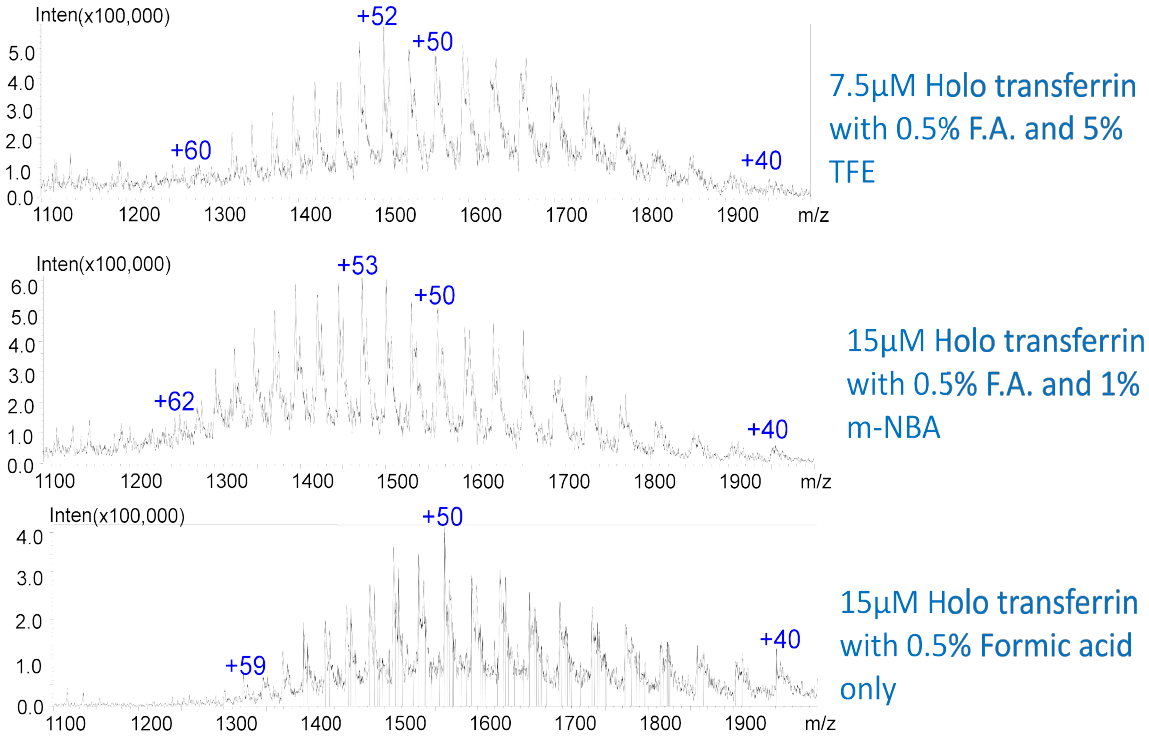

Figure 12: Effect of 0.5% formic acid with different concentration of other supercharging agent on hTrf

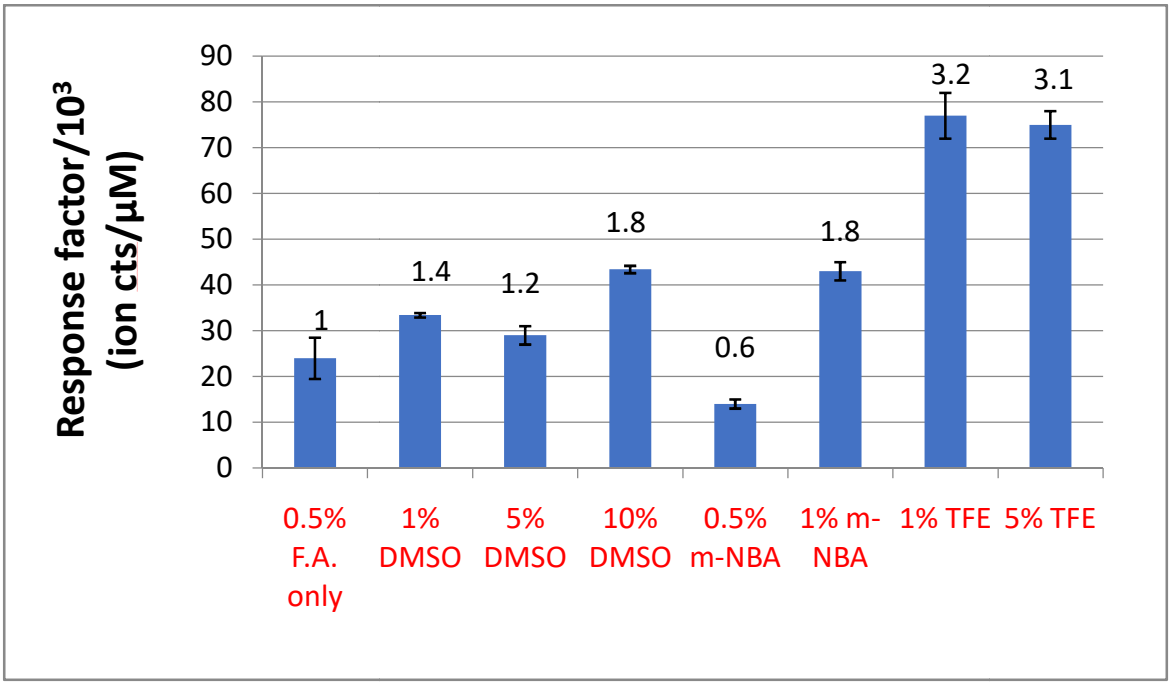

Figure 13: Effect of different supercharging agent on intensity of Holo-Transferrin

In the similar way, by the use of different concentration of supercharging agents on hTrf, we got the different results with different charge state distribution on hTrf. Working with 0.5% of formic acid as the reference the precursor ion obtained is m/z 1561 (+50) with the maximum charge state of +61. With 1% of DMSO the most intense precursor ion found as m/z 1501 (+52) with maximum charge state of +61 and if we see the intensity profile, intensity increased was 1.4-fold higher with 1% DMSO with respect to 0.5% FA only. While with 5% and 10% DMSO the charge state for most intense precursor ions was decreasing, and found as +50 and +48, respectively. But from ut intensity profile the result was 1.22 fold increased with 5% DMSO and 1.8 fold increased with 10% DMSO. Similarly, with 0.5% m-NBA and 1% m-NBA, the precursor ions obtained were m/z 1599 and m/z 1473 with the charge state of +49 and +53 and maximum charge state +58 and +63,respectively, and intensity obtained were altered 0.6-fold and 1.8-fold,respectively.

