CHARACTERIZATION OF HYDROPHILIC POST TRANSLATIONAL MODIFICATIONS BY LC-MS

by

DAVID ALAN FISCHLER

(Under the Direction of Ron Orlando)

ABSTRACT

The purpose of this research is to utilize liquid chromatography-mass spectrometry (LC-MS) to investigate significant questions concerning hydrophilic post translational modifications (PTMs). PTMs are modifications which take place following protein biosynthesis, such as glycosylation and the deamidation of asparagine. There are hundreds of PTMs which have been discovered and possibly many more which have yet to be discovered. The many possible combinations of proteins and PTMs create daunting challenges in proteomic analysis compared to the analysis of the 20 unmodified amino acid residues, and LC-MS is a powerful analytical tool capable of aiding in overcoming these challenges and performing significant analysis of hydrophilic PTMs.

The most diverse PTM, glycosylation, is of great importance leading to possible bio-markers for investigation and the production of possible biotherapeutics. In 2016, a new protocol was published describing a new technique for releasing Asparagine (Asn)-linked glycans from a peptide backbone for the large scale preparation of Asn-linked glycans which is much cheaper and faster than standard protocols. The release of Asn-linked glycans from their peptide backbone is essential in the characterization of the structure of these glycans. Here, the validity

and practicality of this protocol was compared to an established technique, the use of peptide: N-glycosidase F (PNGase F), for the release of Asn-linked glycans on much smaller scale.

Another well-studied modification is the deamidation of Asparagine (Asn) residues to form aspartic acid (Asp) and iso-aspartic acid (i-Asp). The biological consequences of Asn forming i-Asp include the mutation of the secondary structure and conformation of a protein. The biological consequences demonstrate the importance of identifying the formation of deamidation products, especially the formation of i-Asp, accurately. The deamidation of asparagine residues is often detected after trypsin digestion, and when products from this reaction are seen they are often presumed to have been present in the native protein. Here, the possibility of the conditions of trypsin digestion playing a role in causing the deamidation of immunoglobulin G (IgG) peptides was investigated.

INDEX WORDS: Post Translational Modification (PTM), Deamidation, Glycosylation, N-Linked Glycans, Liquid Chromatography-Mass Spectrometry(LC-MS)

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DEDICATION

This research combined with all my time in graduate school has been a winding road with speed bumps and hurdles all along the way. There were times I questioned whether I could make it, if I could make it, but the support I received from my friends and family along the way was immeasurable. Therefore, I dedicate this work to the people that mean so much to me and were instrumental in helping me achieve my goals.

To my mom and dad, thank you for all the unconditional love and support during my years in graduate school and beyond. You really were the best parents a son could ask for. To sister Jennifer and brother Michael, somehow, we did not kill each other growing up, and I am thankful for that. We have seen each other through the good times and the bad, and you both are a sister and brother I would not trade for anyone. Lastly, to Madelyn, who has become so important to me the last few years, thank you for your love and support and bringing out the best in me.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Liquid chromatography coupled to mass spectrometry (LC-MS) provides many opportunities to broaden the understanding of post translational modifications (PTMs). Simply put, PTMs are the possible modifications that can follow protein biosynthesis. The abundance and complexity of the different PTMs add to the challenge in understanding the complete biome.[1-2]

The most common PTM is glycosylation which has proven to play key roles in cell signaling, cell-pathogen interactions, cellular regulation, and much more. The emerging field of glycomics has proven to be a new frontier in discovering a new realm of possible biotherapeutics and bio-markers. The characterization of the structure of glycans is essential, but in order to fully characterize asparagine (Asn)-linked glycans they must be released from the peptide backbone. Asn-linked glycans can be released for analysis typically utilizing one of two methods, enzymatic release techniques or chemical release techniques. A new chemical release technique utilizing the sodium hypochlorite (NaOCl) found in bleach was published in 2016 for a large-scale preparative method to release Asn-linked glycans.[3] In Chapter 1, the validity and practicality of utilizing NaOCl to release Asn-linked glycans for small-scale analysis will be explored by Hydrohilic Interaction Liquid Chromatography-Selective Reaction Monitoring (HILIC-SRM).

The deamidation of Asn, another well-studied hydrophilic PTM, can lead to the formation of iso-aspartic acid (i-Asp) which can alter protein secondary structure, conformation,

and function of a protein by extending the protein backbone by a -CH_2- group. The deamidation of asparagine residues is often detected after trypsin digestion, and when products from this reaction are seen they are often presumed to have been present in the native protein. In Chapter 3, deamidation rates of immunoglobulin G (IgG) peptides will be characterized to determine the effects of the conditions of a trypsin digestion on the deamidation of IgG peptides.

LITERATURE REVIEW

PTMs

PTMs, such as glycosylation and the deamidation of Asn, play important roles in adding heterogeneity to the human biome. There have been hundreds of PTMs discovered, each adding different traits to a protein's structure, conformation, or function.[1-2] Possible functions which can be controlled by a protein's PTMs include protein modulation, solubility, and cell signaling.[4] The the importance of these PTMs that demonstrates the need for them to be investigated, but the limitless possible combinations of PTMs on a protein substrate result in an exponential increase in challenges in proteomic analysis. [5-6] However, the challenges that stem from these combinations of PTMs have also lead to an increase in creative opportunities in the pharmaceutical industry for potential therapeutic targets, possibly with the aid of either monoclonal antibodies (mABs) or Fc-fusion proteins.[7] A lack of knowledge and understanding of PTMs coupled with all of the possible therapeutic benefits emphasizes the importance of PTM analysis.

Glycans and Glycosylation

The most common and the most diverse PTM in eukaryotes, occurring in nearly half of human genes, is glycosylation.[8] Glycosylation is the addition of at least one monosaccharide to a protein substrate (Table 1.1). Over the past several decades the role of glycans and glycosylation have been determined to be of great importance in many areas including the structure, stability, and proper folding of a protein.[9-10] Other roles include the mediation of pathopsychological events, the mediation of cell-cell reactions, mediation of cell-pathogen reactions, as a cellular regulation switch, and as a cellular nutrient sensor.[11-16] Some examples of their importance are the consequences of glycan mutations leading to many carbohydrate-deficient glycoprotein

syndromes (CDGS) which have been identified since the 1980s through glycan profiling, where differences in glycan profiles have been used for diagnosis.[7, 17]

The field of glycan research has emerged in recent years leading to the investigation of the possible role of glycosylation and specific glycans in various types of cancer and other disorder such as CDGS.[7, 18-20] As the importance of glycosylation and their resulting glycans have become known, attempts to possibly produce and use glycans as drugs and other therapeutics have become the proverbial goals.[21-24] Although there are a number of types of glycans present in the biome which are being researched, two of the most common forms of glycosylation being investigated are asparagine (Asn)-linked glycans and oxygen(O)-linked glycans.

Asn-Linked Glycosylation

The most common glycosylation is Asn-linked glycosylation. Asn-linked glycosylation typically occurs on the asparagine residue in the presence of Asn-X-serine/threonine (Ser/Thr), where X is any amino acid except proline. The synthesis of Asn-linked glycosylation begins in the rough endoplasmic reticulum (RER) before terminating in the golgi apparatus.[25-26] Beginning in the RER, a dolichol-phosphate complex is the precursor of the synthesis until the complex is extended to Dolichol-$(phosphate)_2$-$GlcNAc_2$-Man_5. The complex is then "flipped" from the cytoplasm to the lumen of the RER where the complex is further extended with the addition of four Man and three Glucose (Glc) residues by Dolichol-phosphate-Man and Dolichol-phosphate-Glc complexes respectively. The dolichol -phosphate-glycan complex is then transferred to an Asn residue before undergoing protein folding and moving to the golgi apparatus. The protein folding also plays the role of quality control which sends the glycan to the cytoplasm for protein degradation if there are errors in the glycoprotein. As the protein-glycan complex continues

through the golgi apparatus, monosaccharides are constantly removed and added by a variety of transferases and transporters, such as alpha mannosidase II, UDP-Gal and CMP-Sialic Acid Transporter. The completed Asn-linked glycan is terminated in the golgi apparatus as one of the three categories of Asn-linked glycans. (Figure 1.1). All three types of Asn-linked glycans have one distinct feature where they all contain a single core that is present in all Asn-linked glycans, consisting of $[GlcNac]_2[Man]_3$ (Figure 1.2).[27] However, following the core the glycans can differ greatly, and with the links to abnormalities in Asn-linked glycosylation profiles being linked to diseases such as Cancer and Alzheimer's disease, the analysis of Asn-linked glycans is of utmost importance that Asn-linked glycans continue to be researched and analyzed.[28-29]

O-Linked Glycosylation

Another product of glycosylation are O-linked glycosylation. Although O-linked glycosylation is not as prevalent as Asn-linked, O-linked glycosylation is just as important as Asn-linked. O-linked glycans has been shown to be responsible for protein solubility, conformation, stability, and much more.[30] This importance to investigate O-linked glycans also brings up the biggest challenge. Compared to Asn-linked glycosylation, the core of O-linked glycans is much more diverse, as there is no standard core.[31-32] For example, in eukaryotes there are eight different cores for O-linked glycans compared to the single core for all Asn-linked glycans.[33] Further differences between O-linked and Asn-linked glycosylation is the synthesis pathway. While Asn-linked glycans are synthesized in the RER and golgi apparatus, O-linked glycosylation is primarily synthesized only in the golgi apparatus.[11] O-linked glycosylation is an important PTM to understand, but the analysis of O-linked glycans also provides a myriad of investigative challenges which are not present in the analysis of Asn-linked glycans.

Analysis of Glycans

The need to characterize glycans, both Asn- and O-linked glycans, has led to the emergence of the field of glycomics, the study of glycosylation. Advances in glycan release and glycan analysis techniques have emerged over the last several decades to aid in glycan research for the complete characterization of glycans. The advancements in the field of glycomics and the possible roles of glycosylation in cancer and other diseases as possible bio-markers or therapeutic targets demonstrate why the ability to analyze glycosylation and glycans is of utmost importance.[21-24]

Glycan Release

The characterization of Asn-linked glycans typically involves releasing them from their protein, and this glycan release can be accomplished either chemically or enzymatically. One of the oldest glycan release techniques is a chemical release method. Hydrazinolysis has been utilized for the release of both Asn-linked and O-linked glycans; however, although cheaper than enzymatic methods, chemical release techniques are dangerous being highly toxic and volatile. They can also lead to the loss of information that can be found by preserving the peptide backbone, as the peptide backbone is degraded.[34-38] As well-established as the chemical release method is, there are several alternative techniques to release Asn-linked glycans by the use of enzymes.

The enzymatic release of Asn-linked glycans is a time consuming and expensive process utilizing enzymes, such as endoglycosidase H (Endo H). Endo H was one of the first commercially available enzymatic release techniques.[39] Endo H, along with other glycosidases, such as endoglycosidase F, release specific Asn-linked glycans by cleaving glycosidic bonds (Figure 1.3).[40-43] The use of these enzymes has been commonly utilized to cleave N-linked

glycans, and the methods are efficient; however, there are drawbacks. The major drawback to the endoglycosidases is their inability to release intact glycans, but there are alternative release techniques that are solutions to this problem.

A proven alternative to the endoglycosidases is digestion with a peptide: N-glycosidase (PNGase). The PNGases, including Peptide: N-Glycosidase F (PNGase F) and Peptide: N-Glycoamidase A (PNGase A) have been some of the most widely utilized methods for releasing N-linked glycans due to their abilities to cleave complete glycans, by cleaving the bond between the Asn residue and GlcNAc while preserving their original peptide backbone (Figure 1.4).[44-45] Each peptide:N-glycoamidase has unique benefits, but neither is without drawbacks. PNGase A, does not have the ability to cleave larger, more complex glycans, such as those containing sialic acids.[33, 46-48] PNGase F, on the other hand, is capable of cleaving all Asn-linked glycans, with the exception of glycans containing a 1,3-core fucose (typically observed in plants).[49-51] In spite of the cost and time consuming nature of enzymes, PNGase F is one of the most preferred methods for the release of N-linked glycans for simplicity and ability to release intact glycans while preserving the information that can be learned from the peptide backbone.

Derivatization

The ability to analyze released Asn-linked glycans by mass spectrometry provides a great deal of complications compared to the analysis of proteins. Several of these complications are the complexity and diversity compared to a 20 amino acid backbone and a lack of UV absorbance for quantitation analysis. One of the major complications is low ionization efficiency. Where basic amino acids, such as lysine and arginine provide a site of protonation in positive ion mode for proteins, no such site exists on a glycan. There are several derivatization techniques,

which are modifications designed to mitigate some of these complications, and one of these derivatizations is permethylation.

Permethylation has been around since the 1960s, and is one of the oldest and one of the most important derivatization techniques(Figure 1.5).[52] Permethylation protocols have become more established in recent decades, but the end result of replacing hydroxyl groups with more stable, hydrophobic methoxy groups is the same.[53-55] Since permethylation leads to increased hydrophobicity, permethylation also leads to both an increased volatility of the analyte and increased ionization efficiency. This hydrophobicity also increases the suitability of glycans for reverse phase (RP) chromatography, a chromatographic separation technique which will be covered later.[11, 53-54, 56-58] The permethylation of the hydroxyl groups can also bring stability to acidic residues in positive mode.[59] Furthermore, permethylation can aid in prevention of analyte rearrangement in the gas phase, which makes structure identification a more manageable task.[60-61] These major benefits for glycan analysis demonstrate the importance of permethylation as a derivatization tool.

The utilization of permethylation in glycan analysis is beneficial, but there are still some drawbacks to this technique. First, is the compatibility of this method in that this method is not suitable for all analytes.[62] Even when analytes are compatible with the technique the permethylation reaction is tricky, and complete conversion of all hydroxyl groups to methoxy groups may vary from glycan to glycan. However, despite the challenges of using permethylation, permethylation remains a valuable technique in the analysis of glycans.

Another valuable tool, reductive amination, in the derivatization of glycans depends on the structure of the glycan analyte. Reductive amination can be applied if the glycans being analyzed contain a free-reducing end. Reductive amination is the use of an adduct containing a

free amine (NH_2) to bind to the free-reducing end of a glycan (Figure 1.6). Two of the more well-established labelling adducts are the fluorescent tags 2-aminobenzamide(2-AB), and more recently, procainamide (ProA). Both of these labels are powerful tools for analyzing free glycans.

The first major benefit to the use of these labels is their ability to provide absolute quantification. The ultraviolet (UV) analysis of these two labels is very straight forward and reliable. By Beer's Law ($A=\varepsilon bc$); where A is the absorbance, ε is the extinction coefficient, b is the path length, and c is the concentration of the tagged-glycan; the determination of absolute abundance of a glycans tagged with either label is easily determined.[63-69] The second major benefit of these tags, as with the permethylation method above is the increased hydrophobicity of the glycan analyte. Lastly, and to a mass spectrometrist most importantly, are the amine groups in the labels which are not used to bind to the glycan analytes. These amine groups greatly increase the overall ionization efficiency of the glycans by supplying a much more attainable destination for protons in the '+' mode. Glycan labeling, as well as permethylation of glycans, are remarkably powerful analytical tools in the analysis of glycans.

Monoclonal Antibodies (mABs)

One possible source of glycoproteins are mABs, which are antibodies that are identical immune cells that are clones of a single parent cell. First developed in 1975, but not licensed until 1986, mAB offer a way to specifically target mutagens and defects, since they are antibodies that are capable of targeting a single epitope.[70-72] Today, mABs have become some of the highest selling pharmaceuticals in the world. As of 2017(based on SEC filings and company websites), half of the top 10 selling pharmaceuticals are mABs, lead by adalimumab with over $18 billion in sales worldwide. Research continues to focus on improving the efficacy of mABs

by improving their effects, as well as investigating the addition of modifications which could be beneficial.[73-74]

Immunoglobulin G (IgG)

The top selling monoclonal antibody worldwide is adalimumab, which is an IgG that is utilized for the treatment of rheumatoid arthritis and Crohn's disease.[75-77] Overall IgGs make up 75% of human serum antibodies, broken down to four subclasses based on abundance (IgG1, IgG2, IgG3, and IgG4). The basic structure of any IgG is a Y-shaped structure consisting of a heavy chain and a light chain, bound by disulfide bonds (Figure 1.7). Amongst the Y-shape, there are two distinct regions. The first of these two regions is the F_c-region, which is a constant region which does not change amongst a particular class of IgGs. For example, all IgG1s have the same F_c region. The second region is the F_{ab} region, which consists of the variable region for any IgG. This variable region is responsible for the binding specificity of a particular binding analyte.

