DEVELOPMENT OF A BIOANALYTICAL METHOD FOR RAPID AND EFFICIENT N-GLYCAN ANALYSIS OF THERAPEUTIC PROTEINS BY MASS SPECTROMETRIC METHODS

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"I declare that all the information within this thesis has been gathered and presented in accordance with academic regulations and ethical principles and I have according to the requirements of these regulations and principles cited all those which do not originate in this work as well."

Zidan Masoud RAGOUBI

ABSTRACT

M. Sc. Thesis

DEVELOPMENT OF A BIOANALYTICAL METHOD FOR RAPID AND EFFICIENT N-GLYCAN ANALYSIS OF THERAPEUTIC PROTEINS BY MASS SPECTROMETRIC METHODS

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Glycosylation process is a post-translational modification, which plays an active critical role in many cellular events. It also regulates many functions of proteins. Monoclonal antibody (mAb)-derived drugs are used in the treatment of many diseases, and glycosylation affects the activity of such drugs developed. On the other hand, *N*-glycans may change in certain diseases. Therefore, rapid and efficient bioanalytical methods are needed for *N*-glycosylation profiling. In the study, an integrated stage-tip application was developed for simple and rapid *N*-glycosylation profiling of glycoproteins.

By integrating all glycoproteomic and glycomic sample preparation steps into a stage-tip, a fast and inexpensive *N*-glycosylation profiling was ensured. The glycomic

Approach of integrated stage-tip reduces the *N*-glycan profiling time from 2 days to Approximately 2.5 hours. It also allows profiling of immunoglobulin G (IgG) *N*-glycopeptides directly from human plasma. In addition, *N*-glycosylation profiling can be done in the developed method without sorbents C18 or others, such as strong-cation exchange (SCX) at the glycopeptide level.

Key Words : Glycomics, Glycosylation, *N*-Glycan Profiling, Mass Spectrometry, Therapeutic protein.

Science Code : 92509

ÖZET

Yüksek Lisans Tezi

KÜTLE SPEKTROMETRİK YÖNTEMLERLE TERAPÖTİK PROTEİNLERİN HIZLI VE ETKİLİ N-GLİKAN ANALİZİ İÇİN BİYOANALİTİK BİR YÖNTEMİN GELİŞTİRİLMESİ

Zidan Masoud RAGOUBI

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Glikozilasyon, birçok hücresel olayda aktif rol oynayan translasyon sonrası bir modifikasyondur. Aynı zamanda proteinlerin birçok fonksiyonunu da düzenler. Monoklonal antikor (mAb) türevi ilaçlar birçok hastalığın tedavisinde kullanılmaktadır ve glikosilasyon, geliştirilen bu tür ilaçların aktivitesini etkiler. Öte yandan, bazı hastalıklarda *N*-glikanlar değişebilir. Bu nedenle, *N*-glikosilasyon profillemesi için hızlı ve verimli biyoanalitik yöntemlere ihtiyaç vardır. Çalışmada, glikoproteinlerin basit ve hızlı *N*-glikosilasyon profillemesi için entegre bir aşamalı uç uygulaması geliştirilmiştir. Tüm glikoproteomik ve glikomik numune hazırlama adımlarını bir aşama ucuna entegre ederek, hızlı ve ucuz bir *N*-glikosilasyon profili oluşturması sağlandı. Entegre kademeli ucun glikomik yaklaşımı, *N*-glikan profil oluşturma süresini 2 günden yaklaşık 2,5 saate düşürür.

Ayrıca, immünoglobulin G (IgG) N-glikopeptidlerin doğrudan insan plazmasından profilinin çıkarılmasına da izin verir. Ek olarak, *N*-glikozilasyon profili, glikopeptit seviyesinde güçlü katyon değişimi (SCX) gibi sorbentler C18 veya diğerleri olmadan geliştirilen yöntemde yapılabilir.

Anahtar Kelimeler: Glikomik, Glikozilasyon, N-Glikan Profili Oluşturma, Kütle Spektrometrisi, Terapötik Protein.

Bilim Kodu **:** 92509

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CONTENTS

Page

Page

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

SYMBOLS AND ABBREVITIONS INDEX

SYMBOLS

NHS : N-hydroxysuccinimide groups.

APTS : (3-Aminopropyl)triethoxysilane.

ABC : Ammonium bicarbonate.

ACN : Acetonitrile.

Ser : Serine.

The : Threonine.

ABBREVITIONS

MS : Mass Spectrometry.

TOF : Time of Flight.

ESI : Electrospray ionization.

MALDI : Matrix-Assisted Laser Desorption/Ionization.

FDA : Food and Drug Administration.

QbD : Quality by Design.

ADCC : Antibody-dependent cellular cytotoxicity.

CDC : Complement-dependent cytotoxicity.

GC-EI : Gas Chromatography – Electron Ionization.

QTOF : Quadrupole Time of Flight.

AP/LIAD-CI : Atmospheric Pressure Laser-induced Acoustic Desorption Chemical Ionization.

APPI : Atmospheric Pressure Photoionization.

DNA : Deoxyribonucleic Acid.

FWHM : Full-Width Half-Maximum.

HILIC	: Hydrophilic Interaction Chromatography.
HPLC	: High-Performance Liquid Chromatography.
HPAEC	: High pH Anion Exchange Chromatography.
PAD	: Pulsed Amperometric detection.
HIV	: Human Immunodeficiency Virus.
3D	: Three Dimensional.

PART 1

INTRODUCTION

Glycosylation occurs because of a series of enzymatic reactions in the cell. In this modification, complex oligosaccharide structures called glycans bind to proteins. Two glycosylation types, N- and O-linked, are commonly observed in living organisms. The functions of proteins that have undergone this modification are changed. *N*-glycans are known to contribute to these critical functions. In addition, changes in *N*-glycans are observed in diseases such as cancer.

Moreover, most of the therapeutic proteins are glycoproteins, and the structure of their glycan determines the effectiveness of these drugs. For this reason, the analysis of these N-glycan structures in proteins should be done with fast, which is associated with efficient techniques. Mass spectrometry is considered one of the most popular methods in N-glycan analysis, attributable to the aforementioned high mass accuracy and rapid analysis capability. Moreover, there are several preparation steps for mass spectrometric analysis of *N*-glycans. Performing these sample preparation processes in different experimental environments results in sample losses. It also increases the time required for sample preparation processes. Therefore, a fast and efficient bioanalytical method that minimizes the sample loss remains necessary for analyzing *N*-glycans via mass spectrometric techniques. This study aims to develop a strategy that allows all sample preparation processes to be performed in the same experimental setup for the fast and efficient analysis of *N*-glycans via mass spectrometric techniques. This study aims to develop a bioanalytical approach for N-glycan and *N*-glycopeptide analysis of glycoproteins, including protein therapeutics. In addition, it is targeted to:

- Achieve rapid and quantitative identification of N-glycan patterns for pharmaceutical samples.

- Integrate all sample preparation steps for *N*-glycopeptide analysis in the same experimental condition, including denaturation, glycan release, labeling, and purification before their analysis.

PART 2

LITERATURE REVIEW

2.1. GLYCOSYLATION

Glycosylation remains an enzymatic process. Furthermore, glycans can bind to proteins through a reaction in which any hydroxyl occurs with a glycosyl donor. Moreover, glycans provide various functional or structural roles in the secreted proteins and the membrane [1]. In the same way, most of the glycoproteins remain synthesized in the coarse endoplasmic reticulum with the glycosyl binding process. Moreover, there remain mainly three types of glycosylation observed in mammalian proteins. These stay as below;

C-linked glycosylation is uncommon glycosylation in which a sugar is attached to a tryptophan side-chain carbon. O-linked glycosylation occurs when the hydroxyl oxygen of hydroxylysine, threonine, tyrosine, serine, and hydroxyproline side-chains are attached to oxygens. *N*-linked glycan remains attached to nitrogen of an arginine side-chains. Also, N- linked glycosylation requires the inclusion of a lipid known as dolichol phosphate.

2.2. GLYCAN TYPES

N-linked glycosylation remains a prevalent form of glycosylation which remains associated with an essential for folding numerous eukaryotic glycoproteins, cell-cell, and cell-extracellular matrix attachment. Moreover, the *N*-linked

glycosylation process takes place in eukaryotes, especially in the endoplasmic reticulum lumen, which can be seen widely in archaea. However, it can be seen very rarely in bacteria. Additionally, protein's N-linked glycans can influence hoit functions. In addition, the *N*-linked glycosylation, in several cases, can act as an on and off switch [68].

On the other hand, O-linked glycosylation is the binding of a sugar molecule to the oxygen atom of serine (Ser) or threonine (Thr) residues of a protein. After the protein has been produced, O-glycosylation is a post-translational modification that occurs. It is found in the endoplasmic reticulum, the Golgi apparatus, and infrequently in the cytoplasm of eukaryotes. However, it can be seen in the cytoplasm of prokaryotes [3]. In the same way, several diverse sugar types can be added to the serine; otherwise, threonine also affects the protein in various forms via changing protein stability, which is associated with adjusting the activity of the protein. O-glycans, the type of sugars added to the serine, provide a variety of activities in the body, including immune system cell trafficking, foreign material recognition, metabolic cell control, and tendon flexibility cartilage [4]. Changes in O-glycosylation are significant in diseases such as cancer, diabetes, and Alzheimer's disease because of their varied functions [5]. O-glycosylation can be found in all life forms, including archea, eukaryotes, and numerous pathogenic bacteria, including Burkholderia cenocepacia [5], Neisseria gonorrhoeae [6] and Acinetobacter baumannii [7].