Similarly, with 5% TFE the precursor ion obtained was m/z 1501 (+52) with maximum charge obtained was +62 and the intensity obtained was 3.1-fold higher even with 30 μL injection volume with respect to 0.5% FA.

Similarly, with 5% TFE the precursor ion obtained was m/z 1501(+52) with maximum charge obtained was +62 and the intensity obtained was 3.12 fold higher even with 30 μL injection volume with respect to 0.5% FA.

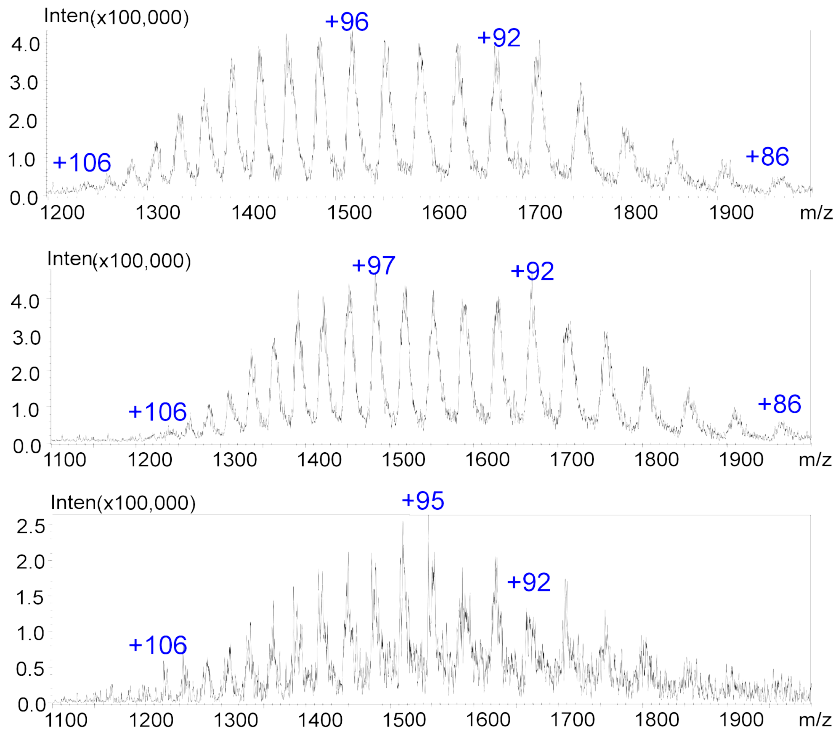

Figure 14: Charge envelope of Anti-Rabbit IgG with 0.5% F.A. & different conc. of DMSO

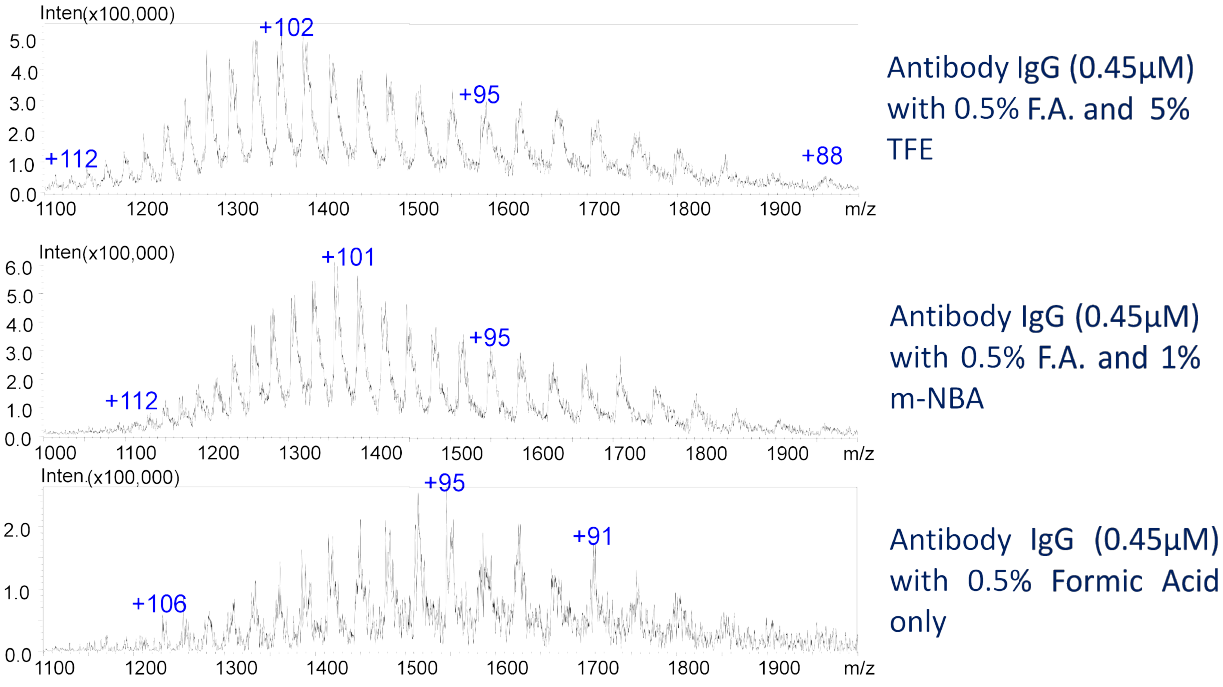

Figure 15: Charge envelope of Anti-Rabbit IgG with 0.5% F.A. &different concentration of different supercharging agents