Deamidation

Other PTMs are the deamidation of either Asn or glutamine (Gln). Deamidation produces either aspartic acid (Asp) and iso-aspartic acid (i-Asp) in the case of Asn or glutamic acid (Glu) and iso-glutamic acid in the case of Gln (Figs 1.8-1.9). Deamidation of both Asn and Gln are well-studied PTMs with the rates of deamidation being investigated under a variety of different conditions, including different pHs and temperatures. [78-79] Although both Asp and Gln are both susceptible to deamidation, the rate of deamidation of Asn is much more rapid than the rate of deamidation for Gln. Another major factor that affects the rate of deamidation and can lead to a much faster rate of deamidation is the neighboring effect. The neighboring affect is where an amino acid consisting of a smaller side chain on the C-terminus side of the deamidating residue

contains minimal steric hindrance leading to a faster rate of deamidation.[80] Therefore, deamidation at a site consisting of a Asn followed be a Gly on the C-terminus side would contain the fastest rate of deamidation.

The possibility of deamidation forming n-Asp also include the possibility of the formation of the i-Asp residue.[81-82] There is also the possibility for the interconversion of n-Asp to i-Asp. The formation of i-Asp produces an additional -CH_2- moiety to the peptide backbone which can alter the secondary structure, conformation, and function of a protein.[83-84] This protein modification has also been shown to have major biological ramifications, such as Alzheimer's disease and other neurodegenerative diseases.[85-88] Due to the complications in a protein's integrity from the deamidation of Asn, any observance of this modification in commercial drugs could raise doubts of the quality and effectiveness of protein-based pharmaceuticals, especially those which are stored under conditions conducive to the deamidation of Asn.[89-92] The possible consequences of i-Asp formation explains the importance for accurate quantitation for the formation of i-Asp.

Attempting to accurately quantitate the two deamidation products independently is a challenge in mass spectrometry. Unlike when they are compared to their Asn counterpart, which differs by 1Da to the molecular, the two deamidated species have the same mass. Using Hydrophilic Interaction Liquid Chromatography (HILIC) has shown the ability to separate a peptide containing n-Asp and a peptide containing i-Asp with baseline resolution.[93] With LC-MS the accurate quantitation of i-Asp is possible.

Instrumentation

Ionization Techniques

Mass spectrometry, at its core, is an instrumental method which allows for the analysis of the mass-to-charge ratios of analytes of interest. In order to perform this analysis, ions need to be produced by providing or removing electrons. This ionization process can be accomplished in several different ways, and two of the most popular ionization techniques are matrix-assisted laser desorption ionization(MALDI) and electrospray ionization (ESI).

MALDI

One of the most common and robust soft ionization techniques, where minimal internal energy is transmitted to the sample during ionization, is the use of MALDI. MALDI is the use of a laser to ionize an analyte of interest which has been co-crystallized with a selected matrix, such as α-Cyano-4-hydroxycinnamic acid and spotted on a MALDI plate. A laser is fired at the co-crystallized matrix-analyte mixture, and the energy from the laser is absorbed by the matrix followed by the desorption and ionization of sample analytes in the matrix. This results in the target analyte being placed into the gas phase. This technique has been utilized for the characterization of many biomolecules, including proteins and permethylated glycans.[94-95]

MALDI is typically coupled to a time-of-flight (ToF) analyzer. A Tof analyzer consists of a long tube under vacuum where an ion (or group of ions) can be accelerated and the time the ion(s) takes to reach a detector can be measured. An ion or group of ions is accelerated at the same time with the same amount of energy. Since the velocity of the ions is constant following acceleration, the m/z ratio can be calculated based on velocity. The m/z ratio of an ion can be calculated by the equation $t = k\sqrt{m/z}$, where t is time for the ion to reach the detector, k is an instrument calibration factor, and m/z is mass to charge ratio. Advancements in ToF analyzers,

by the addition of reflectrons which alter the path of an ion based on mass, has lead to high mass accuracy and mass resolution.[96] The coupling of MALDI to a ToF analyzer produces a robust instrument with high mass resolution and high mass accuracy in the analysis of a variety of biomolecules.[97]

ESI

Another soft ionization technique, just like MALDI, is ESI. ESI was first developed in the 1960s and first coupled to MS instruments in the 1980s. ESI is where a sample analyte, which has been dissolved in a polar solvent, is ionized by aerosolization producing charged droplets which are driven toward the instrument. [98] As these droplets move through the source the solvent is vaporized and the charged analyte ions eventually desorb into the gas phase. The ions are then guided to the mass spectrometer.[99] Just like with MALDI, ESI is capable of analyzing most biomolecules, like DNA and glycoconjugates.[100-102] However, salt and other adducts and/or impurities can provide challenges in their analyses.

There are several major advantages of this soft ionization technique compared to MALDI. One such advantage is the ability for the ionization to yield predominantly multiply charged ions thereby allowing for the analysis of larger molecules.[103-104] Furthermore, this soft ionization is also much less harsh on the labile nature of specific monosaccharides in glycan analysis, such as salicylic acid. Although insource fragmentation is still possible even with ESI, analysis can be performed without permethylation.[105] The second major advantage to ESI is the ability for quantification. Since ESI does not need the analyte to be co-crystallized with a matrix in a heterogeneous mixture, there is little to no discrepancy in what is sampled among replicates. This leads to high reproducibility in ESI, and there is no need to deduce where the sweet spot for

sampling is, as is the case with MALDI.[106] For many reasons, ESI offers many advantages which are major improvements upon ionization capabilities.

Analyzers

Several different variations of mass analyzers are commercially available at many different price points. Depending on the analyzer that is utilized several factors; such as sensitivity, mass accuracy, mass resolution, and even cost can all vary greatly. Each different type of analyzer has benefits and disadvantages, and the type of data needed to be acquired will determine which analyzer is required. Three of the more popular analyzers in PTM analysis are a quadrupole ion trap(QIT), a quadrupole-time-of-flight (Q-ToF), and a triple quadrupole (QqQ).

Linear Trap Quadrupole (LTQ)

LTQs and other QITs are mass spectrometers which have a high sensitivity, due to their ability to capture ions, and are some of the oldest analyzers utilized in mass spectrometry. All QIT instruments are set up similarly with four, precisely placed, parallel electrical rods. Two of these rods share alternating radio frequency(rf) potentials and the other two rods share alternating direct current(dc) potentials. As ions are pulsed through the quadrupole, the alternating potentials filter out ions which do not fit in a narrow m/z window that is set for analysis.[107-108] A major advantage of QIT instruments is their ability to be utilized as a either a true ion trap or just a mass filter. [109] Furthermore, the size, cost efficiency, and robustness of QIT instruments help explain their appeal. However, there is an important disadvantage to QIT instruments. These instruments tend to have low mass accuracy and low mass resolution. Although this drawback has been shown to be improved by attaching an analyzer to the back of the quadrupole, for example a ToF analyzer, for multi-stage MS/MS analysis.[110]

Q-ToF

The benefits of a QIT can be combined with the beneficial mass accuracy and mass resolution which comes with the use of a time-of-flight detector. Although not utilized in this research, Q-ToF instruments were first described in 1996, and the use of a Q-ToF instrument analysis for MS/MS analysis began to be utilized as early as the 1980s.[111-113] The main layout of this instrument is two quadrupoles aligned orthogonally with a ToF analyzer (Figure 1.10). There is also a third quadrupole in front of the main quadrupole analyzer, running an rf-only frequency, which allows for ion focusing by collisional cooling of the ions and which allows for the quality of the ion beam to remain high.[114] Amongst the other two quadrupoles the first quadrupole is the ion filter portion of the instrument and the second quadrupole is the collision cell in an MS/MS analysis. During a full MS analysis, all quadrupoles are rf-only and the analysis is performed in the ToF analyzer. The Q-ToF's high mass accuracy and resolution compared to a normal QIT instrument that allows the Q-ToF instruments to remain one of the more popular analyzers for the analysis of peptides, glycans, and other PTMs.[110]

QqQ

A challenge when attempting to analyze PTMs has always been their low abundance, and this is a challenge that would not be expected to be solved utilizing a QqQ instrument, an analyzer consisting of 2 quadrupole mass filters with a collision cell in between (Figure 1.11). A QqQ has the major drawbacks of low mass accuracy, mass resolution, speed, and sensitivity compared to what can be achieved by a LTQ or Q-ToF instrument for MS1 and product ion experiments. However, a QqQ instrument has the benefit of being able to conduct a selective reaction monitoring (SRM) experiment. An SRM experiment is capable of increasing the sensitivity of low abundant analytes by performing a targeted analysis as selected parent ions pass through the first quadrupole, fragment in the collision cell, and the fragment ions are

selectively filtered prior to the ions reaching the detector.[115-116] SRM experiments have been used to analyze PTMs such as glycans and glycopeptides.[117-120] Besides the benefit of an SRM experiment, the triple quadrupole has the unique advantage of being highly quantitative as the detector counts every ion that reaches the detector above the thresh-hold, which is typically the largest negative aspect of mass spectrometry.[120] These traits allow this analyzer to be uniquely suited for the quantification of low abundant PTMs.

Liquid Chromatography

Although much information can be deduced by mass spectrometry alone, when ESI is coupled to liquid chromatography the analytical capabilities increase by performing a separation of analytes which can better isolate analytes and concentrate analytes prior to mass spectrometric analysis. Three of the most readily utilized chromatographic techniques, which have been utilized successfully for the analysis of PTMs, are RP, porous graphite carbon (PGC), and HILIC. A Halo-Penta-HILIC column was utilized for this research.

RP

RP chromatography is a well-studied chromatographic technique with the premise that a RP column is capable of separating hydrophobic analytes. A hydrophobic analyte, such as peptides, are adsorbed onto the stationary phase and eluted as the mobile phase becomes less aqueous. A mobile phase consisting of acetonitrile and water is typically utilized, which makes this technique compatible with ESI and less likely to be subject to too much backpressure as would be the case with more viscous solvents. The most utilized stationary phase in RP chromatography is the octadecyl carbon chain (C_{18}) silica bonded column, which has covalently bonded alkyl chains that create a hydrophobic stationary phase. The mechanism and driving forces for this adsorption/desorption reaction are still not fully understood.[121] However, this

analytical technique remains popular due to the simplicity to use RP, the versatility of RP, and the capabilities to analyze a large variety of hydrophobic analytes.[63, 122-123]

This technique has been vastly improved leading to more reproducible and predictable retention time.[124] The major improvements include additives to the mobile phase such as ammonium formate or formic acid, to increase the ionization efficiency lower the pH to control which charge states of the analytes.[125-126] Although greatly improved the ability to separate hydrophilic analytes, like glycans, is still difficult without derivatization.[54]

PGC

Although not utilized in this research, PGC chromatography; is an important chromatographic technique which has been used for the analysis of PTMs. The first variation of PGC was first realized in 1979, but an improved process to produce stable and chromatographically reproducible PGC was established in the early 1980s.[127-128] The stationary phase of PGC is made up of sheets of hexagonally arranged carbon atoms which are linked by the same conjugated bonds which are present in any large aromatic hydrocarbon.[129] PGC is very parameter driven with the size of the carbon, the structure of carbon, as well as other column factors such as pH and column temperature capable of drastically affecting retention on the PGC column.[130-131]

PGC is a unique technique which is capable of separating not only non-polar, hydrophobic, analytes, but PGC is capable of separating polar analytes.[132-133] PGC's unique ability to retain polar compounds, such as glycans, was coined the "polar retention effect on graphite." This theory has been described as the delocalization of electrons in the carbon producing a polar surface, which can then retain hydrophilic compounds.[129] However, the mechanism is not entirely understood, and there can be unexpected retention times. Furthermore,

contaminations in the samples can further lead to a loss of retention of sample analyte. Additives, like ion-pairing reagents, have proven beneficial in improving reproducible results.[134] Due to all of the benefits in the analysis of hydrophilic analytes, like glycans, PGC is an important tool in the analysis of these analytes; however, the biggest challenge to PGC is the limitation in method development, as improvements are limited to the development of newer PGC stationary phases.

HILIC

HILIC, one of the newer chromatographic techniques, has been around since 1975 before being fully described in 1990.[135-137] HILIC is a separation technique which shares characteristics from several chromatographic techniques. Although the full mechanism of HILIC is not fully understood, the mechanism involves the binding of your hydrophilic analyte to a thin water layer on the surface of stationary phase, which is then eluted by increasing the percentage of aqueous buffer in the mobile phase. Two of the major characteristics of HILIC are elements of ion exchange chromatography and normal phase chromatography. First, the stationary phase for HILIC is polar, similar to the stationary phase in normal-phase chromatography, which allows for the separation of polar analytes. Second, unlike RP chromatography, HILIC(although not to the extent of ion exchange chromatography) can partially separate charged analytes [138-139] Besides these two prominent characteristics there is also a third major characteristic of HILIC, the mobile phase. The mobile phase of HILIC is most closely linked to RP chromatography. Both RP and HILIC share similar buffer systems, with the elution of analytes on HILIC being characterized as the reverse of RP chromatography, where samples are loaded on a column in high organic buffers and a gradient elution takes place with increasing aqueous buffer.[135, 140-141] The different characteristics of HILIC make HILIC a unique chromatographic technique.

HILIC is generally related to RP chromatography; however, unlike RP chromatography HILIC is more suited for the separation analytes which are hydrophilic, such as glycans. However, similarly to RP chromatography, HILIC is adequately suited to be coupled to ESI with predictable elution times.[142] Recently, retention time prediction models utilizing HILIC for the analysis of peptides, glycans, and glycopeptides have been established.[93, 143-144] The capabilities for the analyses of hydrophilic compounds has allowed HILIC to be utilized in the analysis of not only peptides like RP chromatography, but carbohydrates, other polar pharmaceuticals, and other hydrophilic PTMs. [139, 141, 145]

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Tables and Figures

Table 1.1: Common Monosaccharides Present in Eukaryotic Asn-Linked Glycans. Symbols adopted from Symbolic Nomenclature For Glycans (SNFG).

Monosaccharide	Nomenclature	Symbol
Mannose	Man	
N-Acetylglucosamine	GlcNAc	
Galactose	Gal	
Fucose	Fuc	
Sialic Acid	NeuAc	
Glucose	Glc	

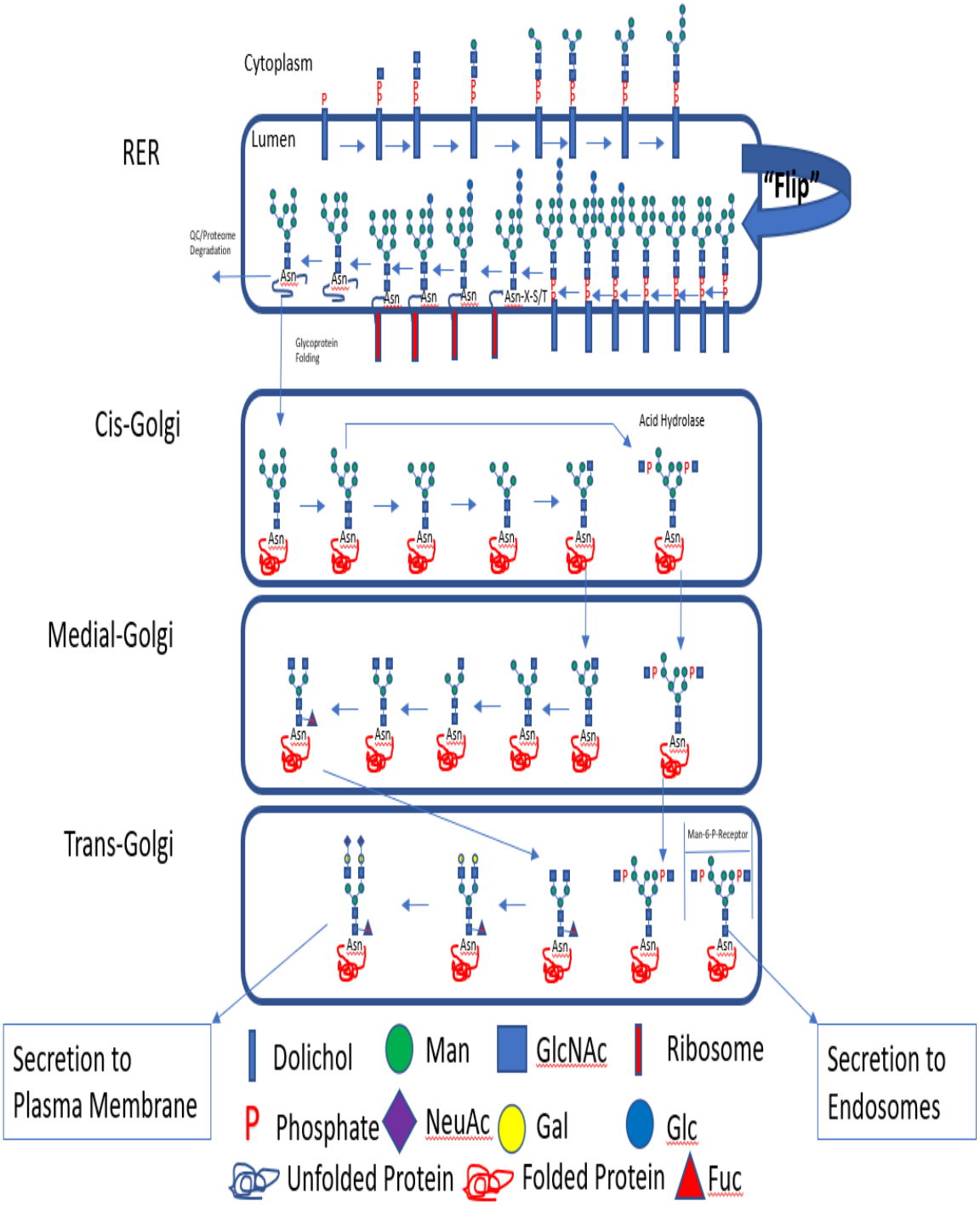


Figure 1.1: Synthesis of Asn-Linked Glycans. The synthetic pathway of Asn-linked glycans beginning in the RER with a dolichol-phosphate complex and terminating in the golgi apparatus.