2.3. GLYCAN BIOMARKERS

A biomarker remains biologically important, unambiguously identifying a particular and based the physiological condition. In the same way, it could be a single measured entity; otherwise, it could be a panel based on an indicator substance [8]. Moreover, a biomarker can be utilized for screening the disease condition and monitoring patients undergoing therapy, which is associated with even identifying the re-occurrence of the disease condition. Moreover, markers remain beneficial based on determining who is at a higher risk of contracting certain diseases. Similarly, advancements in cancer

research enhance expectations for early detection markers-based identification. As a result, cancer is a leading cause of death in almost every country [9]. For instance, in the USA, more than five hundred thousand deaths remain projected being assigned based on cancer. Also, around 1.6 million new cancer diseases remain expected to be diagnosed in 2016 [10]. These harmful effects based on cancer continue even though it can be contained or even cured when cancer is diagnosed early. Moreover, efforts toward identifying biomarkers that can detect cancer at an early stage as well as discriminate a given sort of cancer based on other diseases face numerous challenges. Likewise, the complexity of biological materials from which possible biomarkers are formed, the considerable variability of potential analytes among different conditions, and the limitations of current analytical technologies are only a few of the obstacles [8]. Moreover, glycosylation is a post-translational modification of proteins that changes the glycoproteins' biochemical activity, and this is important in cancer biology. Glycomics and glycoproteomics approaches are less developed than proteomics methods, owing to the inherent difficulties in analyzing them. [11].

2.4. GLYCANS AND THEIR IMPORTANCE

Glycosylation has been associated with numerous diseases. Moreover, several diagnostic biomarkers remain glycoproteins. In addition, most of the protein therapeutics remain glycoproteins [11]. In the same way, *N*-glycans located on monoclonal antibodies influence the drug efficacy [12]. Therefore, it remains obligatory to analyze them via high-throughput methods. Indeed, Food and Drug Administration (FDA) only approved the monoclonal antibodies as biosimilar drugs when they have proper N-glycan distribution [13]. Moreover, fast and efficient bioanalytical methods are needed to analyze these monoclonal antibody-based drugs [13].

2.5. THERAPEUTIC GLYCOPROTEINS

The development of biological pharmaceuticals remains stimulated via the emergence of recombinant protein-based drugs [14], which remains associated with the fastest-growing sector in the pharmaceutical industry today [15]. Moreover, numerous therapeutic proteins associated on the market are antibodies, which remain glycoproteins associated with N-glycosylation; for instance, Etanercept, Infliximab, and Rituximab are N-glycosylated therapeutic proteins [16]. In the same way, the enzymatic production of glycans can be affected by minor changes in process conditions. Thus, the physical, chemical and biological properties of the final product are influenced by N-glycosylation. Moreover, in producing biopharmaceuticals based on the quality via design (QbD) model, an important goal is to adjust carbohydrate residues to enhance efficacy [14, 17]. In addition, typical glycan structures based on a therapeutic humanized IgG1 mAb are shown in Figure 2.1.

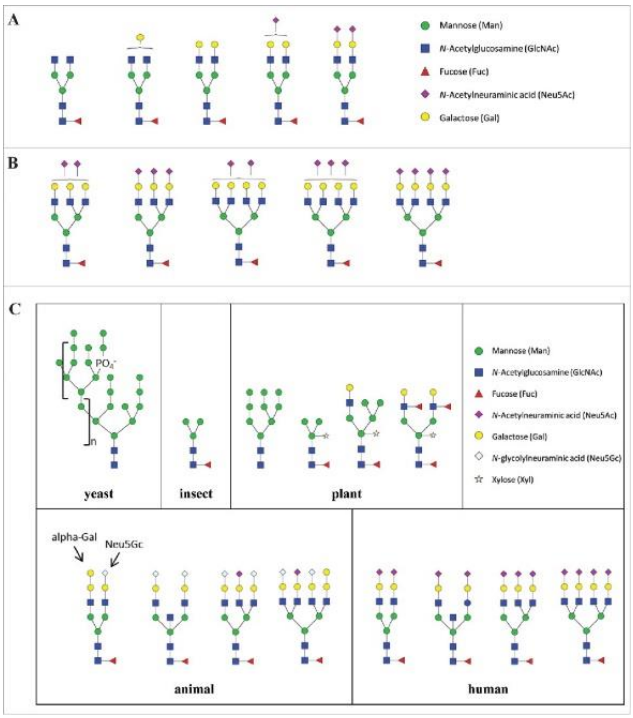

Figure 2.1. Glycan structures on a therapeutic humanized IgG1 mAb (A) and recombinant human erythropoietin (B) [14].

The complexity of protein glycosylation poses a daunting analytical challenge based on a developing therapeutic glycoprotein. In the same way, orthogonal methods are required for characterizing specific features of mAbs. For instance, the glycan content and the structure influence the activity of mAbs. In addition, the two main effectors of mAb's functions are antibody-dependent cellular cytotoxicity (ADCC) associated with complement-dependent cellular cytotoxicity (CDC). Both are influenced by N-glycosylation. Therefore, the precise identification of their carbohydrate structures remains crucial in choosing copies [18]. Moreover, the presence or lack of different sugar residues on the N-glycan cores of monoclonal antibodies enhances microheterogeneity, which influences stability and effector function. Therefore, it remains necessary to analyze these proteins' N-glycans before human utilization [16].

2.6. MASS SPECTROMETRY

Mass spectrometry remains the technique to fulfill the needs of glycomics and glycoproteomics research. Moreover, this technique has been considered in some study more than 20 years ago [64-67], with new advanced modifications. It is still remarkably evolving. Moreover, this approach can be used with a wide range of soft ionization methods, including matrix-assisted laser desorption (MALDI) (Figure 2.2) and electrospray ionization (ESI) [64] [65]. In the same way, the combination of various mass spectrometric techniques can be used to understand glycan structures and functions. In addition, it has numerous advantages, as declared in the literature [64-66].

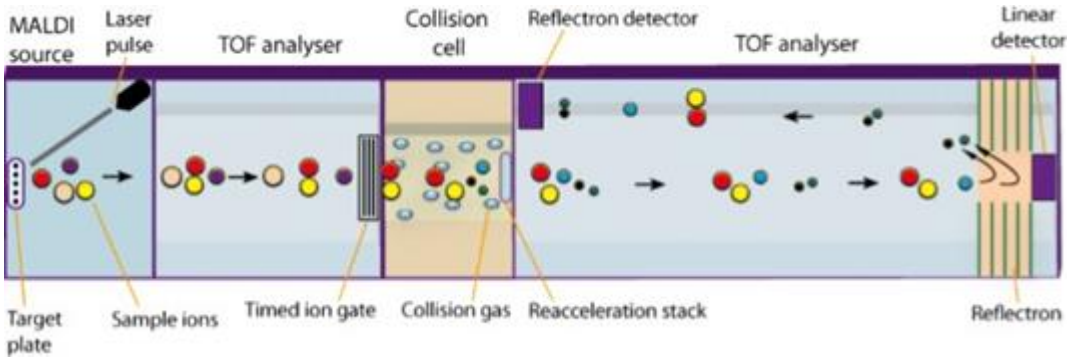

Figure 2.2. Schematic view of a MALDI-TOF/TOF mass spectrometer [19].

2.6.1. Ionization Techniques

There are diverse ionization techniques utilized in mass spectrometers. Moreover, several of them are electrospray ionization (ESI), atmospheric pressure laser-induced desorption chemical ionization (AP/LIAD-CI), atmospheric pressure photoionization (APPI), atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI).

2.6.1.1. Electrospray Ionization (ESI)

In mass spectroscopy, electrospray ionization (ESI) is a common technique for ionizing samples before they are measured (Figure 2.3). Moreover, the electrospray ionization works well with heavier compounds and is often utilized in proteomics, which analyzes proteins.

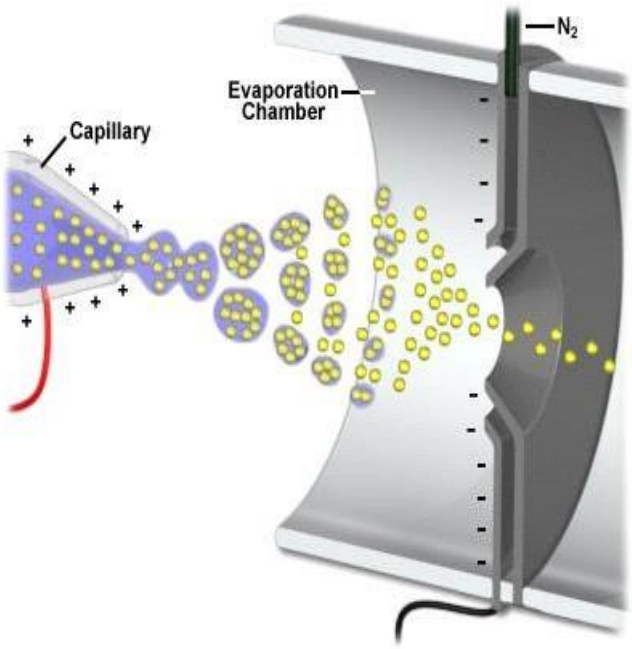

Figure 2.3. Electrospray ionization technique in mass spectroscopy.

The sample is combined with a solvent that is also injected into a capillary tip. Furthermore, this tip is subjected to a very high voltage, creating charged molecules in the solvent. Because they are highly charged, these molecules reject one another almost violently as they are forced into the evaporation chamber through the nozzle. Similarly, when the charged liquid exits the tip first, it briefly forms a Taylor cone before the droplets explode apart from each other into a thin spray.