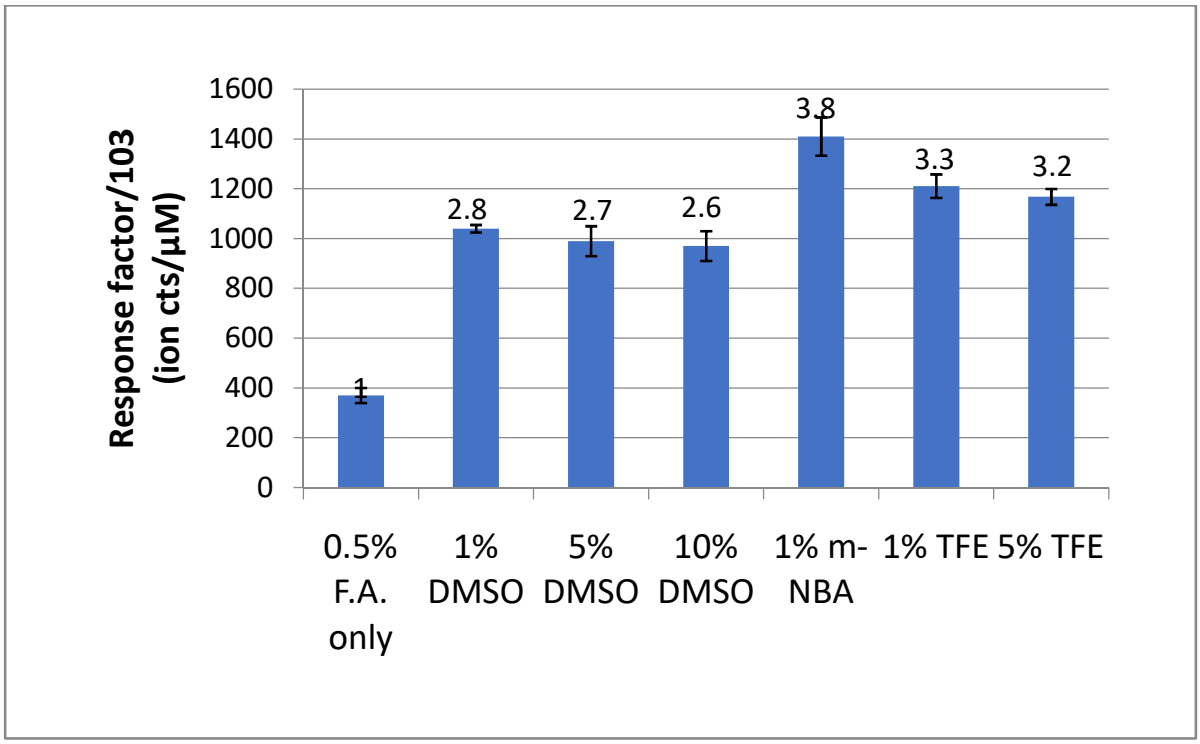

Figure16: Effect of different supercharging agent on intensity of Anti-Rabbit IgG.

In the same way, from **Table 1 and Figures 14, 15, and 16**, results obtained showed us that antibody IgG with 0.5% formic acid only the number of charge distribution was 20 with most intense precursor ion as m/z 1514 (+96) and maximum abundant charge as +106. By the use of 1%, 5% and 10% DMSO, we got charge state of precursor ion as +97, +96, and +92 respectively with maximum charge state of +106 for two and +105 for 10% DMSO which shows gradual decrease in charge state of precursor ion but the intensity profile shows 2.8-, 2.7-, and 2.66-fold increase in intensity, respectively, with reference to use of 0.5% formic acid only. While, with 1% m-NBA, 1% TFE, and 5% TFE, we got the similar results with the precursor ion as m/z 1357 (+101) with the maximum charge state of + 111, but intensity profile showed 3.9-fold increase in intensity with 1% *m*-NBA and 3.3- and 3.2-fold increase in intensity with 1% TFE and 5% TFE .

From all the above results, the 1% and 5% TFE and 1% *m*-NBA are supercharging the proteins very effectively in shifting the charge state as well as increasing intensity. Similarly, 1% DMSO seems good in shifting charge state as well as increasing the intensity but 5% and 10% DMSO were not found good in shifting of charge state. Important to note that these large proteins can be detected using the triple quadrupole, even without addition of a supercharging agent (beyond 0.5% formic acid). We did not expect a large difference between presence and absence of additive because of the presence of formic acid in all solutions. This was included to make the solution more relevant to that encountered in standard mass spectrometric analysis of proteins. There is likely already a significant amount of denaturation of the protein due to the presence of formic acid potentially limiting the effect the supercharging agents might have relative to if they were added to solutions containing proteins in non-denaturing conditions.

For example, TFE are popular for the change in conformation of protein structure but TFE; not previously used as a supercharging agent. But it has been using in different other protein/peptide studies. The proteins are found in different structure naturally depending upon their number of amino acid sequences, their inter and intramolecular interaction as well as their mass complexity. Use of the different additives induces them to change their structure. Some covalently attached to them and chage their structural intrigrity and some of them just alters their intramolecualr interaction and change their conformation. TFE is known to induce alpha-helicity in peptides, so it definitely alters conformation, which in some cases could expose more basic units for supercharging, but also could (especially for BSA) induce a structure that is more favorable for ionization [35, 36, 37].

The work has been also done with sulfolane and 1,2-butylene carbonate as the supercharging agent but result obtained is not very good and useful as above supercharging agents.

Chapter 4.2

Effect of interfacetemperature and flow rate on charge distribution of proteins

Table 2: Effect of interface temperature (heated nebulizing gas) on charge state and response.