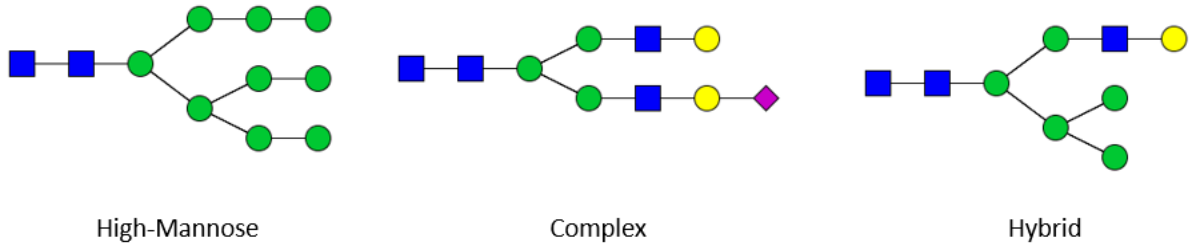


Figure 1.2: Categories of Asn-linked glycans. The three categories of Asn-linked glycans.

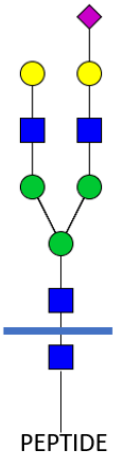


Figure 1.3: Endo H Cleavage Site. Probable cleavage site of Asn-Linked glycan (Blue line) on a glycosidic bond by Endo H.

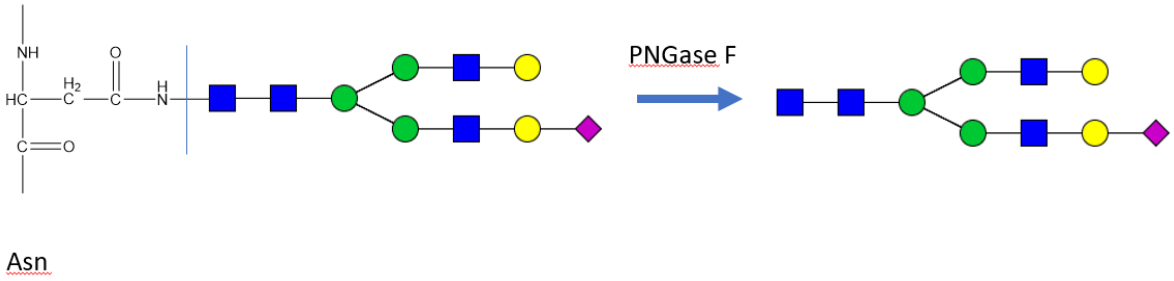


Figure 1.4: Cleavage of Amide bond by PNGase F. Cleavage of an amide bond between the Asparagine residue and the first GlcNAc.

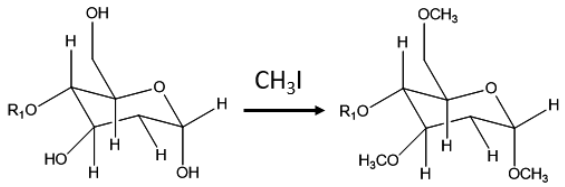


Figure 1.5: Permethylation Reaction. The formation of a permethylated carbohydrate utilizing iodomethane.

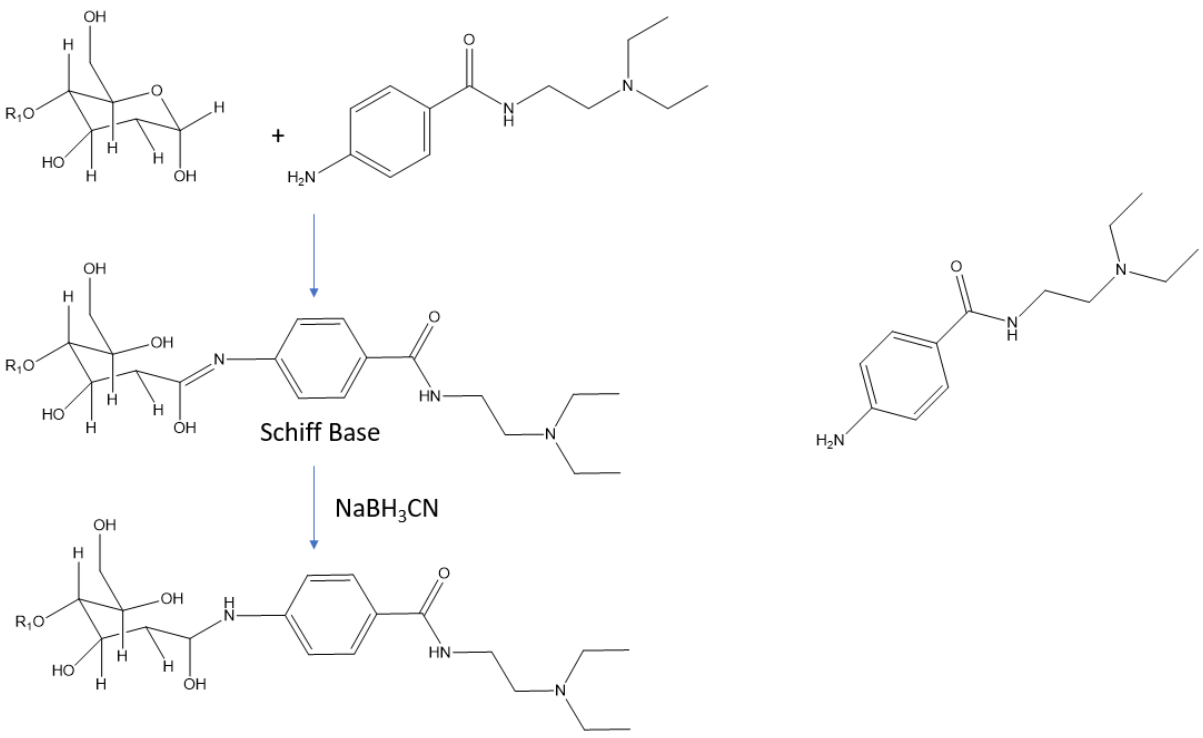


Figure 1.6: Procainamide (ProA) and Reductive Amination Reaction. An example reductive Amination reaction utilizing ProA is shown (left) demonstrating the formation of the Schiff Base and the reduction by $NaBH_3CN$, along with the structure of ProA (Right).

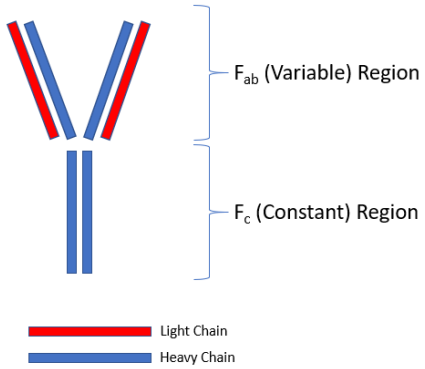


Figure 1.7: Structure of IgG. A general layout of an IgG, showing both the light and heavy chains and their respective orientation in the F_{ab} and F_c regions

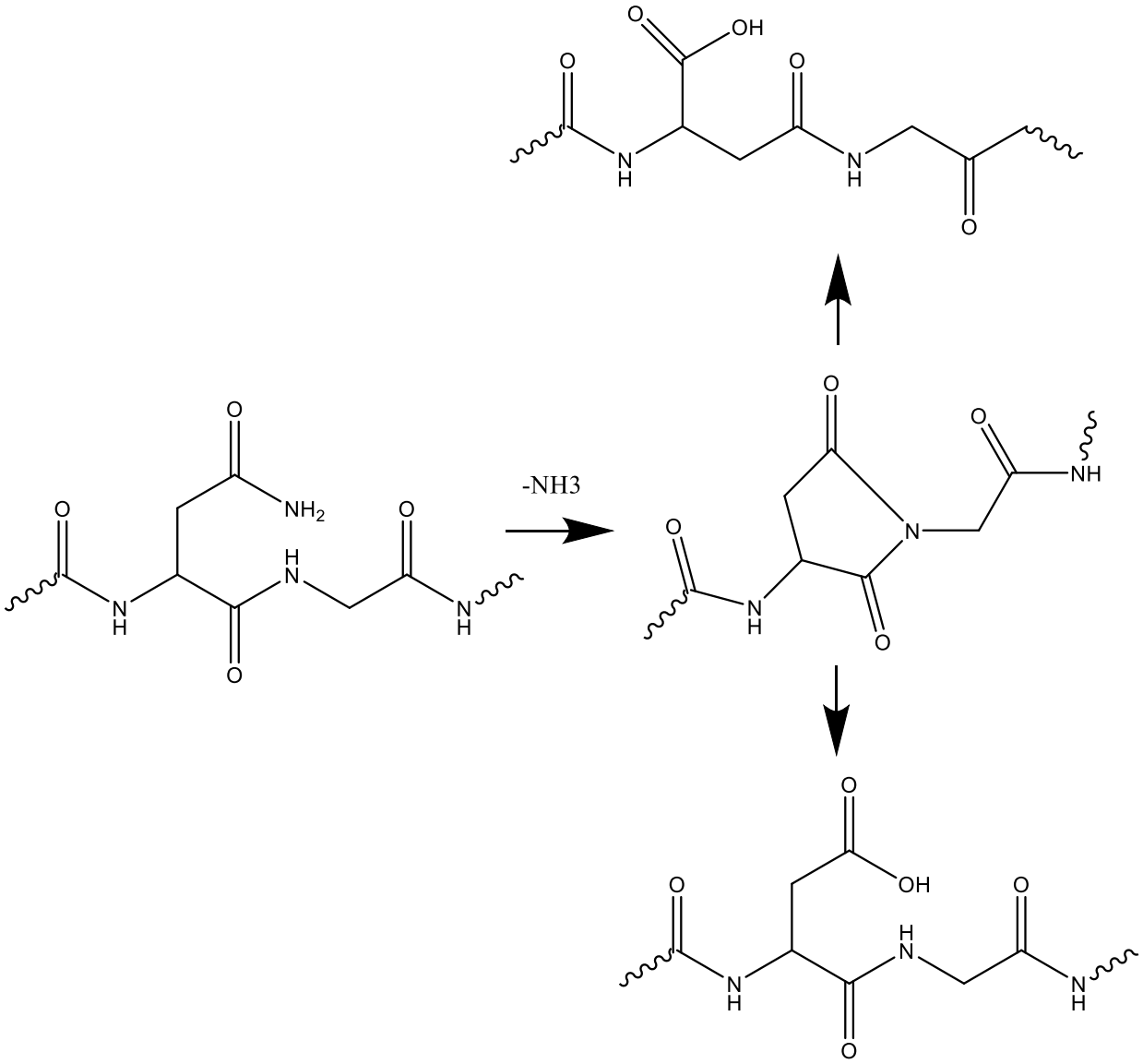


Figure 1.8: Deamidation of Asparagine. The amino acid asparagine loses an ‘NH3’ to form a succinimide intermediate before hydrolyzing to form either aspartic acid or iso-aspartic acid.

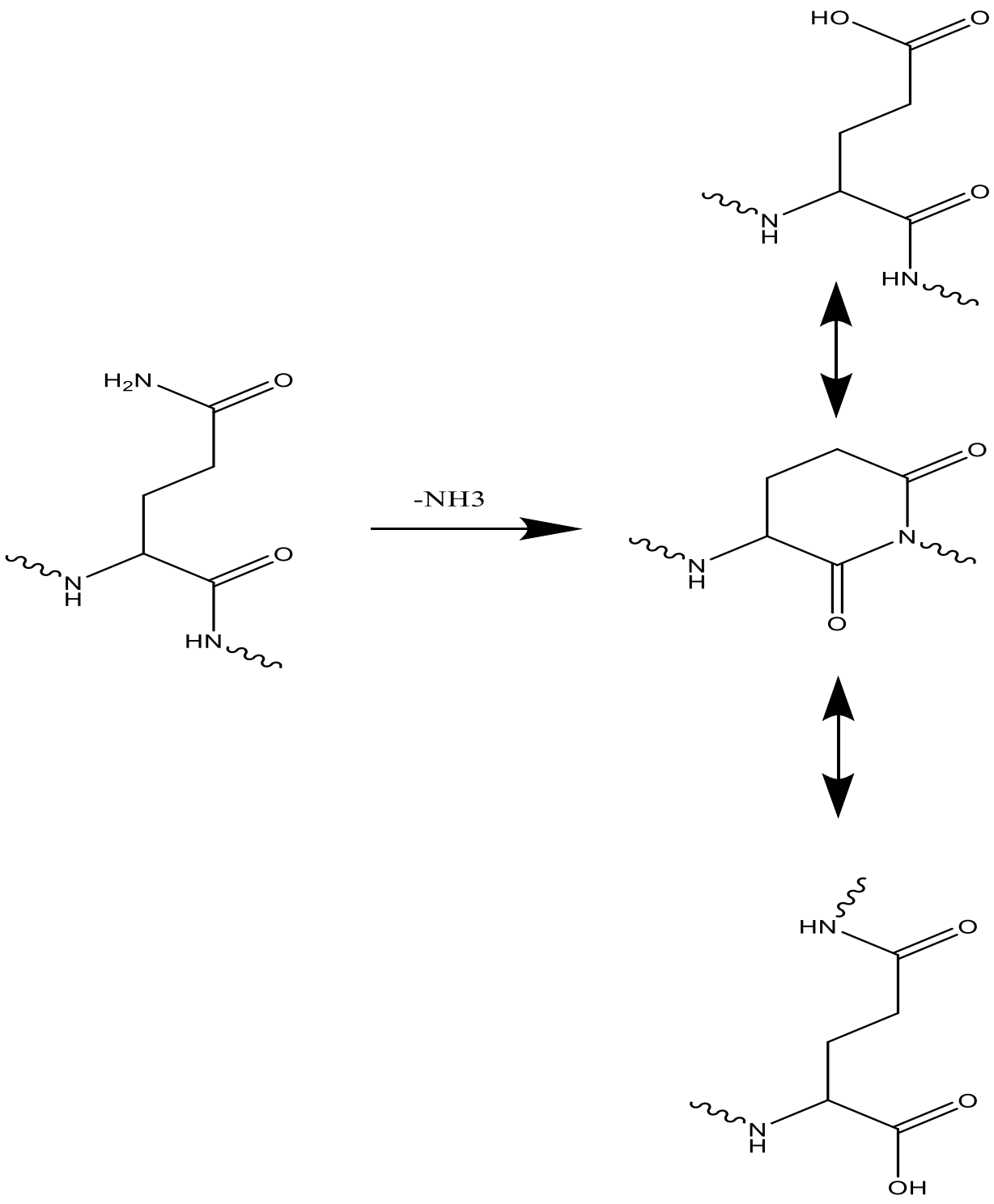


Figure 1.9: Deamidation of Glutamine. The amino acid glutamine loses an ‘NH3’ to form a glutarimide intermediate before hydrolyzing to form either glutamic acid or iso-glutamic acid

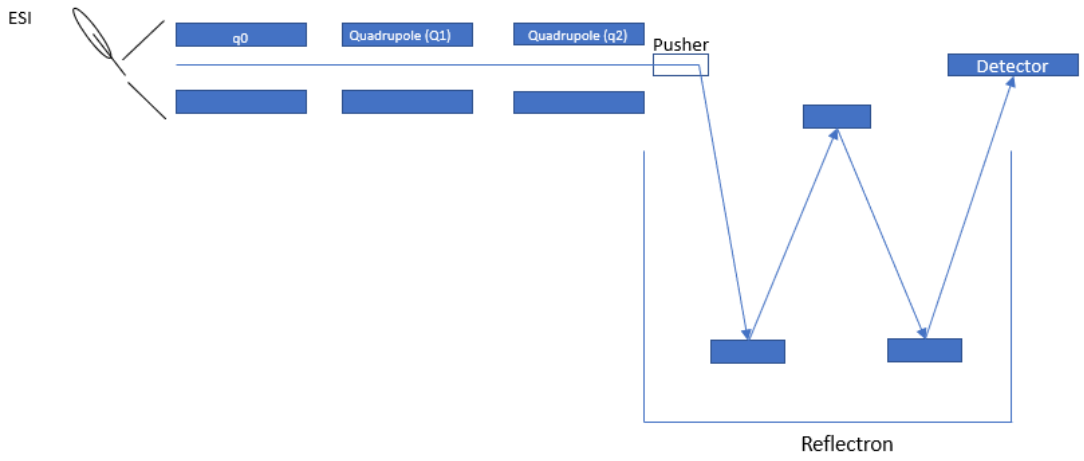


Figure 1.10: Schematic of a Quadrupole Time-of Flight Instrument. A general schematic of a Q-ToF instrument is shown with the q0, Q1, and Q2 quadrupoles coupled to a time-of-flight analyzer, consisting of a reflectron.