The droplets in the spray are divided into several groups. This happens because the solvent within these droplets slowly evaporates (with the help of nitrogen gas injected into the chamber), causing the charges in the molecules within these droplets (identical) to become closer together. When these ions are brought close enough together, they repel each other, forcing the droplets to split into two smaller droplets. In the same way, this process repeats itself until the solvent remains completely evaporated, linked to the droplets that have split off into individual, charged molecules. Furthermore, one of the benefits of this ionization approach is that the molecules

remain intact and are not fragmented; this is what the term "soft ionization" refers to (Figure 2.4.).

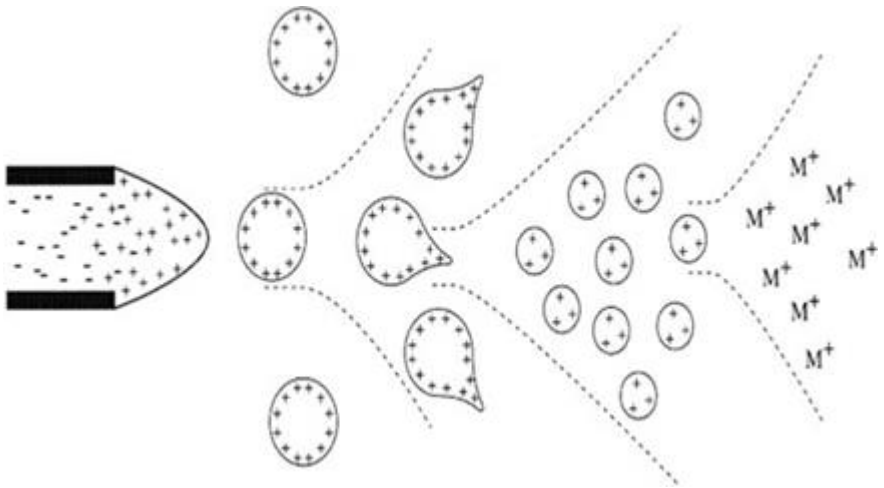

Figure 2.4. Mechanism of electrospray ionization.

2.6.1.2. Matrix-Assisted Laser Desorption/Ionization (MALDI)

In terms of mass spectrometry, matrix-assisted laser desorption/ionization (MALDI) is an ionization technique that creates ions from heavier molecules with minimum fragmentation using a laser energy-absorbing matrix. It has been used to investigate biomolecules such as carbohydrates, proteins, DNA, and peptides [21]. Moreover, these molecules tend to be fragile when ionized via conventional ionization methods. Both MALDI and ESI are relatively soft (low fragmentation) methods of producing ions of intact molecules in the gas phase; however, MALDI typically yields significantly fewer multi-charged ions [22] (Figure 2.5).

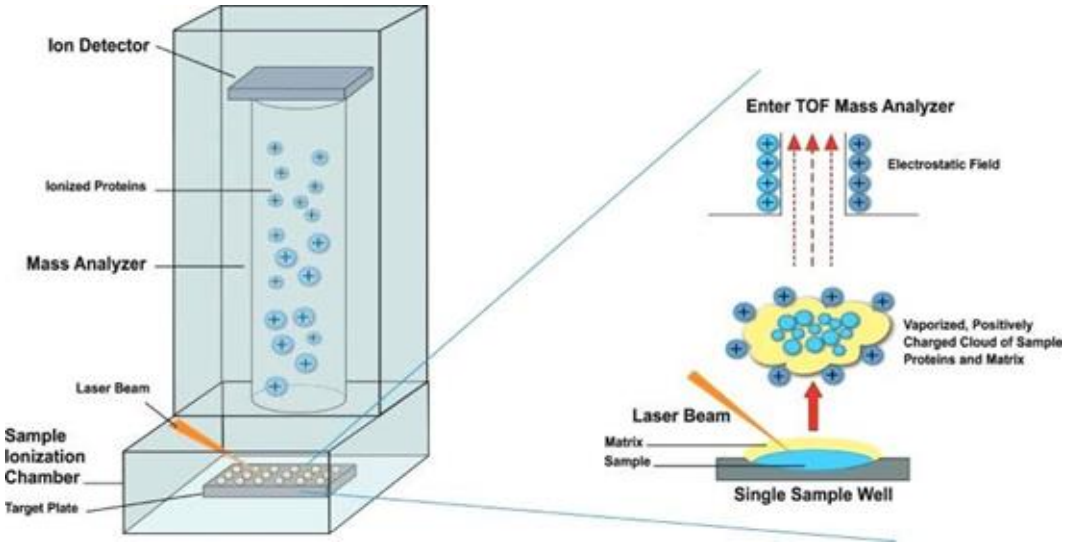

Figure 2.5. Schematic MALDI-TOF mass spectrometer process [19].

2.6.2. Mass Analyzers

2.6.2.1. Quadrupole

A quadrupole is a sequence of ideal configurations for separating ions using electric current or charge based on their gravitational mass [23-26]. Still, it is usually simply a portion of a multipole expansion of a more complicated structure representing various orders of complexity [23, 24].

2.6.2.2. Time-of-Flight

The time-of-flight (TOF) mass spectrometer is the most common type of mass spectrometer used with MALDI, owing to its enormous mass range. Because the pulsed laser takes individual "shots" rather than functioning in continuous mode, the time-of-flight measuring approach is also well suited to the MALDI ionization process. The "ion mirror" on MALDI-TOF equipment deflects ions with an electric field, thus doubling the ion flight path and boosting the resolution. Today's commercial reflectron TOF instruments have a resolving power m/m of well over 20'000 FWHM [26, 27].

However, MALDI-FT-ICR is required when high-resolution measurements are needed [28].

2.7. LIQUID CHROMATOGRAPHY

Various liquid chromatographic separation techniques for N-glycan analysis have been developed throughout the years [30]. Moreover, the most commonly utilized types in the literature are the normal phase, reverse phase, and adsorption (porous graphite carbon), weak anionic exchange [31-34]. High pH anion exchange chromatography (HPAEC), which is commonly used in conjunction with pulsed amperometric detection, can separate and identify carbohydrates at low picomole levels with minimal sample pretreatment (PAD). Carbohydrate hydroxyl groups are slightly acidic in nature, and at high pH (>12), they can ionize to oxyanions [31, 34, 35]. Moreover, the minor changes in pKa allow the diverse hydroxyl groups to match the distinct glycan's associated with the possible addition of negative charges from acidic components, for instance, cyanic acids [36], with very selective separations at stable phases of exchange in HPAEC. Additionally, this technique is less sensitive and selective in contrast to fluorescence-based approaches. It also has concerns with excessive noise and baseline stability, as well as challenges with using high pH buffers [37]. Furthermore, amino acids, peptides, and other oxidizable organic substances may interfere with HPAEC-PAD glycan analyses [38].

HPAEC has been integrated with pulsed amperometric detection (PAD), providing a compassionate and practical framework for separating underivatized carbohydrates [39].

Because glycans have no UV/Vis or fluorescent activity, they must be labeled with fluorophores or chromophores to detect using liquid phase separation methods like chromatography, capillary electrophoresis, or microchip electrophoresis. In addition, most of the sugar residues except sialic acids have no charge to support electrical migration. In the same way, *N*-glycans can be labeled with a wide range of florescence

agents such as 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), 2-aminopyridine (PA), 8-amino naphthalene- 1,3,6-tris-sulfonic acid (ANTS), as well as 8-aminopyrine-1,3,6-tris-sulfonic acid (APTS) [69]. In addition to the most widely used sodium cyanoborohydride, alternative non-toxic chemicals such as sodium triacetoxyborohydride and 2-picoline borane are also available.

2.8. N-GLYCAN ANALYSIS OF GLYCOPROTEINS BY MASS SPECTROMETRY

One of the first steps in analyzing N-glycan is the release of carbohydrates from glycoproteins utilizing enzymes [46]. Furthermore, the procedures from the alkaline medium remain insufficient and otherwise not well defined. Therefore, N-glycan release step is most often applied with Endoglycosidase H (Endo H) as well as Peptide-*N*-glycosidases F, A (PNGase and PNGase A) [47].

2.8.1. HPLC-HILIC-FLD Analysis of Released N-glycans

Hydrophilic interaction chromatography (HILIC) is a technology in the field of high-performance liquid chromatography (HPLC) that is rapidly gaining popularity [53-56]. Moreover, HILIC can separate the most polar chemical compounds using the same technology [55]. When compared to traditional analytical procedures, including ion pairing, ion exchange, and normal-phase HPLC, HILIC saves time and money. Method development and troubleshooting can be complex even for experienced researchers, and HILIC columns have only lately been able to match the performance and reproducibility of better-known reversed-phase chemistries [53-56].

2.9. N-GLYCOPEPTIDE ANALYSIS OF GLYCOPROTEINS BY MASS SPECTROMETRY

2.9.1. Tandem Mass Spectrometry

Tandem mass spectrometry (MS/MS) is an instrumental analysis technique in which two or more mass analyzers are linked, utilizing an additional reaction step to improve their ability to examine chemical samples. The examination of biomolecules such as proteins and peptides is a common use of tandem MS [64-67].