Protein[a]	T (°C)	Z_{avg}	Z_{max}	Z_{base}	Intensity	Response Factor**(ion cts/µM)**[d]
BSA (15 µM)	RT	+47.5	+61	+46	300,000	20,000
	100	+46.5	+59	+45	250,000	16,667
	200	+46.5	+59	+43	330,000	22,000
	300	+46.5	+59	+47	400,000	26,667
	400	+46	+58	+46	400,000	26,667
Holotransferrin (15 µM)	RT	+50.5	61	+51	220,000	14,667
	100	+49	+58	+48	260,000	17,333
	200	+49	+58	+50	380,000	25,333
	300	+49	+58	+50	380,000	25,333
	400	+49	+58	+48	330,000	22,000

[a] Proteins prepared in 50/50 ACN/water with 0.5% formic acid.

Table 3. Effect of flow rate on protein charging and response.

Flow rate→	**0.02 mL/min**			**0.1 mL/min**			**0.2 mL/min**		
Protein	No. of observed charge states	z_{base}	z_{avg}	No. of observed charge states	z_{base}	z_{avg}	No. of observed charge states	z_{base}	z_{avg}
BSA, 0.5% FA only	29	+49	+48	28	+46	+47.5	27	+43	+47
BSA, 0.5% FA and 10%DMSO	27	+50	+47	25	+48	+46.0	21	+46	+44

[a] Proteins prepared in 50/50 ACN/water with 0.5% formic acid and additives

From **Table 2**, to check the effect of interface temperature, we worked on different temperature from room temperature to 400 °C heating gas temperatures in the ESI source. Since, in LC-MS 8050 we can just set-up the interface temperature from 100^0C to 400 ^{0}C, therefore to set-up the room temperature we turned off the interface. The results obtained shows that at lower temperature the shifting of the charge state towards the higher charge state is higher. This would support a mechanism of increased surface tension for the droplets at low temperature.

Similarly, from **Table 3**, to see the effect of flow rate on charge distribution of proteins we worked from 0.02mL/min to 0.2mL/min flow rates and found that at lower flow rate, charge distribution were also shifted to higher charge state values, relative to higher flow rates. This result provides information about the surface tension increases with the decrease in flow rate , therefore generate the higher charge state at low flow rate than that of higher flow rate.

Chapter 4.3

MRM Optimization of different proteins

Multiple reaction monitoring is the multiple channels of SRM. It is a specific, sensitive and low cost effective technique. It can be done by selecting the specific precursor ion and send them for the fragmentation in collision cell for the search of product ions. The fragmentation is done by collision induced dissociation by applying sufficient CID gas pressure and Collision Energy. These CID gas pressure and Collision Energy (CE) are the two most important variables that affect the SRM or MRM. In our lab, Dr. Evelyn H. Wang and her coworkers started the MRM optimization of small proteins in triple quadrupole mass spectrometer [16].

Because of their work advantages, we started working on the high molecular weight proteins using the same top-down approach along with the supercharging agent because the optimization of high molecular weight intact proteins in triple quadrupole mass spectrometer is a challenging job. The concept of supercharging agent in shifting the mass to charge ratio to lower value and increasing the intensity facilitate to work on big proteins. Therefore, MRM optimization is done for different higher molecular weight intact proteins using that concept.

Our initial attempt in optimization of myoglobin (M.W. 17.6kD) in Shimadzu LCMS 8040 was successful. However, optimization of bigger proteins, like BSA, hTrf, IgGin the LCMS 8040 were not effective. The limitation of CID gas pressure on Shimadzu LCMS 8040 maximum limit upto 230 kPa was found to be a limiting factor. Therefore, Shimadzu LCMS 8050 was used for the MRM optimization of these bigger proteins. The presence of collision energy has also great effect in fragmentation and reproducibility of MRM optimization of proteins. Therefore, the CID gas pressure and collision energy were found as two important variables for effective MRM

optimization. For the BSA, hTrf and antibody (IgG), the MRM optimization was done for more than one precursor ions with and without supercharging agents. For the MRM optimization, we have selected at least 6 to 8 different precursor ions for each of the protein. MRM optimization was done for each of them with three most intense product ions so that we can use the most intense product ion for the quantification and other two are for the qualitative purpose for more specificity.

Optimized MRMs of different proteins with their collision energy

Table 4: List of all precursor to the product ion transitions obtained for BSA, hTrf, and Antibody IgG with their Collision Energy (CE).

Protein	Different precursor ion	MRM	Q_1 Bias	Collision Energy (V)
BSA	1385	1385>1438.7	-46	-47
		1385>744.0	-42	-45
		1385>1568.1	-40	-50
	1415	1415>1524.5	-42	-47
		1415>1529.7	-34	-46
		1415>1517.8	-42	-50
	1445	1445>1472.7	-44	-42
		1445>1557.3	-38	-50
		1445>1661.0	-36	-58
	1477	1477>1581.3	-42	-46
		1477>1676.5	-46	-52
		1477>1665.1	-32	-50
	1511	1511>1608.0	-46	-44
		1511>698.2	-30	-60
		1511>1703.0	-50	-58
	1546	1546>1664.4	-32	-53
		1546>691.2	-50	-65
		1546>1642.5	-50	-50
	1501	1501>1547.5	-30	-37
		1501>1083.7	-46	-40
		1501>1355.1	-50	-68

Holotransferrin	1531	1531>1547.8	-30	-35
		1531>1070.5	-48	-74
		1531>1140.5	-46	-52
	1473	1473>1521.3	-50	-39
		1473>1183.5	-48	-54
		1473>1100.7	-46	-53
	1561	1561>1079.5	-50	-65
		1561>1090.5	48	-56
		1561>1648.4	-34	-42
	1445	1445>1082.4	-50	-57
		1445>973.7	-44	-50
		1445>1656.6	-48	-44
	1599	1599>1240.0	-48	-67
		1599>1366.6	-50	-66
		1599>1119.4	-36	-48
Antibody(IgG)	1516	1516>930.5	-42	-75
		1516>1643.5	-44	-58
		1516>1606.5	-34	-41
	1551	1551>744.0	-46	-63
		1551>642.4	-38	-56
		1551>639.0	-50	-67
	1483	1483>1617.0	-48	-45
		1483>916.0	-44	-65
		1483>687.0	-46	-67
	1588	1588>835.0	-34	-55.5
		1588>716.2	-38	-63.5
		1588>798.1	-34	-57.3
	1450	1450>1538.7	-34	-47.8
		1450>1571.2	-30	-43.1
		1450>1602.5	-44	41.5
	1627	1627>698.0	-36	-59.5
		1627>744.0	-38	-62.9
		1627>802.5	-32	-95.4
	1669	1669>835.0	-50	-59.3
		1669>930.0	-38	-75.5
		1669>737.2	-44	-66.3
Antibody with 0.5% formic acid and 1% m-NBA	1357	1357>668.0	N/A	-47
		1357>574.1	N/A	-96
		1357>654.3	N/A	-86