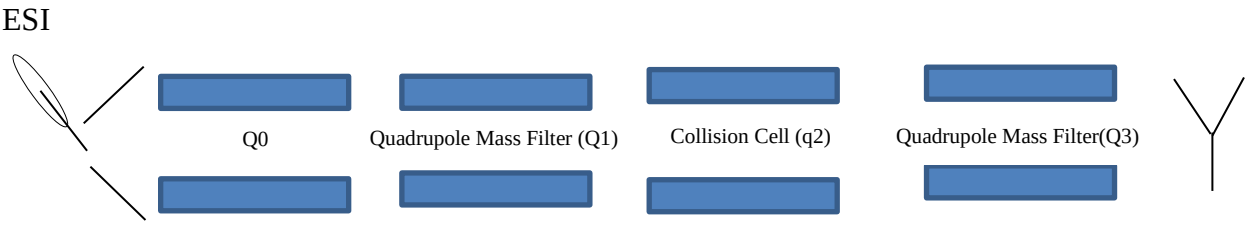


Figure 1.11: Schematic of a Triple quadrupole Instrument. A schematic depicting the set-up used for an SRM experiment where the ions are formed by electrospray ionization and transferred through the q0 to the first quadrupole(Q1). In Q1, the parent ions are filtered, and the selected parent ion is transferred to the collision cell (q2) where they are fragmented by collision induced dissociation. The fragments are transferred to the final quadrupole (Q3) and filtered prior to detection.

CHAPTER 2

N-LINKED GLYCAN RELEASE EFFICIENCY: A QUANTITATIVE COMPARISON BETWEEN NAOCL AND PNGASE F RELEASE PROTOCOLS

Fischler, David A. and Orlando, Ron. To be Submitted to *J. Biomol. Tech.*

Abstract:

There are several methods, both chemical and enzymatic, to release Asparagine(N)-linked glycans prior to structural characterization. One of the most common release methods uses the enzyme peptide: N-glycosidase (PNGase F), which is costly and time consuming. A less expensive and quicker alternative chemical procedure has been reported where sodium hypochlorite (NaOCl) is used to hydrolyze the peptide-glycan bond, yielding the intact glycan with a free reducing terminus.[1] In this study, liquid chromatography-selective reaction monitoring (LC-SRM) was used to quantitatively compare the efficiency of the NaOCl release protocol to that of the PNGase F procedure. These experiments demonstrate that the distribution of glycans released using the two different protocols is very similar; however, the absolute recovery of N-linked glycans was less than 20x lower with the chemical procedure.

Keywords:

Glycomics, N-Linked Glycans , Peptide:N-glycosidase (PNGase F), Release efficiency, Sodium Hypochlorite (NaOCl)

Introduction:

Glycosylation is a post-translational modification that affects both the structure and function of glycoproteins. The glycans attached to proteins are involved with a wide range of biological functions. Specifically, asparagine(N)-linked glycosylation has been demonstrated to be of great importance in the regulation of a protein's folding, structure, and stability as well as intracellular transport.[2-3] Due to the many roles that glycans play, the characterization of their structures is important. One step in the characterization of glycoprotein glycans involves their release from the peptide backbone. Released glycans can be structurally analyzed to identify changes in glycan structure/abundance that may be used for diagnosis of medical disorders, in a field called glycomics.[4-7] The released glycan libraries can also be used to construct micro-arrays to test for glycan binding proteins.[8] The ability to release glycans, plays a major part in understanding the overall biological importance of glycosylation.

The study of N-linked glycans relies on the ability to release the complete, intact glycans from their glycoproteins, and one of the oldest and most well-studied methods for the release of N-linked glycans is the chemical release technique hydrazinolysis.[6] Hydrazinolysis uses anhydrous hydrazine to cleave the intact glycan from the peptide backbone.[6, 9] However, utilizing hydrazine is a very toxic and volatile method. Besides the dangers, the use of hydrazine can also lead to unwanted modifications of the released glycans which need to be rectified post-release, such as loss of N-acetylation and the loss of the free reducing end.[6, 9-11] These drawbacks to the use of hydrazine explains why hydrazinolysis is not a commonly practiced release technique.

Enzymes have also been used to cleave N-linked glycans from their peptides. One class of enzymes are the endoglycosidases which cleave a glycosidic bond in the N-linked glycans. Although efficient, these enogycosidases are limited to the types of glycans they can cleave.

Endoglycosidase H (Endo H), which was one of the first commercially available endoglycosidases, only releases glycans that are high mannose or hybrids.[12] Endo F (usually sold as a combination of Endo F1, F2, and F3) cleaves high mannose and hybrids like Endo H (F1 and F2), while Endo F (F3)is also capable of releasing bi- and tri-antennary complex glycans.[13-16] In addition to the limitations imposed by the type of glycans cleaved, another issue arises from the inability to release complete, intact glycans since these enzymes cleave an internal glycosidic bond which could make characterization of glycans containing a core fucose difficult, as the fucose would remain on the n-acetylglucosamine(GlcNAc) attached to the peptide. Despite the limitations the use of endoglycosidases is still a utilized glycan release method.

Digestion with a peptide:N-glycosidase (PNGase), such as peptide:N-Glycosidase F (PNGase F) and glycopeptidase A (PNGase A), is one of the most commonly utilized methods for the release of N-linked glycans as these enzymes are able to release intact N-linked glycans. Unlike endoglycosidases which cleave the glycosidic bond between two sugars, these enzymes hydrolyze the amide linkage between the core GlcNAc and the asparagine residue of a glycoprotein. PNGase A is less commonly used but, has the ability to cleave high mannose, hybrid, and short complex glycans, with the additional ability to cleave glycans containing a 1,3-core fucose.[17-19] Although a drawback to PNGase A is the inability to release larger, more complex glycans, such as those containing a sialic acid residue.[19-22] PNGase F, similar to PNGase A, keeps the peptide backbones intact and is capable of cleaving nearly all N-linked glycans.[14, 23-25] The one exception is that PNGase F is unable to cleave 1,3-core fucosylated species (usually found in plants).[26] Enzymes such as PNGase F and PNGase A are expensive and time consuming; however, the simplicity and a lack of unwanted reactions has led to the release of glycans with a PNGase to become the preferred method.

A chemical release technique using the sodium hypochlorite (NaOCl) in bleach was recently reported in the literature and suggested as an alternative to PNGase F for the large scale processing of N-linked glycans.[1] Song et. al. demonstrated that the NaOCl chlorinates the glycan-peptide amide bond forming a N-chloroamide. A pericyclic reaction leads to a glycan-isocyanate intermediate, which is further hydrolyzed yielding the intact glycan with a free reducing terminus.[1] The protocol established by Song et. al. is much cheaper and quicker than using PNGase F. The researchers demonstrated that the MALDI-TOF-MS profiles obtained when the NaOCl protocol was used to release N-linked glycans from standard glycoproteins were the same as those obtained using the traditional enzymatic release without unwanted modifications. Here, we evaluated if the NaOCl approach could provide similar results to the PNGase F release on a smaller, non-preparative scale. These comparisons were made on the N-linked glycans released from bovine fetuin using either the NaOCl or PNGase F procedures.

Materials and Methods:

Sample Preparation

Fetal bovine fetuin (1.8mg) (Sigma Aldrich, St. Louis, MO) and 180µg of a maltohexaose (6 GU) standard (Sigma Aldrich, St. Louis, MO) were re-suspended in 600µL of H_2O. Six equal fractions (Three replicates for each preparation) were aliquoted and dried. The glycans were then released following the assigned protocols.

PNGase F: Each sample was re-suspended with 15µg (20:1 (w/w)) of TCPK treated trypsin (Sigma Aldrich, St. Louis, MO) in 60µL of 100mM ammonium bicarbonate buffer. The samples were incubated overnight at 37°C. The samples were then heated for five minutes at 100°C to deactivate the trysin. 1µL(500units) of PNGase F (New England Bio., Ipswich, MA) was then added to the samples, and the samples were incubated for another 12 hours at 37°C. The PNGase

F samples were then cleaned using a C18 extraction column (Avantor Performance Materials, Center Valley, PA) and dried.

NaOCl release[1]: Samples were re-suspended in 15µL of H_2O(20mg/mL), and 2.18µL of Clorox bleach (6.85% NaOCl) was added while stirring. The samples were stirred for 15 minutes at room temperature. Formic acid (0.5µL) was then added to quench the reaction, and the samples were stirred for an additional five minutes. The samples were then spun down on a benchtop microcentrifuge. The supernatant was collected for each sample and dried. The samples were then cleaned on a PD MiniTrap® G10 desalting column (GE Healthcare, Chicago, IL) and a C18 extraction column (Avantor Performance Materials, Center Valley, PA) before being dried.

The samples, from both the chemical and enzymatic release protocols, containing the released N-linked glycans of fetuin, were re-suspended in 20µL of H_2O, and 30µL of procainamide (ProA) labelling solution (400mM procainamide HCL and 1M sodium cyanoborohydride in 70/30 DMSO/acetic acid (v/v)) was added to each sample. The samples were incubated overnight at 37°C and then diluted with 250µL of H_2O before being cleaned on a PD MiniTrap® G10 desalting column (GE Healthcare, Chicago, IL). All samples containing the ProA-labelled N-linked glycans were dried.

LC-MS/MS Settings and Instrumentation

Data was acquired on a Nexera LC-30AC LC System (Shimadzu, Columbia, MD) that was coupled to a 4000 Q Trap MS/MS System (Applied Biosciences, Framingham, MA). The mobile phase consisted of solvents A (94.9%H_2O, 5% acetonitrile (ACN), 0.1%formic acid, and 50mM ammonium formate) and B (100% ACN). All solvents were HPLC grade (Sigma Aldrich, St. Louis, MO). A gradient elution was run from 68%B to 52%B over 16 minutes on a Halo® Penta-HILIC, 2.1X150mm with 2.7µm pore size, (Advance Material Tech, Wilmington, DE) at a

flow rate of 0.4mL/min with the column being heated to 60°C. A selected reaction monitoring (SRM) method was set up where retention times were scheduled based on previous data. For the SRM, a total of six ProA-labelled glycan transitions were scheduled (Table 2.1): a maltohexaose standard (6GU), a bi-antennary, mono-sialylated complex glycan (A2G2S1); a bi-antennary, bi-sialylated complex glycan(A2G2S2); a tri-antennary, bi-sialylated complex glycan (A3G3S2); a tri-antennary, tri-sialylated complex glycan (A3G3S3); and a tri-antennary, tetra-sialylated complex glycan (A3G3S4).[27] All samples were re-suspended in 25µL of H_2O. The sample (12µL) was mixed with 48µL of ACN and 15µL were injected for each run. Each replicate was run in triplicate.

For the glycan degradation analysis, a SRM was also performed, and transitions representing losses of either sialic acids, GlcNAc, mannose, Galactose, or any combination of these monosaccharide losses were investigated.

Data Analysis of SRM

All of the peaks of the HILIC-SRM traces representing the five scheduled glycans along with the peak representing the scheduled internal standard were integrated using Analyst® 1.5 software (Applied Bioscience, Framingham, MA).

Results and Discussion:

A quantitative comparison of the two different N-linked glycan release techniques was performed to gauge the effectiveness of the chemical, NaOCl, technique on a scale more suitable for glycan analysis. Samples were analyzed by LC-SRM to give information on both the glycans' structures as well as their absolute quantities. An analysis of the relative comparison of the released glycans for each method was first done to compare the glycan profile obtained using

the two methods. In addition, an internal standard was utilized so the absolute recovery of all the released glycans combined could be compared amongst the two release techniques.

The LC-SRM analysis produced chromatographic traces of the five most abundant, highly-sialylated, glycans (Figure 2.1). Although the 6GU standard was represented by a single peak the N-linked glycans were observed as multiple peaks. This peak-splitting is due to the branching of sialic acids. Sialic acids can be bound to galactose in either an alpha(2,3) or an alpha(2,6) linkage. As observed in the LC-MS traces in Figure 2.1, the number of peaks represented in the SRM trace is the number of sialic acids present in the glycan plus 1. The only exception of the five analyzed glycans is A3G3S4, where two of the sialic acids are locked into either their linkage position. With the LC-SRM traces acquired the abundance of each glycan could be measured for both relative and absolute quantitation.

A glycan profile was produced by calculating the relative abundance of the N-linked glycans. In order to determine a glycan profile for each technique, the sum of the abundance for each glycan was divided by the sum of the abundance from all N-linked glycans for that replicate and multiplied by 100. The relative percent of all N-linked glycans for all replicates were averaged for both release techniques. The difference in the relative abundance of the five fetuin N-linked glycans between the two different release protocols were within the experimental error of the measurement. Additionally, no evidence of sialic acid loss was observed (Figure 2.2). For this small scale analysis, the relative quantitation of the NaOCl technique appeared to be comparable to the use of PNGase F.

The comparison of the relative quantitation of N-linked glycans described above demonstrates similar results to the published protocol, but a deeper investigation into the absolute recovery of glycans was performed. The absolute recovery of N-linked glycans was

calculated by the sum of the abundance of all five released glycans from a single replicate being divided by the ion counts of the peak representing the 6GU for that replicate. An average of the ratios for each replicate was calculated for each of the release techniques. The average ratios were 3.12 and 0.14 for the PNGase F protocol and NaOCl release protocol respectively (Figure 2.3). These calculations corroborate the conclusions that can be drawn from the LC-SRM traces obtained by glycans released from the two release protocols where the recovery of glycans released by the two methods was significantly different (Figure 2.4). The absolute recovery of glycans was greater than 20x smaller for the NaOCl technique.

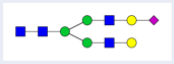
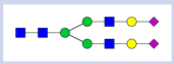
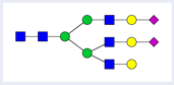
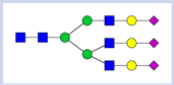
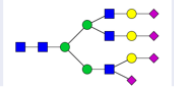
A possible reason for the reduction in N-linked glycan recovery in the NaOCl protocol could be degradation, potentially via a base peeling reaction due to the high pH used in this release. Losses of a GlcNAc residue on the reducing terminus were observed by LC-SRM analysis of the NaOCl released glycans, but these amounts were less than 10% in abundance compared to intact, released glycans (Figure 2.5). Similar LC-SRM analysis of a PNGase F released glycans did not contain peaks corresponding to these degradation products. Further base peeling degradation products were not observed by LC-SRM analysis. While the degradation products of base peeling did lead to minimal sample reduction, the level of these degradation products does not correspond to the large difference determined in the absolute recovery discussed in the preceding paragraph.

The NaOCl release protocol was published for large scale processing of N-linked glycan release, and was shown to release the same N-linked glycans as the more commonly applied PNGase F approach.[1] Although the overall glycan profile of fetuin using the NaOCl protocol showed no significant difference compared to the fetuin glycan profile from PNGase F release, the absolute recovery of glycans was more than 20x smaller from the NaOCl protocol. While the

NaOCl procedure is much cheaper and quicker, the low recovery of glycans using the NaOCl procedure implies that this approach is not suitable with small amounts of sample.