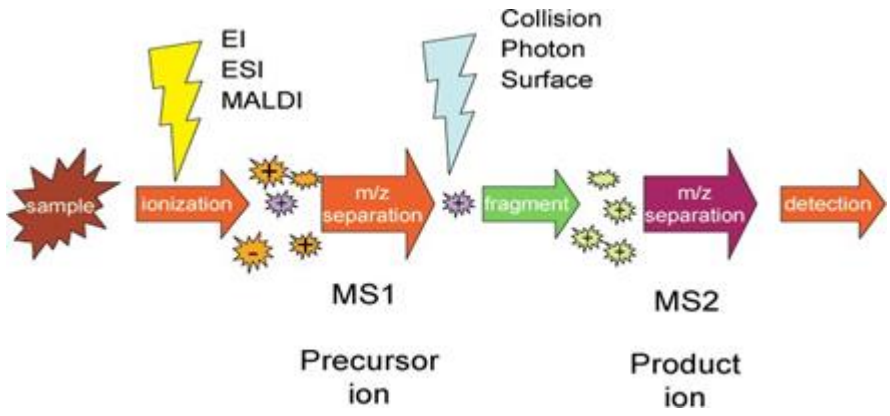

Figure 2.6.Schematic of tandem mass spectrometry.

The molecules of a given sample are ionized in the spectrometer (designated MS1) and have separated in the mass analyzers based on their mass-to-charge ratio [64-67]. Moreover, ions of a specific m/z-ratio coming from MS1 analysis have been selected and then made towards splitting obsessed by a smaller fragment ion via collision-induced dissociation. In the same way, these fragments are then injected into the mass analyzers (MS2), which separate and detect them based on their m/z-ratio [65-67]. Tandem mass spectrometry has included a triple quadrupole mass spectrometer (QqQ), quadrupole time of flight (Q-TOF), and hybrid mass spectrometer [64-67].

2.9.2. Glycopeptide and Glycan Analysis by Tandem Mass Spectrometry

2.9.2.1 Collision-induced dissociation

Several of the essential characteristics of the CID of glycopeptides have already been established in early research by Carr and colleagues [57, 58] using ESI and collision-induced dissociation (CID) on a triple-quadrupole mass spectrometer. With liquid secondary ion MS employing high-energy CID, Burlingame and colleagues [59] have demonstrated similarities in fragmentation behavior and information content. ESI with CID of glycopeptides has developed into a crucial method in glycoproteomics as a result of this groundbreaking study.

CID of N-glycopeptides

By employing a range of tools and experimental setups, CID can fragment electrospray-ionized N-glycopeptides. A technique that is frequently used allows for repeated ion isolation/fragmentation cycles. As proven for a tryptic glycopeptide from horseradish peroxidase, IT-MS/MS of N-glycopeptides is typically dominated by the B-type and Y-type fragmentation of glycosidic links, exposing largely information on the composition and sequencing of the glycan moiety. N-glycopeptides with various glycan structures, such as complex-type sialylated structures, asialo complex type structures from invertebrates exhibiting antenna fucosylation, paucimannosidic structures, oligomannosidic species, as well as peptides carrying a single N-linked N-acetylglucosamine that may carry a fucose, have all been subjected to IT-MS/MS analyses [60].

In general, the MS/MS spectra of N-acetylhexosamine residues appear to be dominated by B-type and Y-type fragmentations of the glycan moiety. The Y-ion resulting from chitobiose cleavage with further loss of an eventually existing core-fucose is therefore a dominating fragment in the case of N-glycopeptides with short or no antennae, preserving a single, N-linked N-acetylglucosamine in a (frequently doubly charged)

peptide ion. Fucoses can also be easily eliminated as neutral losses, especially in their 3-linked form [60].

A sequence of y-ions and/or b-ions are produced as a result of the fragmentation of peptide backbone bonds, which can also be seen in addition to the cleavage of glycosidic connections. However, the majority of biological and medical applications do not utilize these peptide fragment ions for peptide sequence determination in N-glycopeptide analysis by ESI-IT-MS due to their low relative abundance. An alternate strategy combines MS/MS and MS3 studies to characterize N-glycopeptides by IT-MS in greater detail. The cleavage of glycosidic links results in a range of fragment ions when the glycopeptide ion is chosen and fragmented in this method. Following a second cycle of ion isolation/fragmentation (typically carried out in the automatic mode), the peptide ion bearing a single N-acetylglucosamine, which is frequently the most abundant fragment ion, is fragmented. Notably, the N-glycosylation site's y-type and b-type fragment ions frequently retain at least some of the N-acetylglucosamine residue, making it possible to determine the site of glycosylation in many cases [60].

CID of O-glycopeptides

Peter-Katalinic and colleagues [61] have shown the effectiveness of using a quadrupole-TOF mass spectrometer in conjunction with nano-ESI to characterize O-glycopeptides. Nano-ESI-quadrupole-TOF MS/MS has been shown to be a sensitive approach that provides information on glycan structure, glycan attachment position, and peptide sequence for mucin-type glycopeptides with serine- or threonine-linked O-glycans. The characterisation of O-glycosylated peptides containing the Tn-antigen (GalNAcα1-), the T-antigen disaccharide, or other, slightly longer O-glycans based on α-linked GalNAc, connected to serine or threonine residues, has been effectively accomplished using this technique. This strategy can also be used to study O-fucosylation and O-linked N-acetylglucosamine. Ion trap and triple-quadrupole equipment have also been used to examine glycopeptides containing O-linked N-acetylglucosamine [60].

The O-glycosylation site(s) can typically be inferred from the fragmentation mass spectra of mucin-type O-glycopeptides produced with ESI-quadrupole-TOF based on the y-type and/or b-type peptide ions comprising the glycan attachment site. These y-type and b-type ions are typically found in three different forms: (1) in deglycosylated form, (2) with the entire O-glycan chain still attached and (3) with a shortened glycan chain. The fragment ion spectra are quite complicated due to this heterogeneity and the superimposition of several charge phases. C-mannosylation is a different kind of glycosylation that has been examined by nano-ESI-quadrupole-TOF. In many proteins, including the human complement system, mannose is found bound to the C2 atom of the tryptophan indole ring. In contrast to O-glycans and N-glycans, the C-linked mannose in CID seemed to be relatively stable [60].

PART 3

MATERIALS AND METHODS

3.1. MATERIALS

All of reagents, chemicals, and proteins, including human plasma (lyophilized) and human IgG, used in this study were purchased from Sigma Aldrich (Milwaukee, WI, USA). PNGase F enzyme was obtained from Promega, while cotton wool was supplied from the local market.

3.2. PREPARATION OF THE COTTON CONTAINING STAGE-TIPS

A little piece of cotton wool ranged amongst (3 to 4 mm lenght) as well as inserted in a pipette tip with the help of hard thin metal wire otherwise a hard-thin plastic sticks this research has utilized 100 µL capacity pipette tips for preparing cotton containing tips as shown in Figure 3.1.

Figure 3.1. A pipette tip with the help of hard thin metal wire otherwise a hard thin plastic stick.

A small hole was made in the led of a microcentrifuge tube, which is associated with the tip was fixed inside the microcentrifuge tube. Moreover, approximately one-third of the pipette tip was placed inside the tube, as presented in Figure 3.2 below.

Figure 3.2. A small hole was made in the led of an eppendorf microcentrifuge tube.

3.3. INTEGRATED OPERATION OF STAGE-TIPS FOR N-GLYCAN PROFILING

All experiments were carried out in stage-tips containing cotton wool. Fifty µg of sample (IgG and mAb, prepared in 22 µg μL^{-1} in water) was diluted with water in a way that it would make a 9 µl volume in the prepared stage-tips and, 1 µl of denaturation buffer (% 5 SDS) was added to this solution. Then, the stage-tips were incubated at 100 °C for 5 min. Following this, 2 µl of reaction buffer (5X PBS), 2 µl of 5 % Igepal-CA630, and 6 µl of water were added to the final solution. For achieving glycan release from glycoproteins, 1 µl of PNGase F enzyme (50 U μL^{-1}) was added and the stage-tips were incubated at 45 °C for 1 hour for rapid *N*-glycan profiling, while 1 U was used for overnight incubation. Before labeling the released *N*-glycans by procainamide, sodium cyanoborohydride (6 mg mL^{-1} in 100 µl of DMSO/glacial acetic acid, 7/3, v/v) and procainamide (11 mg mL^{-1} in 100 µl of DMSO/glacial acetic

acid, 7/3, v/v) were mixed in a ratio of 1:1 by volume, and 9 µl of this mixture was added to the stage-tips. Then, 1-hour incubation was carried out at 70 °C. After the incubation, the purification of proc-labeled *N*-glycans was performed. To create a proper loading condition (85 % ACN) for the purification of procainamide labeled *N*-glycans, 170 µl of ACN was added to stage-tips. Then, the stage-tips were placed into the centrifuge, and the spinning procedure was started. To be able to load procainamide labeled *N*-glycans onto the cotton wool, the stage-tips were centrifuged three times at 4000 rpm for 2 min. In each cycle, the same loading solution was placed into the stage-tips and centrifuged. After loading, the stage-tips were washed respectively with 200 µl of washing buffer 1 ($ACN/H_2O/TFA$, 85/14/1, v/v/v) and washing buffer 2 ($ACN/H_2O/TFA$, 85/15, v/v/v) by using the centrifuge of the stage-tips at 4000 rpm for 2 min. Finally, elution of procainamide with *N*-glycans label was achieved with 30 µl of water. The elution solvent was loaded two times, and the stage-tips were centrifuged at 4000 rpm for 2 min in the elution step. The batch mode experiments were carried out with the same method described above by using Eppendorf tubes, and the purification of procainamide labeled *N*-glycans were performed using cotton wool-containing stage-tips. All experiments were carried out in triplicates.