Here, we have done the MRM Optimization of six to eight different precursor ions having the comparable intensity for BSA, hTrf, and Antibody IgG as listed in the **Table 4**. In which each of the precursor ions were optimized with three most intense product ions. Among the three most intense product ions, we can use one of the most intense product ion for the quantification of these proteins while other two provide the additional specificity to that proteins. It was very challenging to get the reproducible product ion, therefore optimization was done many times in search for the reproducible and intense product ions. From our results, we found that for higher molecular weight intact proteins we needed the high CID gas pressure. We tried to work on the CID gas pressure of 270 kDa in the beginning for the fragmentation of our high molecular weight proteins but we couldn't get the BSA, Antibody IgG at all but little fragmentation to hTrf that was not enough to get the reproducible product ion. Therefore, we worked on CID gas pressure of 400 kPa for all these proteins from mass range (66 kDa to 150 kDa) which worked for MRM optimization..,From all above these results obtained in **Table 4.** showed that use of the high gas pressure with optimal collision energy is good for MRM optimization of high molecular weight intact proteins. There are some chromatograms showing MRM optimization of more than one precursor ions for BSA in **Figure 17.**

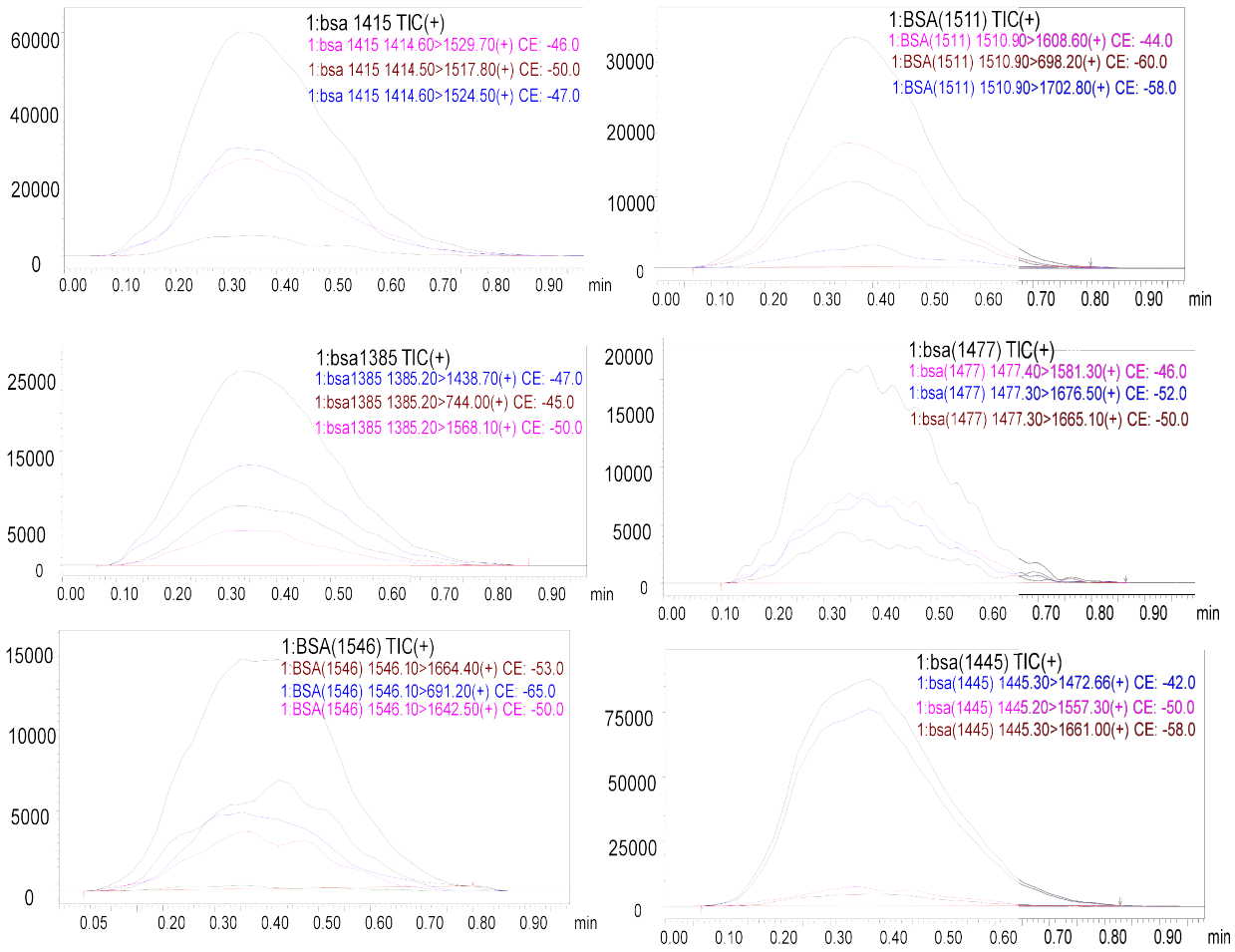

Figure 17: Chromatogram showing the MRM of different precursor ions of BSA with three product ion transition in the presence of 0.5% formic acid only

Chapter 4.4

Summation of MRM

Along with this standard MRM optimization technique, a process for summation of MRM transitions was also investigated. It is a very good approach to done to enhance detection limits of intact proteins and could be done in a different ways.