Tables and Figures:

Table 2.1: Nomenclature, Structure, and Scheduled Transitions for ProA-labelled N-linked glycans of Fetuin and the ProA-labelled 6GU standard

Glycan Nomenclature	Glycan Structure	Parent Ion	Fragment Ion	Retention Time (min)
6GU		1210.7	399.7	30.7
A2G2S1		1076.5	440.8	36.5
A2G2S2		1222.2	440.8	42.5
A3G3S2		1259.1	440.8	40.5
A3G3S3		1550.0	440.8	51.0
A3G3S4		1695.7	440.8	55.0

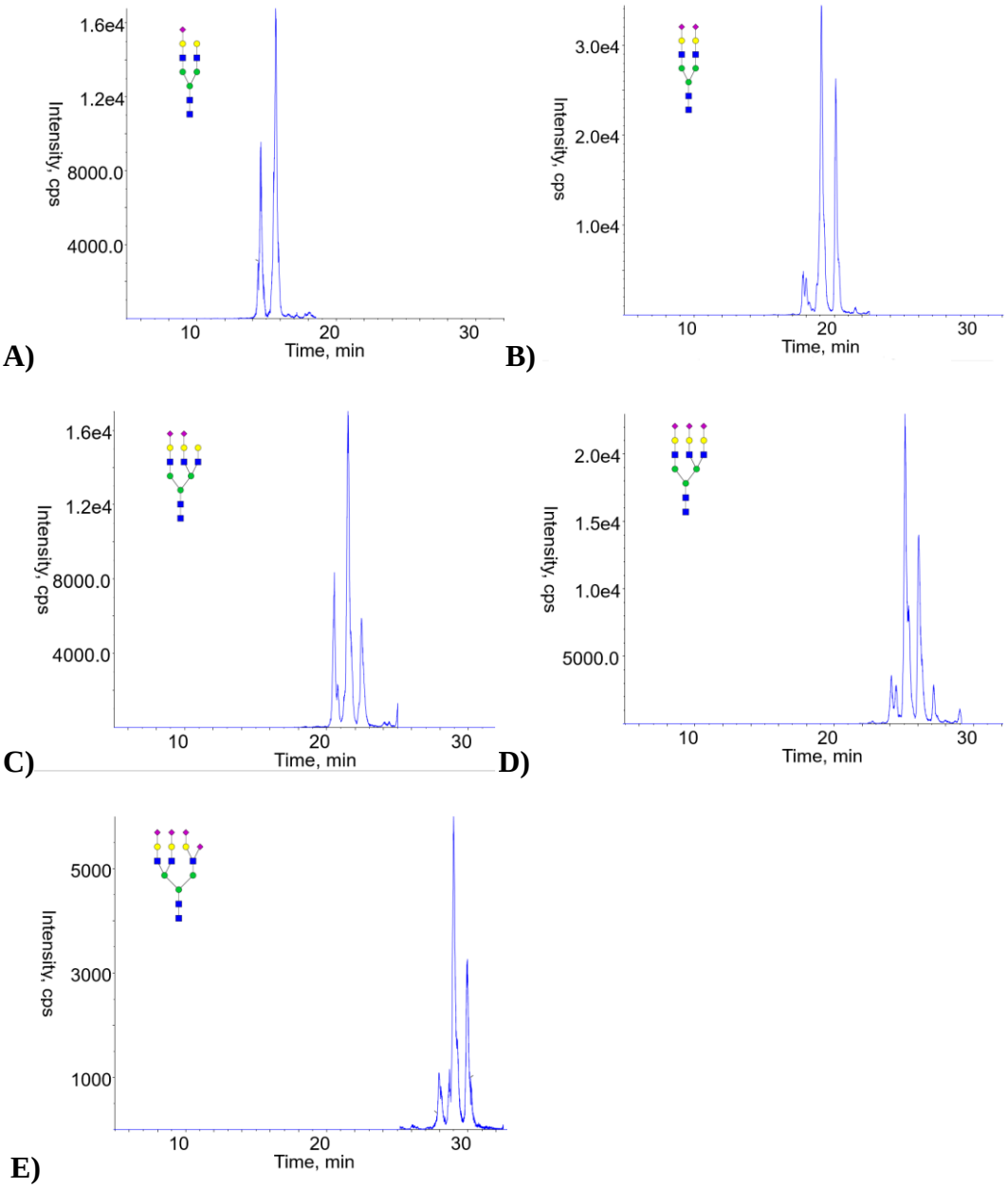


Figure 2.1: SRM Traces of Highly Sialylated N-Linked Glycans of Fetal Bovine Fetuin. A representative XIC trace of each of the five most abundant N-linked glycans (**A**:A2G2S1, **B:** A2G2S2, **C:** A3G3S2, **D:** A3G3S3, and **E:** A3G3S4) demonstrating peak-splitting due to the possible alpha(2,3) or alpha(2,6) linkage of a sialic acid residue.

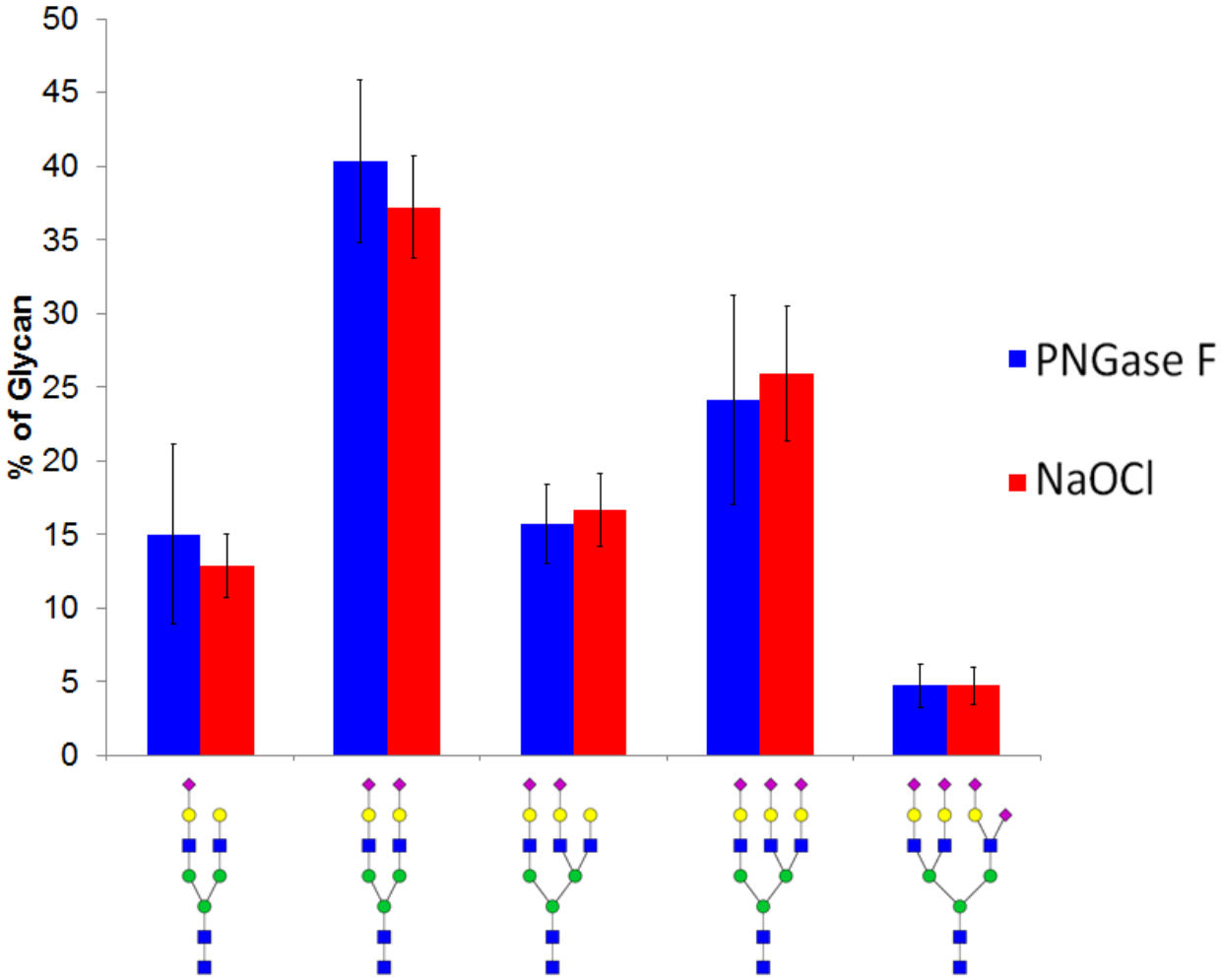


Figure 2.2: Glycan Composition Analysis: PNGaseF vs. NaOCl. The relative glycan profile for each replicate was taken for the 5 most abundant glycans of fetuin. The technical replicates for each were averaged, and the averages for the biological replicates were averaged.

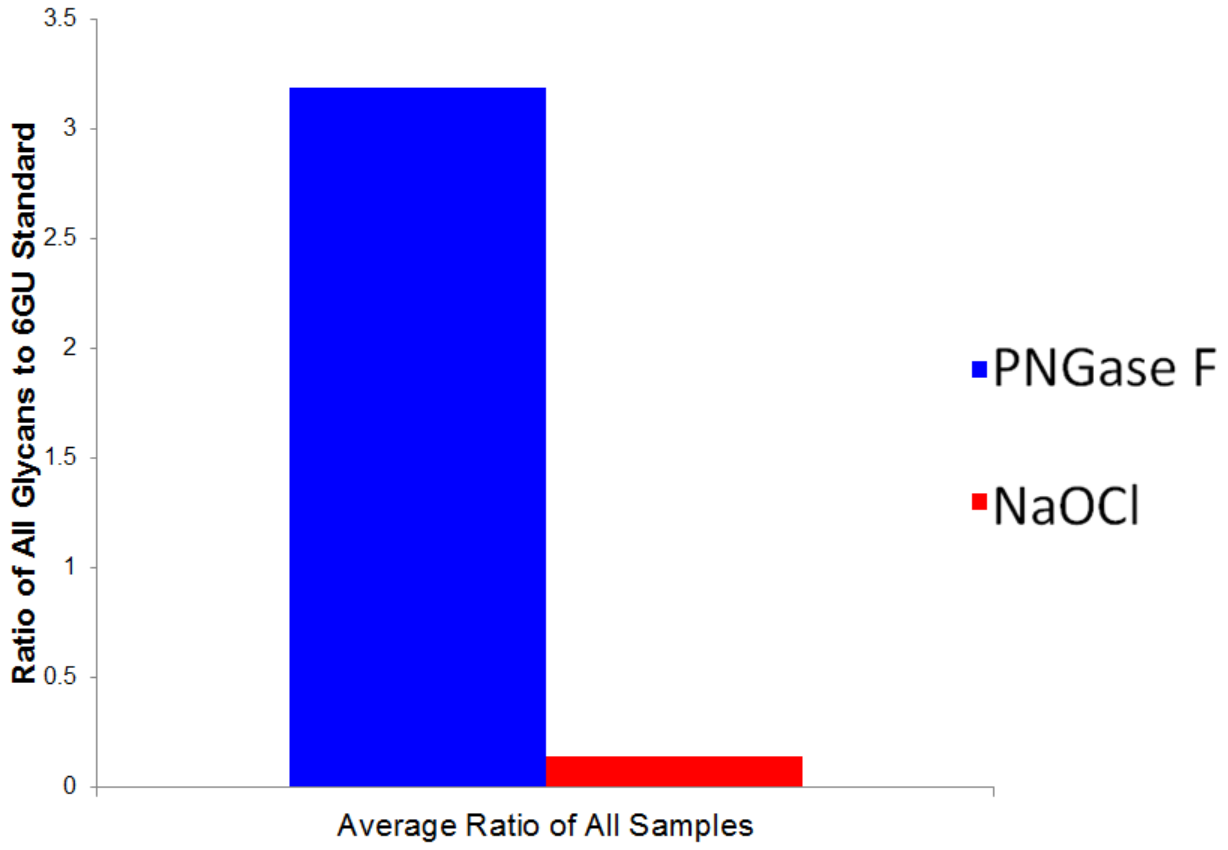


Figure 2.3: Recovery of Glycans compared to the 6GU Standard. The total abundance of the 5 analyzed glycans in a single run were combined and divided by the abundance of the internal standard for that run.

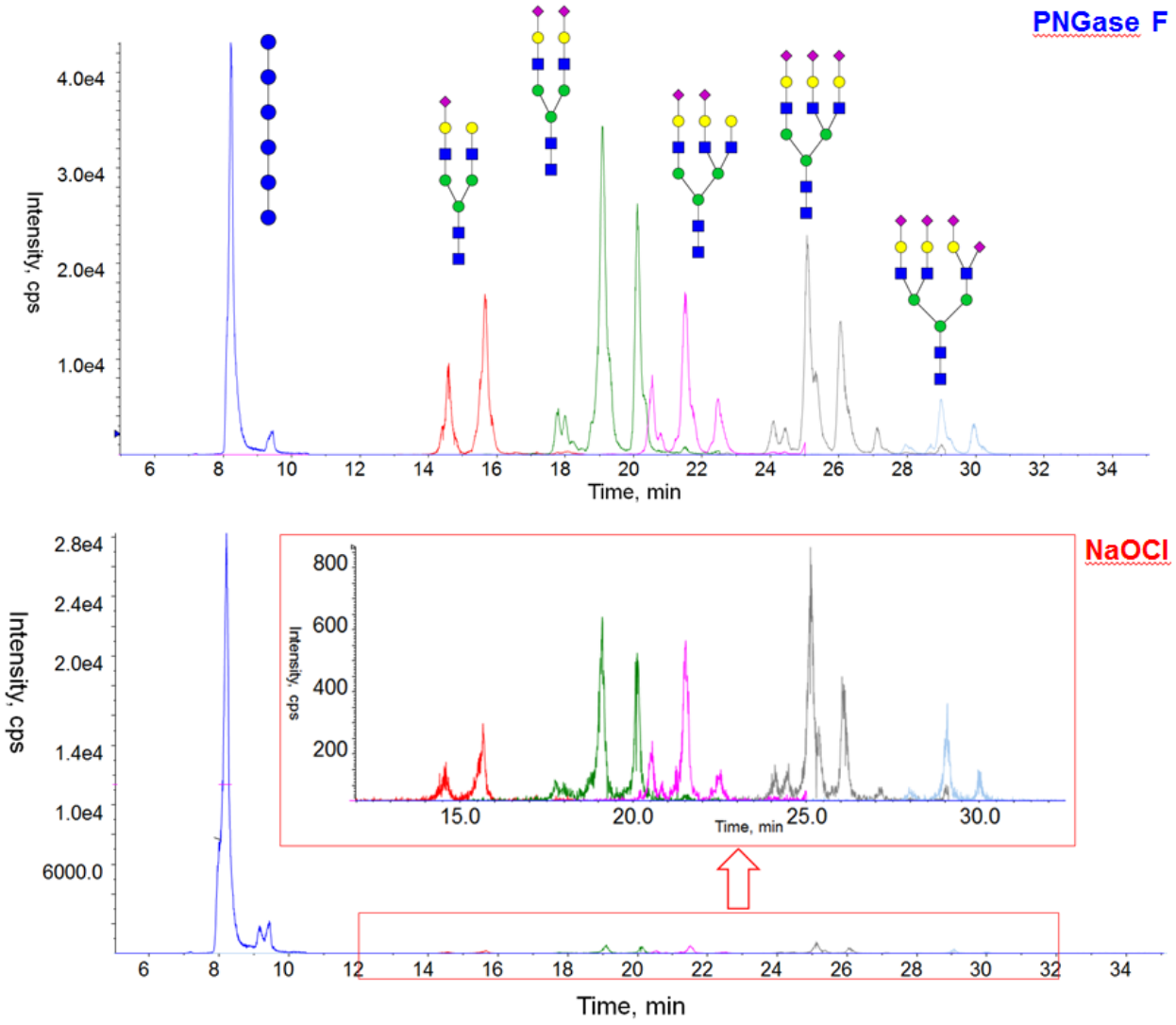


Figure 2.4: SRM LC Traces of the 6GU standard and the N-linked glycans from fetuin released using the PNGase F and NaOCl protocol. LC-SRM traces of the 5 most abundant glycans of fetuin (A2G2S1 (Red), A2G2S2 (Green), A3G3S2 (Pink), A3G3S3 (Gray), and A3G3S4 (Light Blue)) and 6GU(Dark Blue) from LC-SRM analyses following the PNGase F release (Top) and NaOCl release (Bottom) protocols.