3.4. INTEGRATED OPERATION OF STAGE-TIPS FOR N-GLYCOPEPTIDE PROFILING

10 µg of IgG (10 mg mL^{-1}) and 5 µL of human plasma (60 mg mL^{-1} in 1X PBS, prepared from lyophilized human plasma) were inserted into the stage-tips containing cotton-HILIC. The final volume of the samples was set to 10 µL by 25 mM of ammonium bicarbonate (ABC). For reduction and alkylation of the proteins, solutions were prepared in 25 mM ABC. First, 10 µL of 10 mM DTT was added to all samples. And the samples were incubated at 56 °C for 20 min. Then, 5 µL of IAA was added to the samples, and alkylation was achieved at room temperature for 15 min. A trypsin enzyme (prepared in 0.1 mg mL^{-1}) was inserted into samples at the ratio of protein: enzyme, 30:1, w: w. the final volume of the samples was then set to 35 µL by 25 mM of ABC. The samples were then incubated with trypsin overnight at 37 °C for

proteolytic digestion. On the following day, the ACN percentage in the sample was brought to 85 by pure ACN (196 µL) for the enrichment of glycopeptides (loading solution). Then, the spinning procedure, including loading, washing, and eluting steps, was started. The stage-tips were centrifuged three times at 4000 rpm for 2 min by loading solution. The same washing and elution steps were also applied as described previously in the glycomic application. The elution volume was 25 µL. The eluted samples were directly analyzed by MALDI-MS.

3.5. MALDI-MS ANALYSIS

A RapiFlex MALDI-TOF/TOF-MS system with SmartBeam 3D laser technology (Bruker Daltonics, Bremen, Germany) was used for *N*-glycopeptide analysis. All analyses were carried out in positive ionization mode with a 1000-4000 Da mass range. The acceleration potential was set to 25 kV. Purified sample solutions (1 µL for each) were spotted directly on the MALDI target plate and left to dry. Each sample spot was then covered with 1 µL of 5 mg mL^{-1} 2,5-DHB (2,5 dihydroxybenzoic acid) prepared using an equal volume of water and ACN. Crystallized samples were analyzed by collecting 10000 lasers at a frequency of 4000 Hz.

3.6. HPLC-HILIC-FLD-QTOF-MS/MS ANALYSIS OF N-GLYCANS

An Agilent 1200 series HPLC including Agilent 1260 fluorescence detector (Agilent Technologies, Santa Clara, USA) equipped with a Bruker TIMS-TOF-MS/MS system was used for procainamide-labeled *N*-glycan analysis of the samples. For the analytical separation of Proc-labelled *N*-glycans via HPLC, Waters Glycan BEH Amide 2.5 µm (2.1 mm ID x 15 cm L) column (Waters, United Kingdom) was utilized. The emissions and excitation wavelengths of the FLD detector were set to 370 and 310, respectively. ACN (mobile phase A) and 50 mM ammonium formate (pH 4.3, mobile phase B) solutions were used in the analysis. Analytical separation of proc-labeled *N*-glycans was performed applying a linear gradient (75-53% mobile phase A) for 60 min. 25 µL of the purified Proc-labelled *N*-glycans were mixed with 75 µL of ACN to achieve

proper loading conditions before analysis. The injection volume was 40 μL, and the flow rate was 0.25 mL min^{-1}.

Mass spectrometric analysis of proc-labeled *N*-glycans was performed by applying the following parameters: source temperature 150 °C; drying gas 6 L min^{-1}; nebulizer gas 1.7 bar; and capillary voltage 4.5 kV. MS analyses were conducted in the mass range of 500-2500 (m/z) with a frequency of 1 Hz. The two most abundant precursor ions were chosen, and MS/MS spectra acquisitions were carried out at a rate of 0.5-2 Hz.

3.7. DATA ANALYSIS

Glycan structures were identified and confirmed using Protein Scape Software V4 (Bruker Daltonics, Germany), and glycan searches were utilized for proc-labeled glycans. In addition, most of the MS/MS spectra were checked manually to determine specific *N*-glycan structures, such as core-fucosylation and bisecting described previously[19, 20]. Search parameters are given in Supplementary Information Figure S1. Relative abundances (%) of proc-labeled *N*-glycans were calculated using the total area normalization approach.

The glycopeptides onto IgG were determined by comparing our data obtained experimentally with the literature [21, 22]. The areas of the detected *N*-glycopeptides were exported from FlexAnalysis software (Bruker Daltonics, Germany). To calculate the relative abundances of glycopeptides, the total area normalization approach was used.

PART 4

RESULT AND DISCUSSION

4.1. EVALUATIONS OF THE DEVELOPED METHOD

The HPLC-HILIC-FLD is used as a golden standard approach for profiling *N*-glycans [78, 79]. In this approach, *N*-glycans must be released enzymatically first and then labeled for quantitative analysis by using fluorophores [24]. However, since the operations are performed in stages, sample losses may occur. In addition, some chemicals such as detergents, salts, etc., which can affect analysis, are used in these processes. Therefore, labeled *N*-glycans must be purified before their HPLC-HILIC-FLD analysis. This additional stage prolongs the sample preparation steps for analysis. In this study, it was aimed to develop a fully integrated stage-tip application to minimize sample loss caused by multiple steps in *N*-glycan release approaches such as PNGase F treatment, glycan labeling or derivatization, and purification. This stage-tip application provides an efficient integrated pretreatment system for *N*-glycan profiling, including glycan purification by cotton-HILIC which is the cheapest material for *N*-glycopeptide enrichment and *N*-glycan purification[21]. In the approach, procainamide was selected as a labeling agent because it improves sensitivities in *N*-glycan analysis performed by instruments containing FLD and MS detection [25, 26].

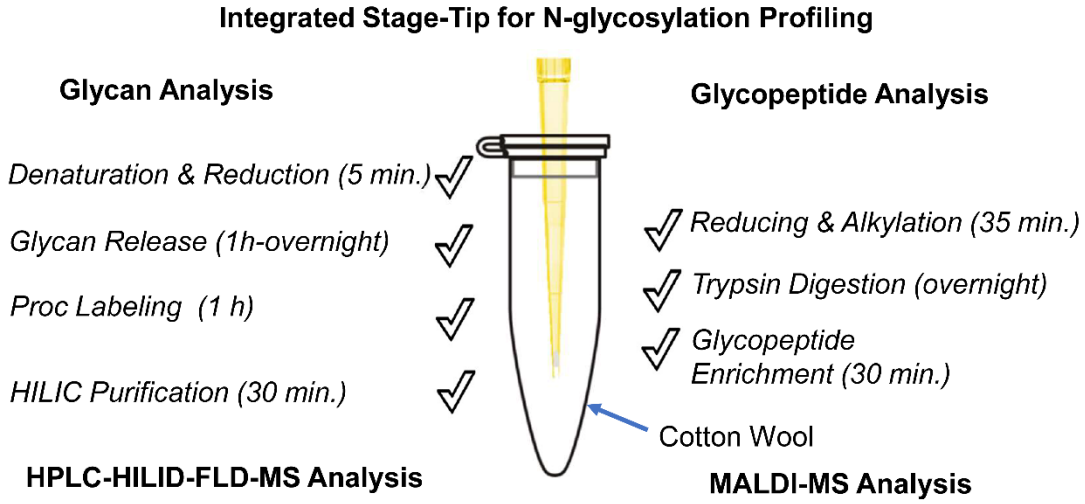

Figure 4.1. The workflow illustration of the method.

Many monoclonal IgG antibodies used for the treatment of various diseases are currently being produced and developed by biotechnology companies. The biological activity of IgGs is modulated by *N*-glycans located in the Fc region of the protein[27]. Therefore, *N*-glycan analyses of monoclonal IgG antibodies are necessary for the development and production of these therapeutic drugs[28]. In addition, *N*-glycosylation profiling of IgG derived from human serum or plasma is one of the most preferred applications to detect *N*-glycan changes in different diseases[29]. Thus, human IgG was used as a model glycoprotein to test the proposed approach. The described protocol was applied as explained in the method section. The resulting procainamide labeled *N*-glycans were analyzed by an HPLC-HILIC-FLD-MS/MS system.

4.2. N-GLYCOSYLATION PROFILING OF IGG BY INTEGRATED STAGE-TIP-BASED GLYCOMIC APPROACH

Figure 4.2 presents the detected *N*-glycans onto IgG using the integrated-stage-tip application. 19 *N*-glycans were determined by the analysis of the samples, and they are shown in Table 4.1. All these *N*-glycans were found to be core-fucosylated by monitoring specific fragments indicated in the literature previously[30]. In addition,

five *N*-glycans were determined as bisected by following the tandem MS fragments presented previously [19].