- ✓ Summation of replicate same precursor to product ion transition.
- ✓ Summation by sequential precursor to product ion transitions.

We have done in both ways one by adding the different precursor to the product ion intensity simultaneously by first taking the most intense precursor ion and adding one from left and right the intensity of MRM of another precursor to product ion transition alternatively that can be called summation of sequential precursor to product ion transition. Similarly, the summation of 10 MRM of same precursor to product ion transition was also done. We found that the summation of MRM concept would be most effective for increased sensitivity. It was clearly shown that multiple transitions from multiple charge state envelopes could be further investigated to increase sensitivity and specificity in future applications. The MRM optimization is normally done in the presence of 0.5% formic acid but few has done with 0.5% Formic acid as well as the supercharging agent with the variable collision energy from -1 V to -100 V in the starting and optimized the voltage and exact collision energy later. For the bigger proteins, we needed the set-up of high CID gas pressure of 400 kPa. The MRM optimization of different individual precursor ions is introduced below.

Graphical representation of sequential summation of MRMS of different of different precursor to product ion transitions of different proteins is given below. The **Table 5 and Figure 18** is the representation of sequential summation of MRMS of more than one precursor ions with their most intense product ion transitions for BSA that gave us an idea about how summation work is helpful to increase sensitivity of that analyte to triple quadrupole instrument.

Table 5: Precursor ions of BSA with their product ion and summation of their intensity

Precursor	Product ion transition	Most intense product	Avg. Int. of most intense product ion	Summation of intensity
1445	1472.7	1472.7	77959	77959
1477	1581.3	1581.3	7836	85795
1415	1524.5	1524.5	28836	114,631
1511	1608.6	1608.6	17719	132,350
1385	1438.7	1438.7	13164	145,514
1546	1664.4	1664.4	6828	152342

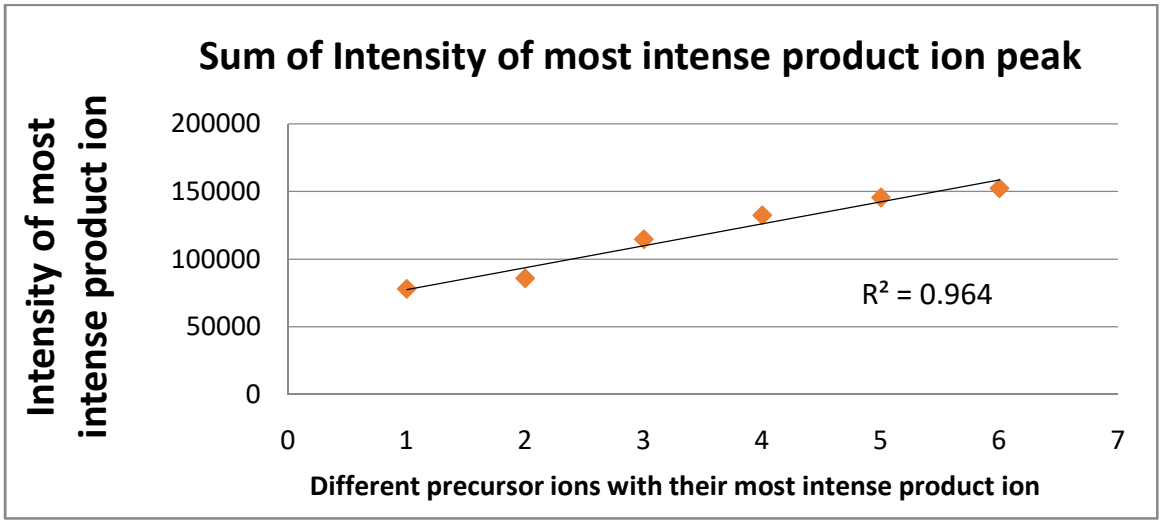

Figure 18: Graphical representation of sequential summation of MRM of BSA

Graphical representation of sequential summation of MRMS of Holo transferrin

Similarly, Table 6 and Figure 19 represent the of sequential summation of intensities of different precursor ions with their most intense product ion and it was done by adding the intensity of the precursor ions left and right to the most intense precursor ions alternatively.

Table 6: List of precursor ion of Holotransferrin with their product ions and summation of their intensity in MRM mode

Different precursor ions of Holo-Transferrin	Most intense product ion	Summation of Intensity
1501	1547.60	144752
1531	1547.90	206032
1473	1521.30	227443
1561	1079.50	229729
1445	1082.40	255058
1599	1240.00	256274

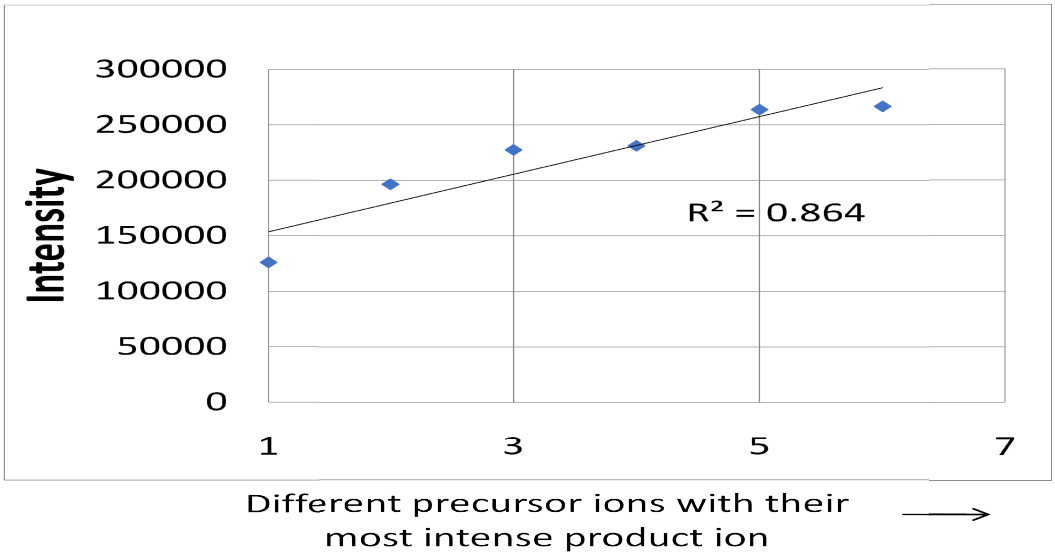

Figure 19: Graphical representation of summation of three most intense product ions of different precursor ions of Holo transferrin

Graphical representation of sequential summation of MRMS of Anti-rabbit IgG

Similar work was done for antibody IgG for the sequential summation of different precursor to their most intense product ion transition and represented in Table 7 numerically and by Figure 20 graphically.