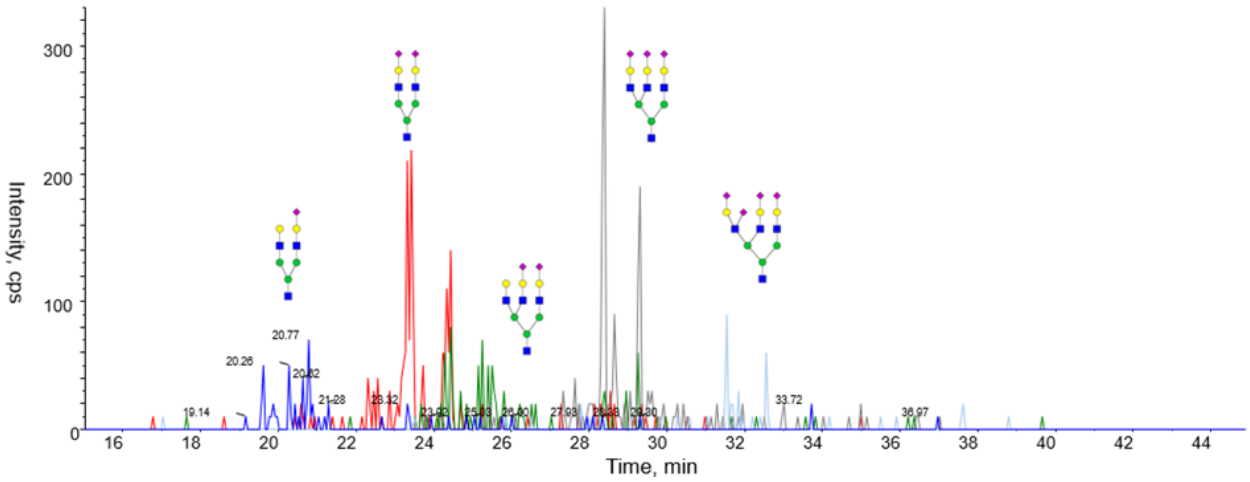


Figure 2.5: Loss of GlcNAc residue on the Reducing ends following NaOCl release of N-linked Glycans. A representative LC-SRM trace of the 5 most abundant glycans minus a GlcNAc residue (A2G2S1 (Dark Blue), A2G2S2 (Red), A3G3S2 (Green), A3G3S3 (Gray), and A3G3S4 (Light Blue)) is demonstrated following the LC-SRM analysis on the NaOCl release sample.

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CHAPTER 3

THE EFFECTS OF THE TRYPSIN DIGESTION CONDITIONS ON THE DEAMIDATION RATES OF ASPARAGINE RESIDUES OF IMMUNOGLOBULIN G (IGG) PEPTIDES

Fischler, David A. and Orlando, Ron. To be Submitted to *J. Biomol. Tech.*

Abstract:

The Deamidation of the side chains of asparagine (Asn) is a well-studied post translational modification. The amine group on the side chain is replaced by a hydroxyl group. One of the products of deamidation of Asn is an iso-aspartic acid (i-Asp) residue, which adds an additional -CH_2- moiety to the peptide backbone. This modification can alter the secondary structure, conformation, and function of the protein. Deamidation of Asn residues is often detected after trypsin digestion, and in instances where products from this reaction are seen they are presumed to have been present in the native protein. The pH used for digestion can lead to deamidation, which can increase the difficulty in the quantitation of the level of deamidation products in the intact protein. The rate of Asn deamidation under tryptic digest conditions was evaluated in this research to see if the digest conditions could cause the detection of erroneously high values for protein deamidation products.

Keywords:

Post Translational Modifications (PTM), Deamidation, Isomerization, Asparagine, Trypsin, Immunoglobulin G (IgG).

Introduction:

The deamidation of the side chain of asparagine (Asn) is a well-known and well-studied post translational modifications (PTM). The amide of the residues' side chain is replaced with a hydroxyl group leading to a 1 Da increase in the mass (Figure 3.1). This reaction results in the formation of either normal aspartic acid (n-Asp) or iso-aspartic acid (i-Asp).[1-2] The i-Asp residue produced in deamidation, or by the isomerization of n-Asp, produces an additional -CH_2- moiety in the peptide backbone, which can have major repercussions on the secondary structure and conformation of a protein. [3-4] Further biological consequences stemming from the additional -CH_2- in the peptide backbone can include protein unfolding and the alteration of a protein's function.[5-6] Additionally, the increased levels of i-Asp have also been linked to the onset of Alzheimer's disease and other neurological disorders.[7-10] The presence of i-Asp formation in a biotherapeutic is a major concern in the pharmaceutical industry since the presence of i-Asp can affect a drug's efficacy and is known to be immunogenic.[11-15] The ability to accurately quantify the amount of i-Asp is of utmost importance.

The possible formation of i-Asp from the isomerization of n-Asp was investigated. Two peptides were investigated which each contained a likely site of the isomerization of n-Asp. Neither peptide demonstrated detectable i-Asp formation during these experiments. The formation of i-Asp from the isomerization of n-Asp is slow enough that the isomerization of n-Asp does not affect the accurate quantitation of the deamidation products of Asn.

Asn-containing peptides containing at least one Asn followed by a glycine (Gly) residue on the c-terminus of Asn were analyzed for the quantitation of the deamidation products. The -Asn-Gly- motif was chosen due to the fact most of the common sites of deamidation, including an Asn followed by either a Gly, a serine, a threonine, or a n-Asp have been well-studied with

the most likely site being the Asn-Gly motif based on size and steric hindrance.[16] Three peptides with possible site(s) of deamidation consisting of the Asn-Gly motif will be the focus of this study.

The identification and accurate quantitation of the products of Asn deamidation in intact proteins provides a challenge for mass spectrometry. In mass spectrometry, the mass of both i-Asp and n-Asp are the same making the accurate quantitation of both i-Asp and n-Asp independent of each other difficult. However, utilizing Hydrophilic Interaction Liquid Chromatography (HILIC), the baseline separation of the unmodified peptide containing Asn, the same peptide containing n-Asp, and the same peptide containing i-Asp is possible.[17] Utilizing HILIC, coupled to mass spectrometry, allows for the accurate quantification of the abundances of i-Asp and n-Asp independently.

The deamidation of asparagine residues is often detected after trypsin digestion, and when products from deamidation are seen they are often presumed to have been present in the native protein.[18] However, the pH used for digestion can facilitate deamidation thereby making the quantitation of the abundance of deamidation products in the intact protein difficult. Here, the rate of deamidation of Immunoglobulin G (IgG) peptides undergoing tryptic digestion were evaluated using HILIC-Selective Reaction Monitoring (HILIC-SRM) to determine if the digestion conditions could cause erroneously high values.

Materials and Methods:

Sample Preparation

IgGs (4.2mg), isolated from human serum (Sigma Aldrich, St. Louis, MO) using a protein G column (GE), were mixed with 2.1nmol [Glu^1]-Fibrinopeptide B human (Glu-Fib) (Sigma Aldrich, St. Louis, MO) and re-suspended in 600µL of H_2O. Six equal fractions (Three

trials for each pH condition) were aliquoted and dried. Three fractions were utilized for pH 8.0 and three fractions were utilized for pH 6.0.

pH 8.0:

Each sample was resuspended in 160µL of 100mM ammonium bicarbonate (Sigma Aldrich, St. Louis, MO) (pH=8.0) and 200µL of 10mM DL-dithiothreitol (Sigma Aldrich, St. Louis, MO) in 100mM ammonium bicarbonate was added. The samples were incubated at room temperature for 1 hour. 55mM iodoacetamide (Sigma Aldrich, St. Louis, MO) in 100mM ammonium bicarbonate was added, and the samples were incubated 1 hour at room temperature in the dark. An aliquot of 14µg (50:1, protein: trypsin) of trypsin (Promega, Madison, WI) was then added to each vial, and the samples were incubated at 37°C. At each time point (0.5 hour, 1 hour, 2 hours, 4 hours, 10 hours, 15 hours, and 22 hours) a 100µL aliquot was removed and frozen immediately in a dry ice/acetone bath. The samples were then lyophilized.

pH 6.0:

The same procedure was followed as the pH 8.0 samples with the exception that the 100mM ammonium bicarbonate was replaced by 50mM ammonium acetate (pH=6.0).

Liquid Chromatography-Mass Spectrometry (LC-MS) Settings and Instrumentation

Data was acquired on a Nexera LC-30AC LC System (Shimadzu, Columbia, MD) that was coupled to a 4000 Q Trap MS/MS System (Applied Biosciences, Framingham, MA). The mobile phase consisted of solvents A (94.9%H_2O, 5% acetonitrile (ACN), 0.1%formic acid, and 50mM ammonium formate) and B (100% ACN). All solvents were HPLC grade (Sigma Aldrich, St. Louis, MO). A gradient elution was run from 78%B to 62%B over 20 minutes on a Halo® Penta-HILIC, 2.1X150mm with 2.7µm pore size, (Advance Material Tech, Wilmington, DE) at a flow rate of 0.4mL/min with a column temperature of 60°C.

A selected reaction monitoring (SRM) method was set up where retention times were scheduled based on previous data for several peptides that should or should not contain possible sites of deamidation (Supplementary Table 3.S1). All samples were re-suspended in 35µL of 70% ACN, 30%, H_2O, 0.1%FA. A sample of 15uL was injected for each run.

Data Analysis of HILIC-SRM

Peaks representing all scheduled IgG tryptic peptides and the peak representing the scheduled internal standard (Glu-Fib) were integrated using Analyst® 1.5 software (Applied Bioscience, Framingham, MA) to determine the abundance of each peptide. Each peptides' abundance was divided by the abundance of the internal standard Glu-Fib for that replicate, giving a Relative Response (RR).

Kinetic Modelling Software

An additional method of analysis of deamidated peptides was the kinetics modelling simulator Tenua. Tenua is an open-source chemical kinetics simulation program based on KINSIM by Barshop, Wrenn, and Frieden.[19]

Results and Discussion

The goal of this project was to determine the rates of deamidation of Asn residues as well as the rate of trypsin digestion to determine if the deamidation of Asn residues can occur during the digestion. A trypsin digestion was performed on IgG proteins at pH 8.0, and the resulting tryptic peptides with Asn, i-Asp, and n-Asp were analyzed by hydrophilic interaction liquid chromatography-selective reaction monitoring (HILIC-SRM) at various time points to evaluate how their abundances changed over time. A similar digestion experiment and kinetics analysis was performed where the conditions of the trypsin digestion were at pH 6.0 as this pH is known to minimize deamidation of Asn residues.

The relative response (RR) of each analyzed peptide was calculated by comparing each peptide's abundance to an internal standard, [Glu^{1}]-Fibrinopeptide B human (Glu-Fib). Using RR provides the ability to compensate for run-run variations in instrument performance since both the analyte peptide and internal standard are detected in the same LC-SRM experiment.

Trypsin digestion of a protein follows pseudo first-order kinetics. The slope of a natural log versus RR plot for a first-order reaction corresponds to the rate constant (k) for the reaction, in this case the k for the production of each tryptic peptide. The HILIC-SRM data of three peptides (EPQVYTLPPSR, TTPPVLDSDGSFFLYSK, FNWYVDGVEVHNAK) that do not contain a likely site(s) of deamidation were investigated, and ln (RR) versus time plots were created for each peptide (Figure 3.2). The early time points(<10hours) of these plots have a linear trend which is expected for a first-order reaction. The slopes of the inclines at these early time-points were measured to determine the rate of peptide production for each of the three replicates of each peptide. The k of all three replicates of a single peptide were averaged for each peptide (Table 3.1). The data suggests that the rates of peptide production for these three peptides are on average 0.23 hr^{-1}. The lack of change in the RR for these three peptides after the 10 hour time point, suggests that the digestion is complete as these peptides are no longer being produced.

Three IgG peptides (VVSVLTVLHQDWLNGK, VVSVLTVVHQDWLNGK, and GFYPSDIAVEWESNGQPENNYK) that contain probable sites of Asn deamidation were also investigated. In the HILIC-SRM traces of the three peptides, the abundance of both deamidation products increased with digestion time. As demonstrated by the HILIC-SRM traces of the peptide VVSVLTVLHQDWLNGK (Figure 3.3), there is only one peak in the HILIC-SRM trace that corresponds to the unmodified peptide at the earliest time point (0.5 Hours). After 4 hours of

digestion peaks corresponding to the deamidation products of the native peptide (i.e. Asn to either n-Asp or i-Asp) were detected. The HILIC-SRM peaks corresponding to the deamidation products are even more prominent after 22 hours of trypsin digestion. The increased level of deamidation products as the length of digestion increases suggests that the conditions used for the digestion cause deamidation of Asn.

The RR of each product peptide was averaged for each time point for the three peptides that undergo deamidation (Figure 3.4). As demonstrated in Figure 3.4, two of the peptides (VVSVLTVLHQDWLNGK and VVSVLTVVHQDWLNGK) had no detectable deamidation products at the earliest time point (0.5 hours), and the lack of deamidation at the early time point suggests that these Asn residues had not undergone deamidation in the native protein. One peptide (GFYPSDIAVEWESNGQPENNYK) contained deamidation products at the earliest time-point (0.5 hours) suggesting that the site had undergone partial deamidation in the native protein. These results suggest that the conditions of a trypsin digestion(pH=8.0) can lead to elevated levels of deamidation products.

The formation of i-Asp can also arise from the isomerization of n-Asp. In order to investigate if the formation of i-Asp is attributable to the formation of i-Asp from deamidation of Asn rather than the isomerization of n-Asp, two possible sites of n-Asp isomerization (consisting a n-Asp-Gly motif) were investigated. The HILIC-SRM traces of both peptides (FNWYV**D**GVEVHNAK and TTPPVLDS**D**GSFFLYSK) demonstrated that i-Asp was not detectable after 22 hours of trypsin digestion (Figure 3.5). The lack of i-Asp suggests that the rate of isomerization of n-Asp to i-Asp is slow enough that isomerization did not cause interconversion of the deamidation products.

A kinetic analysis of the deamidation of Asn residues during a trypsin digest has challenges. For a proper kinetics analysis, the mass spectrometer should be set up to optimally detect both the native and deamidated peptide products. For the quantitation of the formation of the deamidation products and the consumption of the Asn-containing peptide, SRM experiments were performed to measure the abundance of the three product peptides. The same transition was utilized for the three product peptides for the SRM experiments. Since the mass difference of the native peptides and the deamidated peptides differ by 1Da, the detection efficiency of fragment ions could differ greatly between the native and deamidated products. This possible limitation of the SRM technique used here could create a challenge for the quantitation of the deamidated peptides and a thorough kinetics study.

The kinetics of peptide deamidation were analyzed by natural log versus time plots. The natural log of the RR versus time after the addition of trypsin was plotted, for three peptides which contained a site of deamidation of the Asn residue, beginning with the 10-hour time point (Figure 3.6). Data obtained after the 10-hour time point was used to simplify the analysis since the trypsin digestion is not complete before this time point. The slopes of a natural log of the RR versus time plot for a first order reaction, such as deamidation, correspond to the k. For the deamidation reaction, a rate constant can be obtained for both the rate of formation of the deamidated products and for the rate of consumption of the Asn-containing peptides. The rate of consumption of the reactant should be equivalent to the sum of the rates of formation of the deamidated products. However, that expectation was not observed in the natural log versus time plots (Table 3.2). A possible explanation is that the second mass analyzer was not set to pass the proper fragment ion and thus the detection efficiency of the deamidated species was significantly lower than the native species.

The experimental data was further modeled in the kinetic simulator Tenua (Figure 3.7). The modelling produced values of k for the formation of the deamidated products. As expected, the values of k determined from the modelling software were consistent with those obtained from the ln (RR) versus time plots (Table 3.2). Additionally, the models for each of the three peptides fit reasonably well (Figure 3.8). The peptide GFYPSDIAVEWESNGQPENNYK was the weakest fit of the three peptides. The weak fitting of the GFYPSDIAVEWESNGQPENNYK peptide was likely due to the existence of a second possible, albeit much slower, site of deamidation on the peptide GFYPSDIAVEWESNGQPENNYK.

A trypsin digest with a lower pH (pH=6.0) was investigated after attributing significant deamidation to the conditions(pH=8.0) of the trypsin digestion since the rate of deamidation is known to decrease with pH. No deamidation products were observed for two of the three peptides previously analyzed after 22 hours of trypsin digestion at a pH of 6.0 in the HILIC-SRM traces (Figure 3.9). The lack of deamidated products suggests that the decrease in the pH of the digestion conditions slows the deamidation of Asn significantly. The peptide GFYPSDIAVEWESNGQPENNYK had detectable peptides with n-Asp and i-Asp under these conditions after 0.5 hours; however, there was no detectable increase in the abundance of the n-Asp and i-Asp containing peptides after 22 hours (Figure 3.10). The presence of deamidated peptides at the initial (0.5 hours) time point further supports that deamidation is present at this site in the intact protein. Performing the digestion at a pH of 6.0, resulted in no further deamidation on the peptide GFYPSDIAVEWESNGQPENNYK during the digestion. For all three peptides, the rates of deamidation of Asn are slow enough that the deamidation attributable to the digestion conditions are no longer present in the HILIC-SRM traces after 22 hours of trypsin digestion.