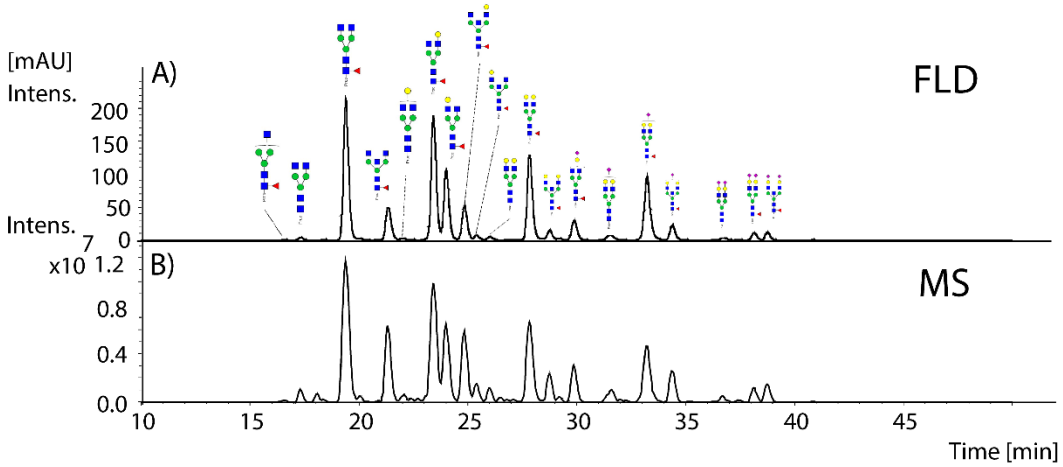

Figure 4.2. *N*-glycan profiling of IgG by using integrated-stage-tips. (A) HPLC-HILIC-FLD and (B) BPC chromatograms of *N*-glycans onto IgG are shown.

Table 4.1. The detected procainamide labeled N-glycans using integrated stage-tips.

Row	Composition	m/z meas.	z	m/z calc.	Δ MH+ [Da]	Rt [min]	Score	FragCov. [%]
1	Hex3HexNAc3dHex1-proc	740.32	2.00	740.33	-0.02	16.18	59.38	47.32
2	Hex3HexNAc4-proc	768.83	2.00	768.84	-0.01	16.90	88.10	95.59
3	Hex3HexNAc4dHex1-proc	841.86	2.00	841.87	-0.01	19.27	83.37	76.23
4	Hex3HexNAc5dHex1-proc	943.40	2.00	943.41	-0.02	20.80	86.40	91.24
5	Hex4HexNAc4-proc	849.86	2.00	849.86	-0.01	21.00	93.71	103.41
6	Hex4HexNAc4dHex1-proc	922.89	2.00	922.89	-0.01	22.85	76.00	63.13
7	Hex4HexNAc4dHex1-proc	922.89	2.00	922.89	-0.01	23.29	80.43	70.63
8	Hex4HexNAc5dHex1-proc	1024.43	2.00	1024.43	-0.01	24.15	84.70	82.90
9	Hex4HexNAc5dHex1-proc	1024.43	2.00	1024.43	-0.01	24.92	71.50	56.00
10	Hex5HexNAc4-proc	620.93	3.00	620.93	-0.01	25.51	105.25	130.61
11	Hex5HexNAc4dHex1-proc	669.61	3.00	669.62	-0.01	27.14	98.80	104.73
12	Hex5HexNAc5dHex1-proc	737.30	3.00	737.31	-0.01	28.21	89.35	86.04
13	Hex4HexNAc4NeuAc1dHex1-proc	712.63	3.00	712.63	-0.01	29.25	82.39	70.00
14	Hex5HexNAc4NeuAc1-proc	717.96	3.00	717.96	-0.01	30.87	89.24	86.09
15	Hex5HexNAc4NeuAc1dHex1-proc	766.64	3.00	766.65	-0.02	32.42	78.74	65.22
16	Hex5HexNAc5NeuAc1dHex1-proc	834.34	3.00	834.34	-0.02	33.86	71.74	53.53
17	Hex5HexNAc4NeuAc2-proc	814.99	3.00	814.99	-0.01	36.17	92.74	92.17
18	Hex5HexNAc4NeuAc2dHex1-proc	863.67	3.00	863.68	-0.02	37.74	74.15	60.45
19	Hex5HexNAc5NeuAc2dHex1-proc	931.37	3.00	931.37	-0.01	38.42	49.72	28.97

4.3. INFLUENCE OF IgG AMOUNT BY THE ANALYSIS OF THE N-GLYCANS USING THE STRATEGY

The presented approach was tested using different amounts of IgG. Figure 4.3 shows the HPLC-FLD chromatograms obtained when different quantities of IgG were used. When the amount of IgG was reduced to 12.5 µg, almost all of the *N*-glycan peaks were detected in the HPLC-FLD chromatogram; however, 50 µg or above was optimum for quantitative analysis.

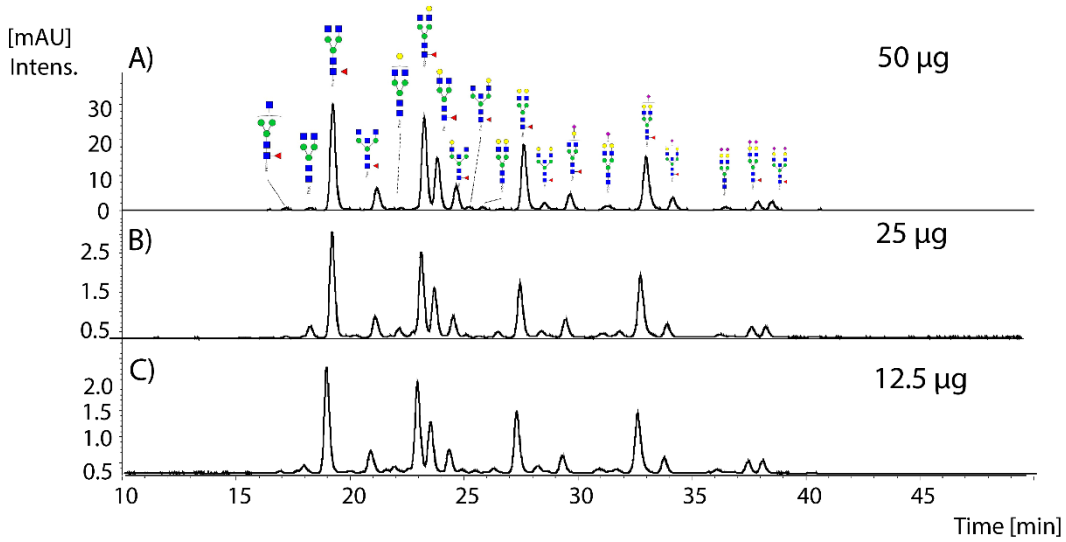

Figure 4.3. *N*-glycan profiling of different amounts of IgG via integrated-stage-tips by using HPLC-HILIC-FLD. (A) 50 µg (B) 25 µg, and (C) 12.5 µg.

4.4. EVALUATION OF GLYCAN RELEASE INCUBATION TIMES IN THE STRATEGY

Rapid sample preparation for *N*-glycan profiling is desired in biopharmaceutical industries because the analysis of many glycosylated biopharmaceuticals is needed during drug development and quality control[31]. Therefore, the incubation time used in *N*-glycan release was also evaluated in terms of fast *N*-glycan profiling. Figure 4.4 shows the HPLC-FLD chromatograms of IgG *N*-glycans for two different incubation times. It was found that one-hour incubation time applied for *N*-glycan release was sufficient for *N*-glycan profiling by HPLC-HILIC-FLD.

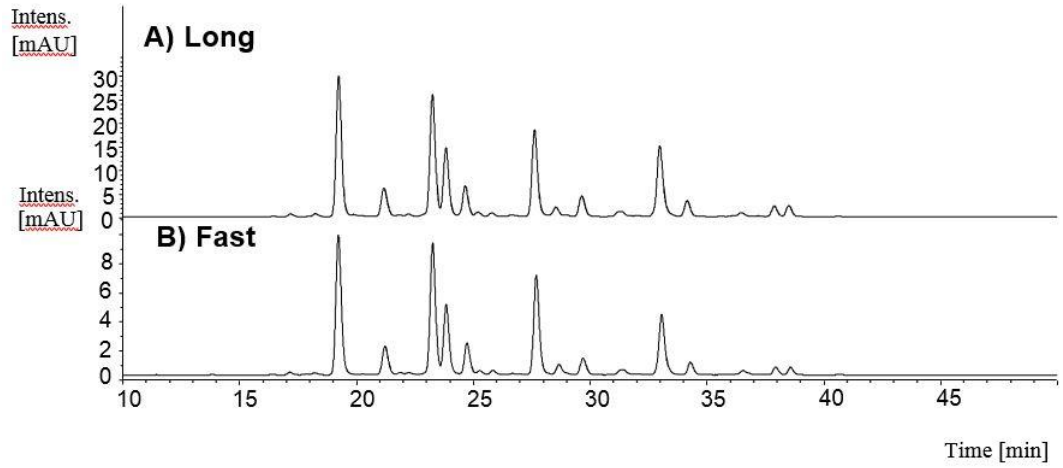

Figure 4.4. *N*-glycan profiling of IgG *N*-glycans via integrated-stage-tips with different incubation times by using PNGase F enzyme. (A) overnight incubation (B) 1 h incubation.

4.5. EVALUATION OF RELATIVE ABUNDANCES OF N-GLYCANS IN THE STRATEGY

The total area normalization approach was used to calculate the relative abundances of N-glycans. First, the relative abundances of *N*-glycans, calculated using different amounts of IgG, were compared. Figure 4.5 presents the relative abundances of *N*-glycans obtained from different amounts of IgG. It was determined in the analysis that there were minor differences between the relative abundances of *N*-glycans. When the IgG amount was decreased to 25 µg and 12.5 µg, the *N*-glycan profiles were observed to change. This may be due to the fact that the tiny peaks detected in the chromatogram could not be accurately quantified. When 200 µg, 50 µg, 25 µg, and 12.5 µg of IgG was employed, the average %CV of the method was determined to be 8.1, 14.3, 16.6, and 37.5, respectively (Table 4.2.). In addition, *N*-glycan profiling was performed by the fast method (FM, Figure 4.4.). The mean %CV value was determined to be 6.4 for the rapid application of the proposed approach. The repeatability of the proposed method was found to be satisfactory in the fast application of integrated stage-tip.