Table 7: List of the precursor ions of Antibody (IgG) with their product ions and summation of intensityin MRM Mode.

Precursor	Product ions	Summation of Intensity
1516	930.5	6161
1551	744.0	13178
1483	1617.0	31,538
1588	835.0	41,918
1450.20	1538.0	65,174
1627	698.0	75170

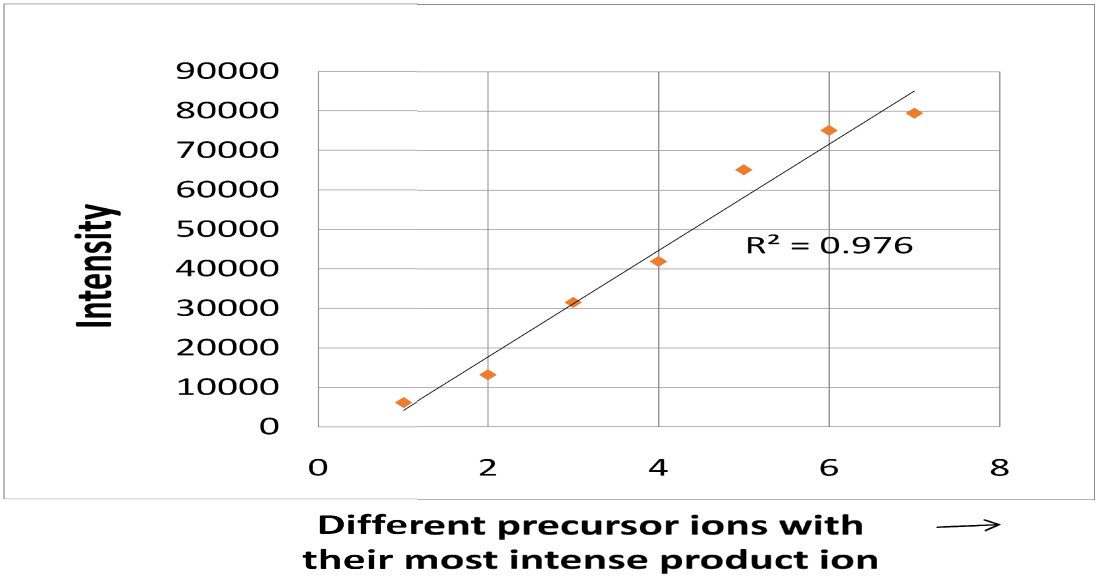

Figure 20: Graphical representation of summation of one of the most intense product ion of different precursor ions of Anti-Rabbit IgG.

Summation of replicates of same precursor to the product ion transitions

Another way of doing summation is by adding 10 same MRM transitions i.e by summing up same precursor to product ion transitions several times. The table 8 below is the representation of summation of 10 same precursors to product ion transitions. The chromatograms in **Figure 21** show the summation of 10 MRMs of same precursor to product ion transition for three precursor ions of BSA.

Table8: List of the six precursor ions of BSA with the MRM of 10 same precursors to product ion transitions and summation of their intensity.

Precursor ion	Product ion	Intensity of summation of 10 product ions			Average Intensity of most intense product ion	Summation of the intensity of the different peaks
		1	2	3		
1445	1472.65	718,292	695,313	701,055	704,887	704,887
1477	1581.30	45,447	43,993	42,470	43,970	748,857
1415	1524.50	139,109	139,196	139,088	139,131	887998
1511	1608.60	108,084	106,574	105,392	106,683	994,671
1385	1438.70	72,000	70,000	82,000	74,666	1,069,336
1546	1664.40	17,264	15,310	17,362	16,645	1,085,981

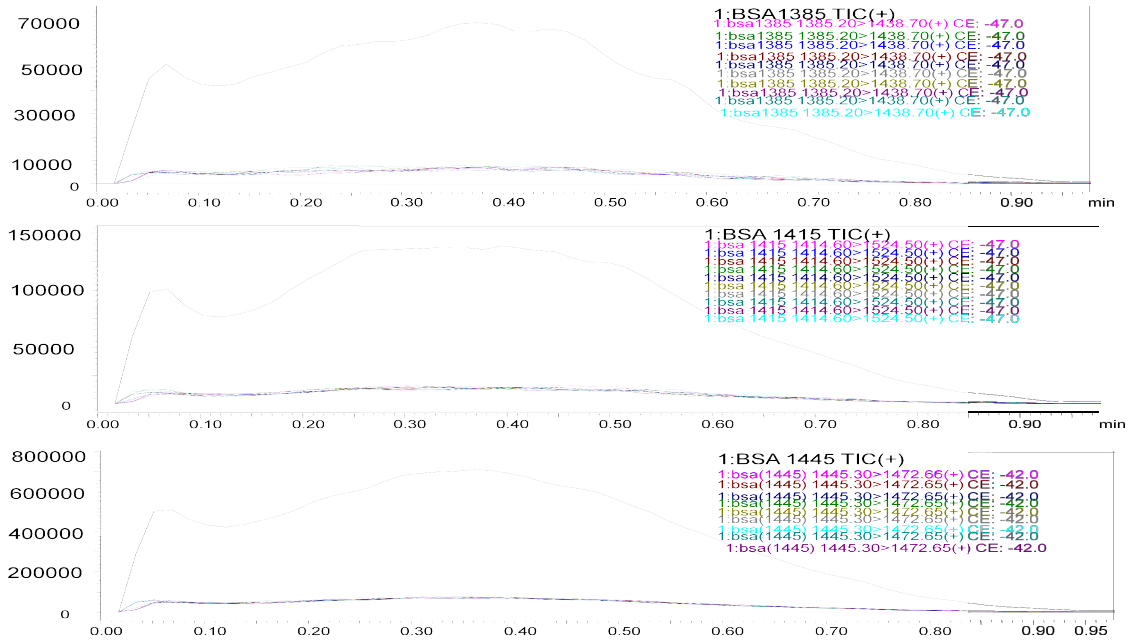

Figure 21: Chromatogram representing summation of 10 MRM of same precursor to product ion transitions