The rates of peptide production during the trypsin digestion at pH 6.0 were also investigated. If the rate of peptide production is significantly slower and requires increased incubation times, the solution of lowering the pH may not be beneficial. An identical analysis was performed on the same three peptides, from the initial rate of trypsin digestion analysis, which lacked possible site(s) of deamidation (Table 3.1). The data suggests that the rates of the peptide production are significantly faster when the digestion is performed at a pH of 6.0. Since lowering the pH of the digestion slows down deamidation and increases the speed of proteolysis, the lower pH is a beneficial alteration to a trypsin digestion protocol.

The possible role trypsin digestion conditions could play in the deamidation of Asn residues was investigated. This study concluded trypsin digestion of an intact protein at a pH 8.0 can cause deamidation of Asn residues. Increasing the incubation time resulted in a larger the abundance of unwanted, deamidated products. These conclusions suggest that potentially inaccurate quantitation of Asn deamidation products on native proteins following a bottom-up analysis is of real concern. However, altering the pH during a trypsin digestion demonstrated the possibility of halting the deamidation process during the trypsin digestion. The ability to curtail the effects of deamidation that come from the conditions of the trypsin digest would allow the presence of n-Asp and i-Asp, when they are native to the complete intact protein, to be properly identified and quantitated.

Tables and Figures:

Table 3.1: Reaction Coefficients (k) of Trypsin Digestion for pH 6 and pH 8.

P-value <0.05 for all peptides between pH 6.0 and pH 8.0

Peptide	k at pH 6.0 (hr^{-1})	k at pH 8.0 (hr^{-1})
EPQVYTLPPSR	**0.96 ± 0.03**	**0.23 ± 0.03**
FNWYVDGVEVHNAK	**0.62 ± 0.08**	**0.26 ± 0.01**
TTPPVLDSDGSFFLYSK	**0.82 ± 0.08**	**0.19 ± 0.02**

Table 3.2: Rates of Deamidation formation (n-Asp and i-Asp) and Consumption (Asn) for Asn-Containing Peptides by Software Modeling (Tenua) and ln (RR) vs Time Plots.

Peptide	**Rate Constant (k) from Tenua (hr^{-1})**	**Rate Constant from ln (RR) vs. Time (hr^{-1})**
VVSVLTVLHQDWLNGK		
n-Asp	0.04	0.04
i-Asp	0.06	0.06
Asn		0.06
VVSVLTVVHQDWLNGK		
n-Asp	0.06	0.06
i-Asp	0.05	0.05
Asn		0.09
GFYPSDIAVEWESNGQPENNYK ('NG' Motif)		
n-Asp	0.04	0.05
i-Asp	0.09	0.10
Asn		0.01

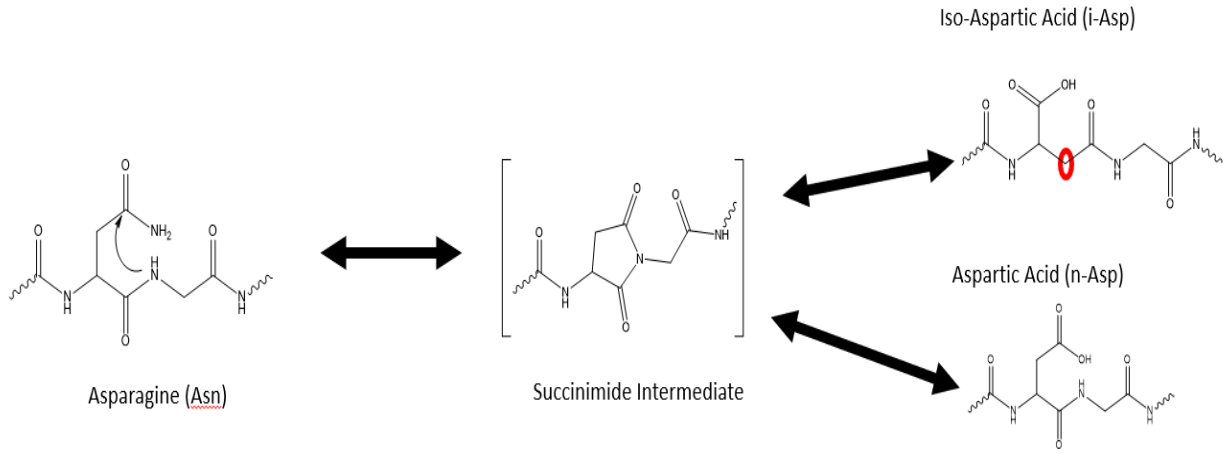
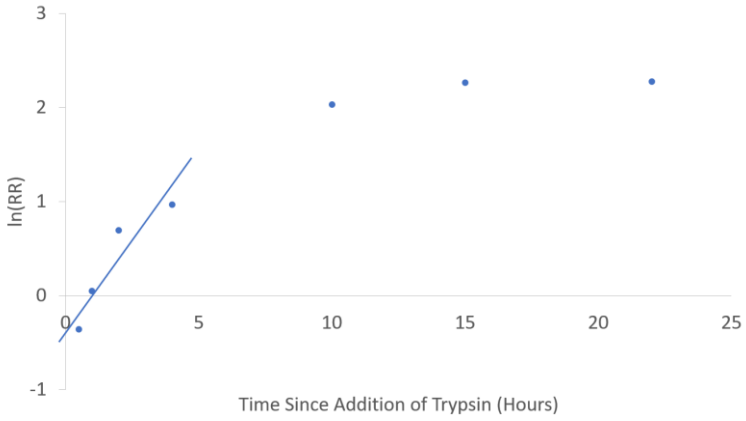
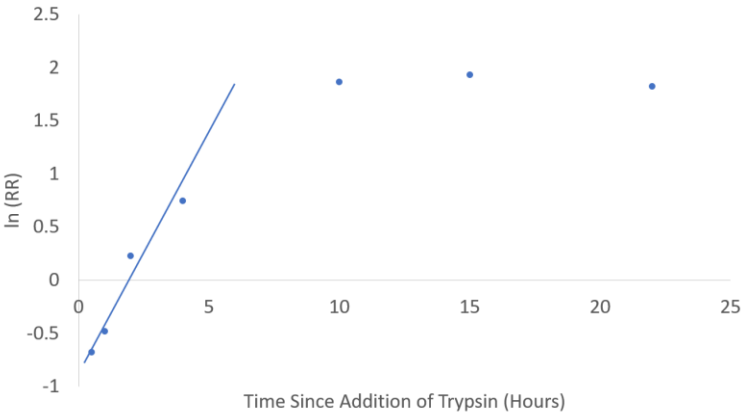


Figure 3.1: Deamidation of Asparagine (Asn). The mechanism of the deamidation of Asn, producing both normal aspartic acid(n-Asp) and iso-aspartic acid (i-Asp). The additional -CH_2- moiety is represented by a red circle.

A)



B)



C)

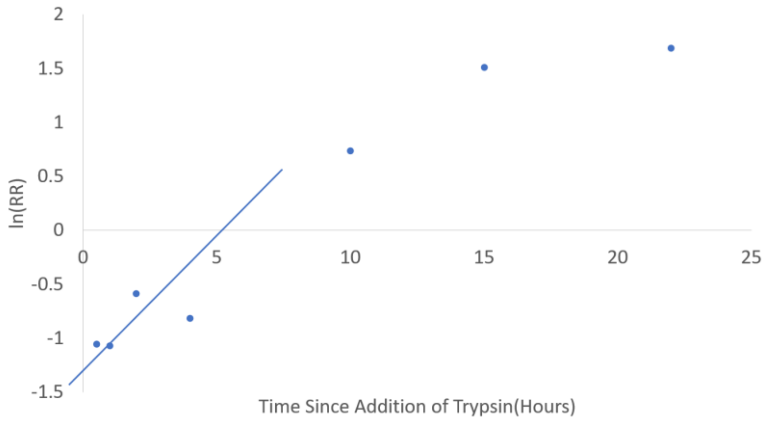


Figure 3.2: Trypsin Digestion of Peptides Lacking Possible Site(s) of Deamidation at pH=8. Average first order plots of the natural log of the RR versus time for three peptides lacking a possible site(s) of Asn deamidation (A: EPQVYTLPPSR, B: FNWYVDGVEVHNAK, and C: TTPPVLDSDGSFFLYSK) containing best-fit line based on the slope of the inclines.

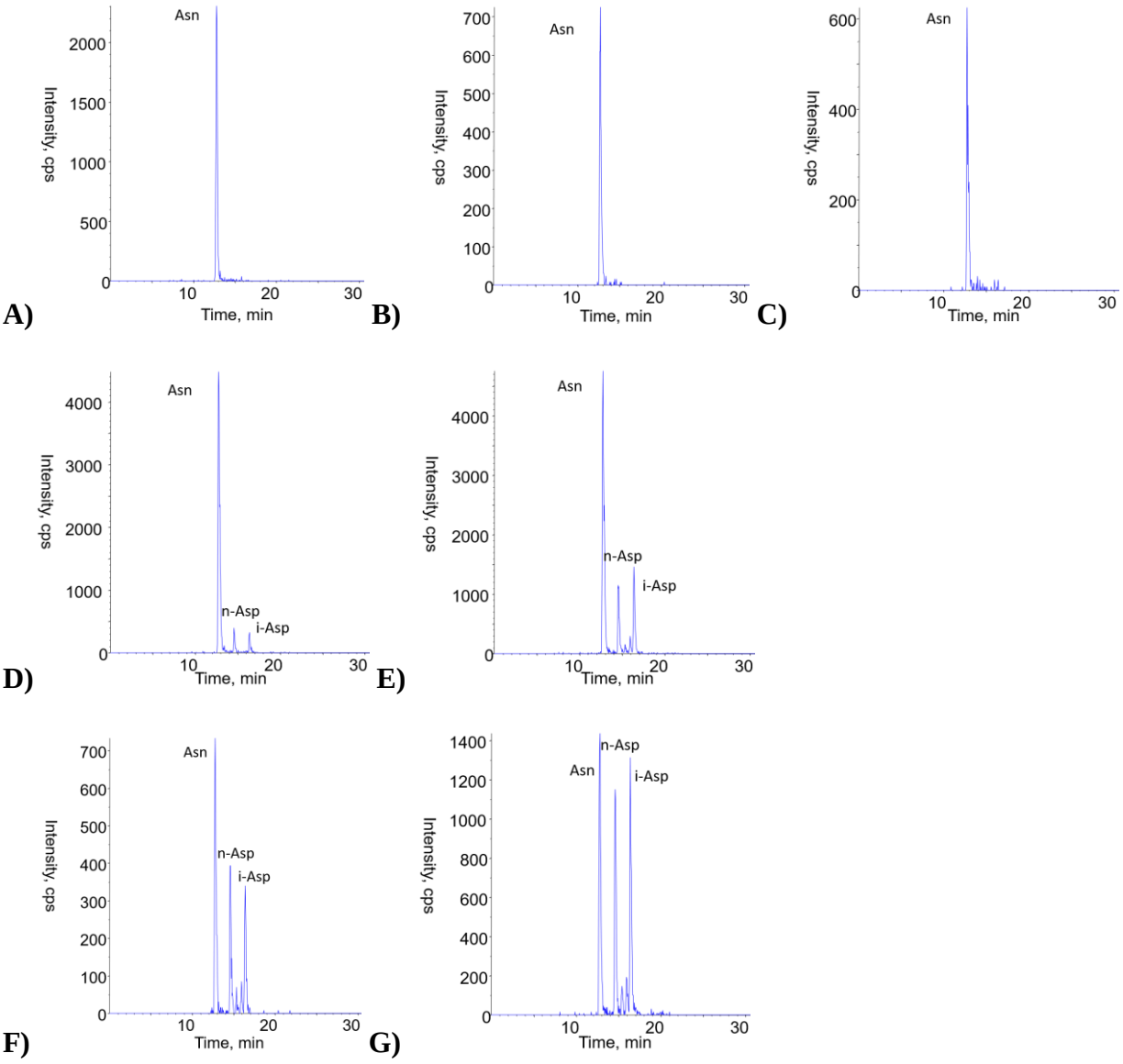
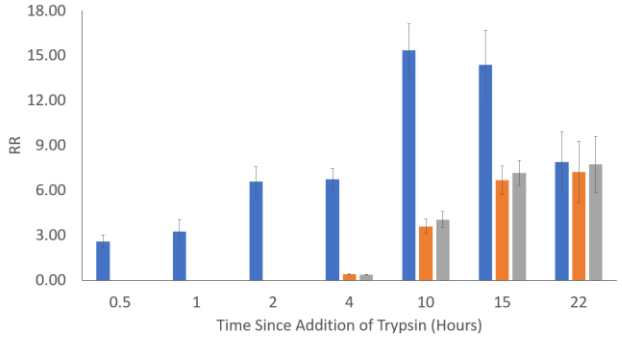
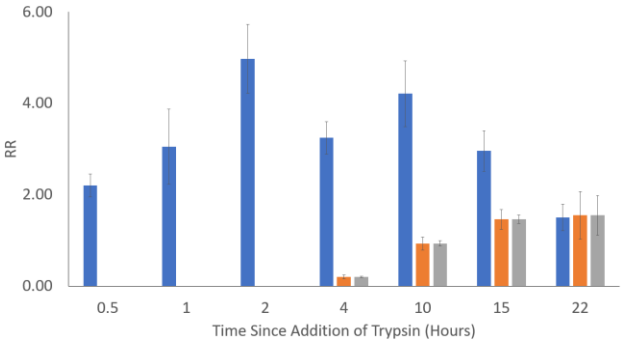


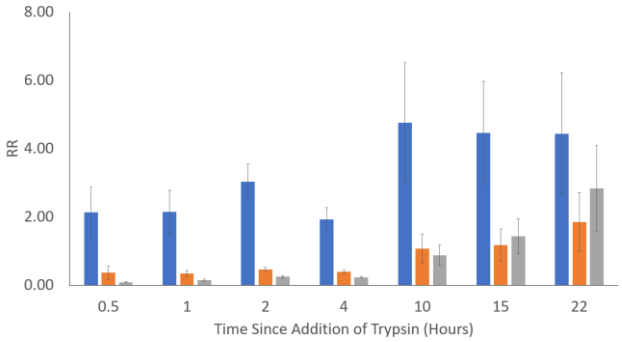
Figure 3.3: HILIC-SRM traces of Deamidated Peptides. HILIC-SRM traces of a single, representative peptide (VVSVLTVLHQDWL**N**GK; Parent Ion: 905.0 Fragment Ion: 997.7) containing a site of deamidation at several different time-points (A: 0.5 hours, B: 1 Hour, C: 2 Hours, D: 4 hours, E: 10 Hours, F: 15 Hours, and G: 22 hours) following the addition of trypsin.



A)



B)



C)

Figure 3.4: The Relative distribution of Deamidated Products in Peptides Consisting of site(s) of Deamidation, demonstrating Native Deamidation versus Deamidation Following Trypsin Digestion at pH 8.0. A relative comparison of the Asn, n-Asp, and i-Asp containing peptides for each for each individual IgG peptide (**A**: VVSVLTVLHQDWL**N**GK, **B**: VVSVLTVVHQDWL**N**GK, and **C**: GFYPSDIAVEWES**N**GQPE**N**NYK) consisting of at least 1 site of deamidation. The three peptide products following a trypsin digestion for each peptide are represented (A peptide consisting of Asn (Blue), n-Asp (Orange), and i-Asp (Gray)). Error bars represent standard error of the mean.