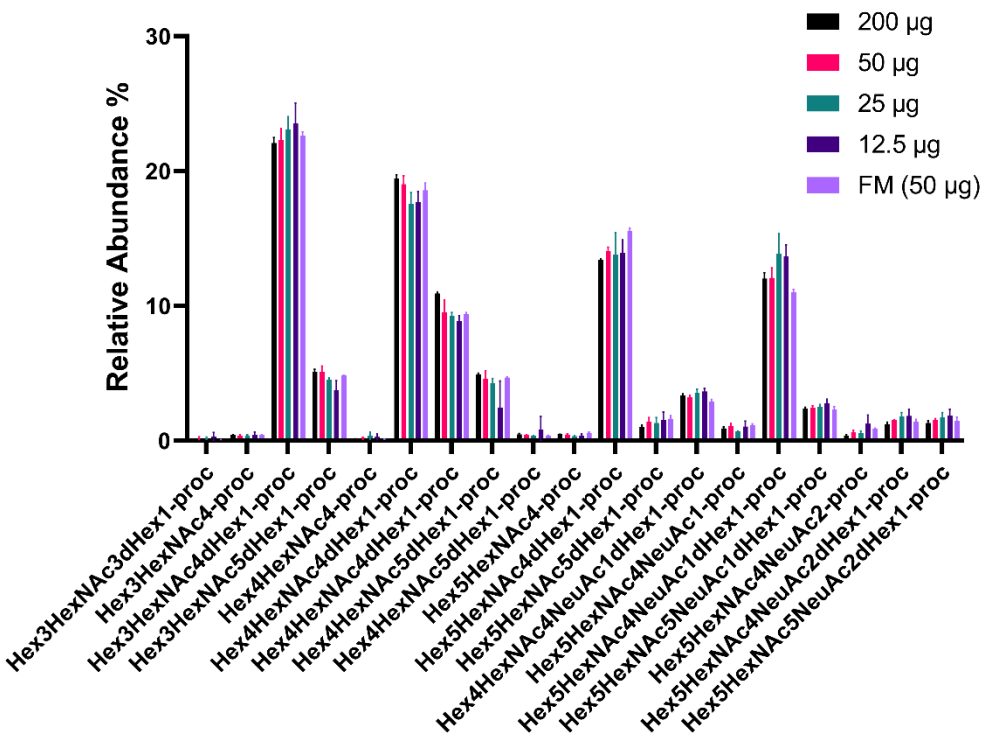

Figure 4.5. Relative abundances of IgG *N*-glycans obtained from integrated-stage-tip applications.

Table 4.2. Relative abundances of N-glycans for different amount of IgG using integrated stage-tips.

		200 µg		**50 µg**		**25 µg**		**12.5 µg**		**(FM) 50 µg**	
Row		Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV
1	Hex3HexNAc3dHex1-proc	0.1	9.3	0.2	56.4	0.2	46.9	0.3	104.6	0.1	6.1
2	Hex3HexNAc4-proc	0.4	5.8	0.4	14.8	0.4	21.4	0.4	46.3	0.4	5.4
3	Hex3HexNAc4dHex1-proc	22.1	1.9	22.3	3.8	23.1	4.0	23.5	6.6	22.6	1.3
4	Hex3HexNAc5dHex1-proc	5.1	3.2	5.1	8.4	4.5	3.6	3.8	18.4	4.8	0.5
5	Hex4HexNAc4-proc	0.1	20.0	0.2	34.7	0.4	66.1	0.3	76.8	0.1	4.7
6	Hex4HexNAc4dHex1-proc	19.4	1.5	19.0	3.4	17.5	4.9	17.7	4.5	18.6	2.9
7	Hex4HexNAc4dHex1-proc	10.9	1.1	9.5	9.6	9.3	2.7	8.9	4.6	9.4	1.3
8	Hex4HexNAc5dHex1-proc	4.9	2.1	4.6	14.0	4.2	8.8	2.5	80.4	4.7	1.2
9	Hex4HexNAc5dHex1-proc	0.5	16.9	0.4	13.0	0.4	1.6	0.8	126.7	0.4	6.7
10	Hex5HexNAc4-proc	0.5	3.5	0.4	16.8	0.3	15.0	0.4	30.7	0.6	11.5
11	Hex5HexNAc4dHex1-proc	13.4	0.6	14.1	2.1	13.8	11.8	13.9	6.8	15.5	1.6
12	Hex5HexNAc5dHex1-proc	1.0	14.0	1.4	23.1	1.3	31.5	1.5	39.8	1.6	15.2
13	Hex4HexNAc4NeuAc1dHex1-proc	3.4	3.1	3.2	5.9	3.5	7.4	3.6	6.9	2.9	5.0
14	Hex5HexNAc4NeuAc1-proc	0.9	12.3	1.1	19.1	0.7	3.9	1.0	38.5	1.1	8.7
15	Hex5HexNAc4NeuAc1dHex1-proc	12.0	3.7	12.1	6.1	13.8	11.1	13.7	6.3	11.0	1.9
16	Hex5HexNAc5NeuAc1dHex1-proc	2.4	3.5	2.4	7.1	2.5	8.0	2.8	11.4	2.3	9.1
17	Hex5HexNAc4NeuAc2-proc	0.4	24.4	0.6	24.1	0.6	26.7	1.3	51.9	0.9	4.9
18	Hex5HexNAc4NeuAc2dHex1-proc	1.2	14.4	1.5	1.7	1.8	18.5	1.8	27.5	1.4	13.5
19	Hex5HexNAc5NeuAc2dHex1-proc	1.3	12.9	1.5	7.7	1.7	21.7	1.9	24.5	1.5	19.4
	Mean %CV		8.1		14.3		16.6		37.5		6.4

4.6. N-GLYCOSYLATION PROFILING OF IGG BY INTEGRATED STAGE-TIP-BASED GLYCOPROTEOMIC APPROACH

In the glycoproteomic application of integrated stage-tip, IgG and human plasma were digested using a classical bottom-up proteomic approach, described in the method section, to profile intact IgG glycopeptides by MALDI-MS. Because ordinary peptides suppress the signals of N-glycosylated peptides in MALDI-MS analysis, enrichment of IgG *N*-glycopeptides is recommended [22, 32]. This approach allows rapid enrichment of IgG *N*-glycopeptides directly after proteolytic digestion by the stage-tips containing cotton-HILIC. In particular, this minimizes sample loss in the enrichment processes applied before the analysis of IgG *N*-glycopeptides with MALDI-MS. Figure 4.6 shows the IgG N-glycopeptides detected by MALDI-MS obtained with integrated stage-tip applications. N-glycopeptides onto purified IgG isolated from human serum are displayed in Figure 4.6.A, while the MALDI-MS spectrum obtained from the analysis of human plasma glycoproteome is shown in Figure 4.6.B. When the mass range of IgG *N*-glycopeptides of the spectra was examined, 28 and 17 *N*-glycopeptides onto IgG were determined directly in purified IgG and human plasma, respectively (Table 4.3.). This allows analysis of IgG N-glycopeptides by MALDI-MS without isolation of IgG by specific methods, such as affinity purification from human plasma in particular. An important step, which is often used in the analysis of IgG *N*-glycopeptides, is eliminated by this approach. Thus, it may not be necessary to use the IgG isolation process, which is both costly and time-consuming, thanks to the integrated stage-tip approach developed for N-glycosylation analysis. Profiling of intact IgG glycopeptides was successfully achieved by the developed integrated stage-tip application (Table 4.3.). This application can also be integrated with direct *N*-glycopeptide profiling of IgG performed by nLC-MS/MS.