The work on MRM summation was done either by the summation of replicate or by sequential addition of different precursor to product ion transitions. Both are prone to enhance detection limits and it would be the nice way to enhance the detection limit of sample having very low detection limit. In the sequential addition of different precursor to the product ion transition, result obtained shows that it is a very effective way to increase the intensity but there will be increase in error too in sufficient amount as in the hTrf which is not following the high linearity. As well as, we have to optimize all different precursor ions for this type of summation work. Therefore, we found that the summation of 10 MRM of same precursor to product ion transition would be most effective way to represent for increased sensitivity. It was clearly shown that multiple transitions from multiple charge state envelopes could be further investigated to increase sensitivity and specificity in future applications.

Chapter 4.5

Estimated Limit of Detection:

The limit of detectionwas evaluated to check instrument sensitivity to particular type of sample. It is usually done by working with variable concentration of sample, usually by drawing calibration curve. Here, we have considered a minimum amount of sample concentration that is detected by the instrument which is higher than that of limit of blank as the limit of detection. We have used the MRM method file (summation of the 10 same product ion) for the calculation of limit of detection. For this case the noise is alwaysalmost zero. Therefore, value of detectable level of concentration is easy to find out. Similarly, the limit of quantization is calculated by 3.3 times of the LOD. Here, the limit of detection of BSA, Holotransferrin and Antibody is obtained by diluting stock solution into different series of concentration and it is comparable to the value previously obtained by our senior lab mates Evelyn H. Wang. and co-workers [16]. The table with the limit of detection value is given below:

Table 9: Limit of Detection obtained for BSA, Holo-Transferrin, and Anti-rabbit IgG.

Sample	Lowest Concentration of the sample measured(LOD)	Limit of Quantation (LOQ)
BSA with 0.5% formic acid and 5% TFE	4.43 µg/mL (66.7nM)	14.62 µg/mL (220.11 nM)
Holotransferrin with 0.5% formic acid and 5% TFE.	23.4 µg/mL (300nM)	77.22 µg/mL (990nM)
Antibody IgG (1483) with 0.5% Formic Acid only	6.7 µg/mL 45 nM	22.11 µg/Ml 90 nM
Antibody IgG (1357) with 0.5% F.A. and 1% m-NBA.	6.7 µg/mL 45 nM	22.11 µg/mL 90 nM

Chapter 5

Conclusions

In conclusion, Shimadzu LC-MS 8040 and 8050 instruments were used for characterization and optimization of lower and higher molecular weight proteins BSA, hTrf and antibody IgG. Furthermore, MRM optimization of these proteins by Shimadzu LCMS 8050 was found to overcome CID gas limitation of LCMS 8040.The effect of different supercharging agents like DMSO, m-NBA, and TFE was found interesting. Since, DMSO and m-NBA were already widely using as the supercharging agents previously and our results also show that 1% DMSO and 1% m-NBA were good in supercharging of proteins as well as increasing intensity of intact proteins more effectively by 3- to 4-fold with respect to 0.5% formic acid only.But the large difference in results in presence and absence of additive was not observed potentially due to presence of formic acid in all solutions. Formic acid could denature proteins in significant amount thatcould limit the effect of supercharging agents relative to the presence of 0.5% formic acid because they were added to solutions containing proteins in non-denaturing conditions. However, supercharging agents were found to effectively enhance the signal.

Our study revealed interesting effect of TFE as supercharging agent. TFE is usually known for the conformational change of proteins and peptides. However, their use as supercharging agent has not been studied. In our study, we observed positive effect of TFE as supercharging agent. Its effect was found to be greater in BSA, increasing the intensity by more than 15 fold just with the presence of 5% TFE. Similarly, use of 0.5% m-NBA did not show any supercharging and intensity increase of any proteins but use of 1% m-NBA is effective to all the proteins in shifting the most intense precursor ion towards the higher charge state as well as

increasing the intensity of them. Similarly, the use of the sulfolane and 1,2-butylene carbonate did not give any exciting results but it was decreasing the charge state as well as intensity by their use.

Another important aspect of our work is MRM optimization of different precursor ions ofthe studied proteins. Reproducibility of getting product ions in higher intensity was challenging. The MRM results of proteins obtained showed that MRM optimization is possible with high CID gas pressure at 400 kPa along with optimal collision energy around -50 V.

Similarly, work done with different interface temperature showed that there is effect of interface temperature on charge distribution of proteins. We found that at room temperature, charge shifts towards higher charge state. On the other hand, intensity was found increasing with temperature. In addition, flow rate also affected the results. At lower flow rate, the droplet formation is bigger that might increase the surface tension of droplet and as a result, it gives higher charge state. This is based on the Charge residue model.

Chapter 6

Future work

Future plans include working on effect of other supercharging agents like 1,2-butylene carbonate, *N,N,N,N*-tetraethyl sulfomide(TES), and 2-Methyl-2-Oxazoline(MOZ), among potentially others, andon different proteins. In addition, effect of combinations of supercharging agents (automated, if possible) would be interesting follow up work. Our primary future goal isto develop method to optimize proteins from real samples along with their separation using liquid chromatography (LC). This would certainly enhance the scope of our current study. Similarly, our current results with MRM optimization towards increasing sensitivity of the peaks have inspired us to continue the related work. More specifically, we seek to work on MRM summation closer to the limit of detection level (LOD) of the instrument.

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