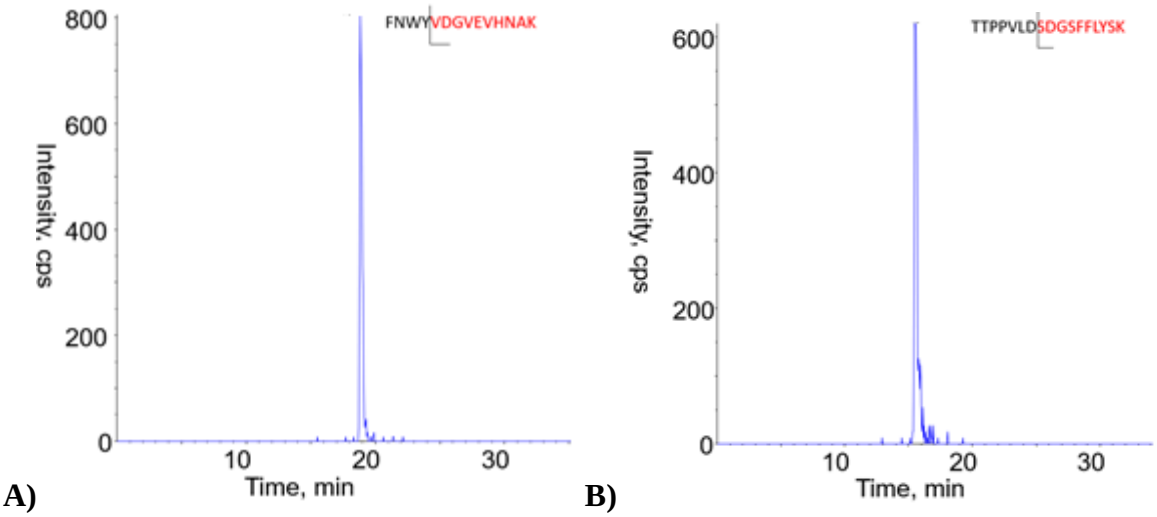
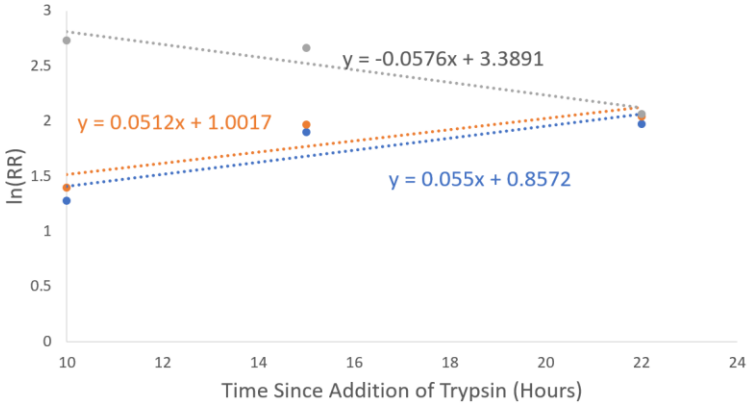
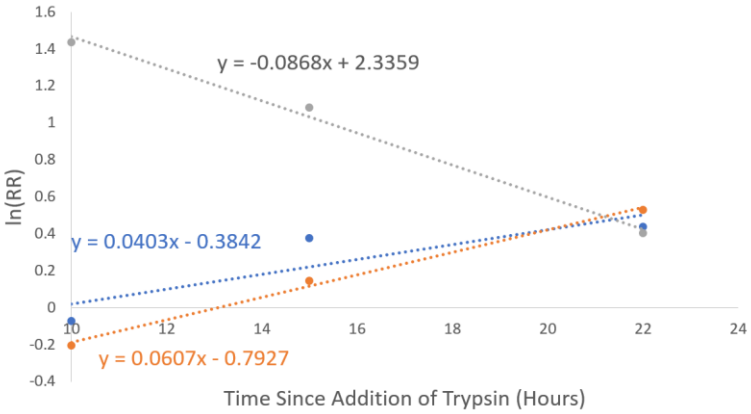


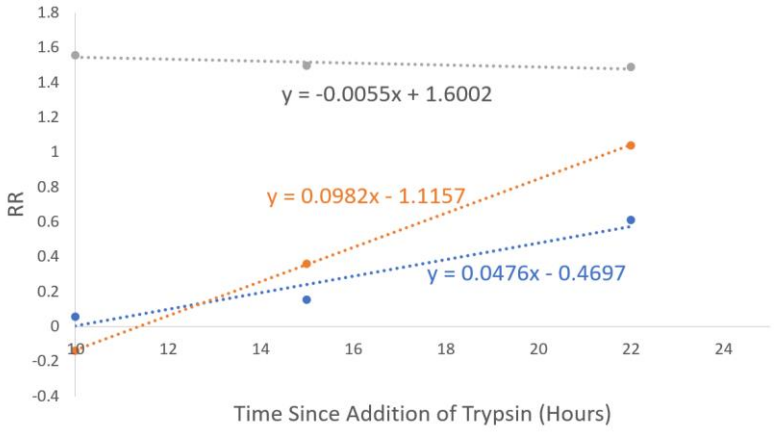
Figure 3.5: HILIC-SRM traces of Peptide Consisting of Possible Site(s) of n-Asp Isomerization. SRM traces of two peptides with 1 possible site of n-Asp isomerization (**A**: FNWYVD*GVEVHNAK and **B**:TTPPVLDSD*GSFFLYSK) after 22 hours following the addition of trypsin at pH 8. Transitions(A: Parent Ion: 839.4 Fragment Ion: 1067.6 and B: Parent Ion: 937.5 Fragment Ion: 1150.5.



A)

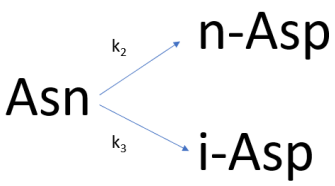


B)



C)

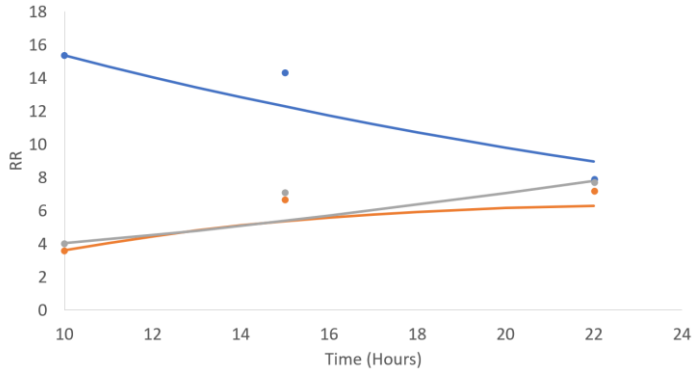
Figure 3.6: **LN(i-Asp and n-Asp) vs Time Plots.** ln(RR) vs. time plots for the deamidation products(Asn (Gray),i-asp(Orange),n-Asp(Blue)) of three peptides (A: VVSVLTVLHQDWLNGK, B: VVSVLTVVHQDWLNGK, and C: GFYPSDIAVEWES**N**GQPE**N**NYK) with a site of deamidation.



A)

Figure 3.7: Tenua Kinetic Models for Parallel, First Order Reaction (A) and For Continuous, Parallel First Order Reactions(B). k_2 and k_3 are the rate of rate of formation of n-Asp by deamidation, and rate of formation of i-Asp by deamidation respectively.

A)



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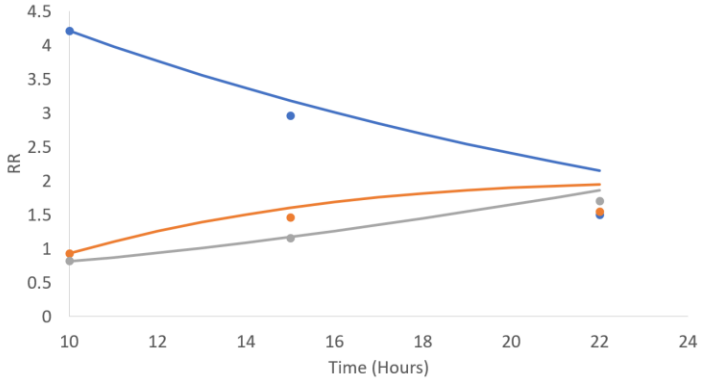


C)

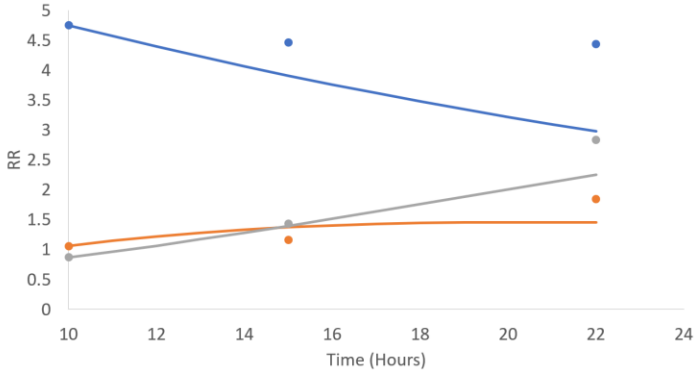
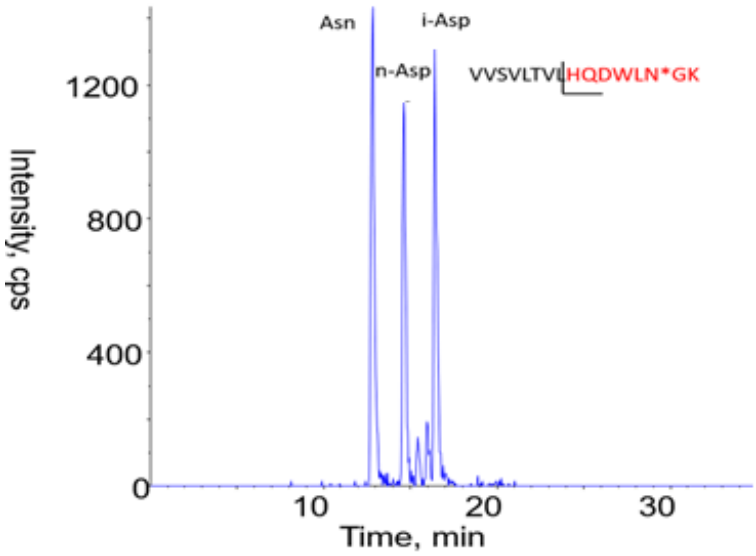
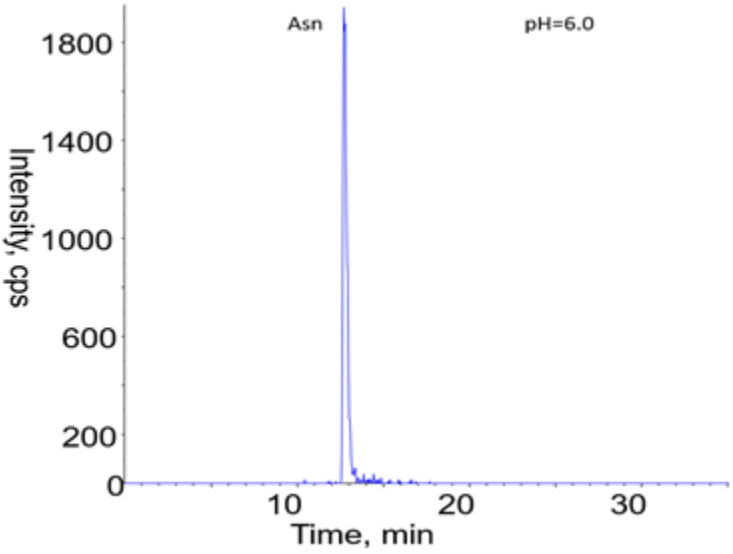


Figure 3.8: Kinetic Simulations from Tenua. Kinetic data from Tenua of three deamidated peptides (A: VVSVLTVLHQDWLNGK B: VVSVLTVVHQDWLNGK C: GFYPSDIAVEWES**N**GQPE**N**NYK) was simulated (Asn=Blue line, n-Asp=Orange Line, i-Asp=Gray Line). Experimental data was overlaid over their respective models (Asn=Blue Markers, n-Asp=Orange Markers, i-Asp=Gray Markers)



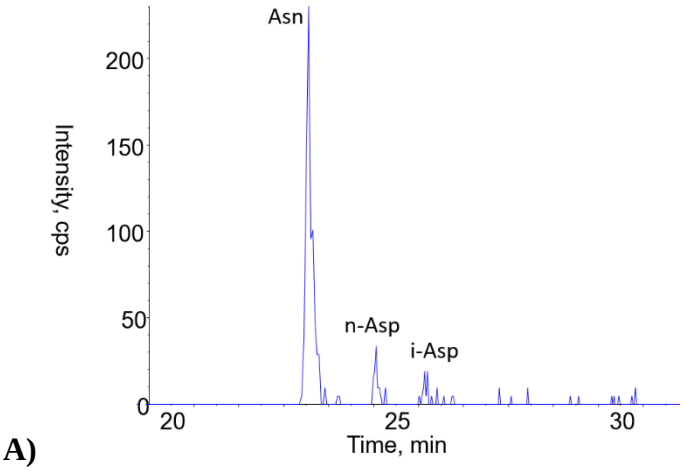
A)



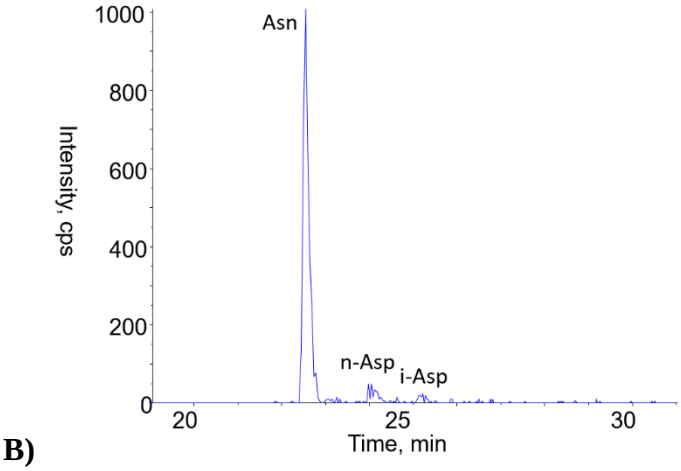
B)

Figure 3.9: HILIC-SRM traces of Representative Peptide with at least 1 Possible Site of Deamidation following Trypsin Digestion performed under Different Digestion Conditions.

SRM traces of a single peptide with 1 site of deamidation (VVSVLTVLHQDWL**N**GK) after after 22 hours following the addition of trypsin under different conditions (**A**: pH=8.0 and **B**: pH=6.0). SRM Transition: Parent Ion: 905.0, Fragment Ion: 997.7.



A)



B)

Figure 3.10: HILIC-SRM traces of Peptide Suspected of Native Deamidation following Trypsin Digestion under different conditions (pH 6.0 and pH 8.0). SRM traces of a single peptide with 1 site of deamidation (GFYPSDIAVEWES**N**GQPE**N**NYK) after 0.5 and 22 hours following the addition of trypsin at pH 8.0. (**A**: 0.5 Hours and **B**: 22 Hours). SRM Transitions, Parent Ion: 1273.1, Fragment Ion: 1089.3.

Supplementary Materials

Table 3.S1: Scheduled Transitions for Analyzed IgG peptides and the GluB standard.

'N'- Possible Site of Asparagine Deamidation

D*- Possible Site of Aspartic Acid Isomerization

Peptide	Parent Ion	Fragment Ion	Retention Time (min)
Glu-Fib	785.5	684.4 (y6)	23.0
GFYPSDIAVEWESNGQPENNYK	1273.1	1089.3 (y17)	25.0
VVSVLTVLHQDWLNGK	905.0	997.7 (y8)	14.5
VVSVLTVVHQDWLNGK	898.0	997.7 (y8)	15.3
EPQVYTLPPSR	643.8	456.5 (y4)	17.5
TTPPVLDSD*GSFFLYSK	937.5	1150.5 (y10)	15.5
FNWYVD*GVEVHNAK	839.4	1067.6 (y10)	18.8

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CHAPTER 4

CONCLUSIONS

The purpose of this work was to analyze specific hydrophilic post translational modifications (PTMs) utilizing liquid chromatography-mass spectrometry (LC-MS) in order to investigate significant questions pertaining to hydrophilic PTMs. Hundreds of PTMs have been discovered and analyzed due to their potential biological significance. LC-MS has been proven to be a powerful analytical tool for the abilities to perform structural analysis and characterization. In this work two specific examples of hydrophilic PTM analysis, N-linked glycans and deamidation, were investigated.

Chapter 2 investigated a recently published protocol which described a procedure for chemically releasing Asparagine (N)-linked glycans from their peptide backbone for large scale processing utilizing sodium hypochlorite (NaOCl). This newly published protocol was quantitatively compared to peptide: N-Glycosidase F (PNGase F) for the release of N-linked glycans for a small scale glycan anlaysis. Initially, a glycan profile of the the five most abundant glycans of fetal bovine fetuin were compared, and the results indicated that the profiles of released N-linked glycans from both techniques were indistinguishable from eachother. However, in the analysis of the absolute recovery of the released glycans, the recovery of glycans where the NaOCl was performed released greater than 20x less N-linked glycans than the PNGase F procedure. Although NaOCl is a much cheaper and fster alternative the low recovery of glycans using the NaOCl procedure implies that this approach is not suitable with small amounts of sample.

Chapter 3 explored the potential of the conditions of trypsin digestions to be complicit in the identification of deamidation of asparagine (Asn) in proteomics analyses. Often the presumption is that deamidation of Asn present in a bottom up analysis of a protein is indicative of deamidation which is native to a the protein. This includes the formation of iso-aspartic acid (i-Asp), which can have significant biological consequences. However, the conditions of a trypsin digest, including high pH, are conducive to deamidation. The conditions could possibly cause the quantitation of i-Asp formation to produce erroneous results. Chapter 3 demonstrated that the conditions of a trypsin digestion do in fact play a significant factor in the deamidation of asparagine (Asn) residues, forming both aspartic acid (Asp) and i-Asp residues in immunoglobulin G (IgG) peptides during a trypsin digestion. Furthermore, altering the pH of the digestion conditions was shown to significantly decrease the process of deamidation.