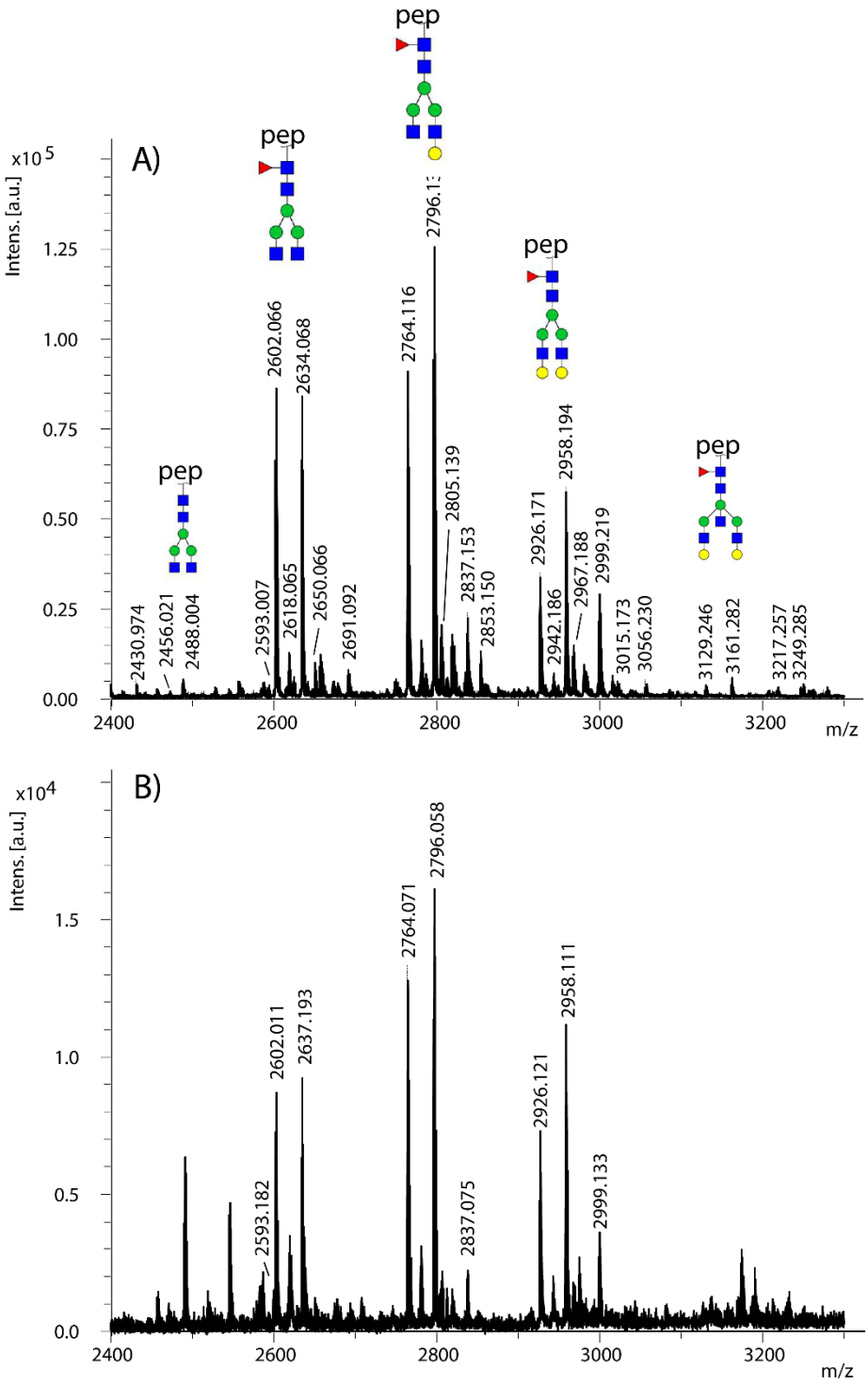

Figure 4.6. MALDI-MS analysis of IgG *N*-glycopeptides using integrated-stage-tips obtained from (A) purified IgG and (B) human plasma.

Table 4.3. The detected IgG N-glycopeptides using integrated stage-tips from purified IgG and human plasma.

								Relative Abundances %			
	Peptide Sequence	**Glycan Structure**	**N-Glycopeptides**	**N-Glycopeptides**	**Mass Error**	**Detected *N*-glycopeptides**		**Purified IgG**		**Human Plasma**	
No			**Theoretical Mass (m/z)**	**Observed Mass (m/z)**	**(Da)**	**Purified IgG**	**Human Plasma**	**Mean**	**%CV**	**Mean**	**%CV**
1	EEQFN*STFR (IgG2)	Hex3HexNAc3Fuc1	2398.977	2399.014	0.037	+		0.19	12.68		
2	EEQYN*STYR (IgG1)	Hex3HexNAc3Fuc1	2430.966	2430.974	0.008	+		0.34	55.76		
3	EEQFN*STFR (IgG2)	Hex3HexNAc4	2455.998	2456.021	0.023	+	+	0.16	15.46	1.50	7.30
4	EEQYN*STYR (IgG1)	Hex3HexNAc4	2487.988	2488.004	0.016	+		0.77	17.26		
5	EEQYN*STYR (IgG1)	Hex4HexNAc3Fuc1	2593.019	2593.037	0.018	+	+	0.14	3.73	0.24	141.42
6	EEQFN*STFR (IgG2)	Hex3HexNAc4Fuc1	2602.056	2602.066	0.01	+	+	13.40	14.19	11.86	24.10
7	EEQFN*STFR (IgG2)	Hex4HexNAc4	2618.051	2618.065	0.014	+	+	1.76	3.71	3.54	93.91
8	EEQYN*STYR (IgG1)	Hex3HexNAc4Fuc1	2634.046	2634.068	0.022	+	+	10.08	1.97	9.35	23.60
9	EEQYN*STYR (IgG1)	Hex4HexNAc4	2650.041	2650.066	0.025	+	+	1.13	15.64	3.30	30.64
10	EEQYN*STYR (IgG1)	Hex3HexNAc5	2691.067	2691.092	0.025	+		1.45	41.29		
11	EEQFN*STFR (IgG2)	Hex4HexNAc4Fuc1	2764.109	2764.116	0.007	+	+	15.26	15.33	14.31	14.18
12	EEQFN*STFR (IgG2)	Hex5HexNAc4	2780.104	2780.122	0.018	+	+	2.05	4.87	3.50	22.94
13	EEQYN*STYR (IgG1)	Hex4HexNAc4Fuc1	2796.099	2796.13	0.031	+	+	16.96	3.35	14.99	21.67
14	EEQFN*STFR (IgG2)	Hex3HexNAc5Fuc1	2805.135	2805.139	0.004	+	+	3.54	7.96	2.44	1.26
15	EEQYN*STYR (IgG1)	Hex5HexNAc4	2812.094	2812.129	0.035	+	+	0.80	18.04	0.99	21.68
16	EEQYN*STYR (IgG1)	Hex3HexNAc5Fuc1	2837.125	2837.153	0.028	+	+	2.97	8.87	2.81	6.08

17	EEQYN*STYR (IgG1)	Hex4HexNAc5	2853.12	2853.15	0.03	+		2.55	54.27		
18	EEQFN*STFR (IgG2)	Hex5HexNAc4Fuc1	2926.162	2926.171	0.009	+	+	6.22	14.46	11.23	9.75
19	EEQFN*STYR (IgG4)	Hex5HexNAc4Fuc1	2942.157	2942.186	0.029	+	+	0.98	17.12	2.54	74.11
20	EEQYN*STYR (IgG1)	Hex5HexNAc4Fuc1	2958.152	2958.194	0.042	+	+	8.53	12.82	10.96	16.36
21	EEQFN*STFR (IgG2)	Hex4HexNAc5Fuc1	2967.188	2967.192	0.004	+	+	2.86	6.26	3.27	43.45
22	EEQYN*STYR (IgG1)	Hex4HexNAc5Fuc1	2999.178	2999.219	0.041	+	+	4.23	18.08	3.18	29.61
23	EEQYN*STYR (IgG1)	Hex5HexNAc5	3015.173	3015.204	0.031	+		1.38	54.98		
24	EEQFN*STFR (IgG2)	Hex4HexNAc4Fuc1NeuAc1	3055.204	3055.208	0.004	+		0.13	24.66		
25	EEQFN*STFR (IgG2)	Hex5HexNAc5Fuc1	3129.241	3129.246	0.005	+		0.48	87.29		
26	EEQYN*STYR (IgG1)	Hex5HexNAc5Fuc1	3161.231	3161.282	0.051	+		0.79	14.80		
27	EEQFN*STFR (IgG2)	Hex5HexNAc4Fuc1NeuAc1	3217.257	3217.237	-0.02	+		0.16	4.79		
28	EEQYN*STYR (IgG1)	Hex5HexNAc4Fuc1NeuAc1	3249.247	3249.285	0.038	+		0.69	1.93		

The number of new-generation stage-tip applications in the rapid and precise determination of glycosylation sites has increased in the literature [14, 17, 33]. In these studies, different adsorbents such as different C18 or SCX have been usually included in application. The reason for this is to ensure the removal of salts in the sample preparation, which can create extra costs in terms of stage-tip preparation. In this study, we developed a strategy using only cotton wool for both purifications of glycans and enrichment of glycopeptides without desalting steps from a complex medium such as human plasma. This makes the developed method much more convenient than other methods in terms of cost. In addition, the glycoproteomic approach can be further shortened by combining it with next-generation proteolytic digestion processes, such as microwave-assisted approaches. Moreover, by integrating membranes, such as polyvinylidene fluoride (PVDF), into the stage-tips, analyses of much lower amounts of glycoproteins can be performed with different mass spectrometric methods.

PART 5

CONCLUSION

In this study, a bioanalytical method with integrated-stage-tips was developed for rapid and efficient profiling of *N*-glycans and *N*-glycopeptides. The developed method did not only include quick sample preparation methods but was also inexpensive. It allowed *N*-glycan profiling of IgG with a total sample preparation period of 2.5 hours. In addition, it was able to be used for efficient analysis of IgG *N*-glycopeptides by MALDI-MS. It was observed that IgG *N*-glycopeptides were detectable from human plasma even when analyzed directly after the application of integrated-stage-tips. Based on the results, it can be said that the developed method allows the analysis of IgG from human plasma without purification performed by methods such as affinity chromatography. Thus, many clinical samples can be quickly analyzed and evaluated regarding glycosylation by using this method.

REFERENCES

1. Bertozzi, C. R., Freeze, H. H., Varki, A., & Esko, J. D., "Glycans in biotechnology and the pharmaceutical industry", ***Cold Spring Harbor,*** USA, 44-57(2009).

2. Jung, S. T., Kang, T. H., Kelton, W., & Georgiou, G., "Bypassing glycosylation: engineering aglycosylated full-length IgG antibodies for human therapy", ***Current Opinion in Biotechnology,*** 22(6): 858-867 (2011).

3. Uddin, M., & Ashraf, G. M., *"Quality control of cellular protein in neurodegenerative disorders",* ***IGI Global,*** Saudi Arabia, 23-31 (2020).

4. Hounsell, E. F., Davies, M. J. & Renouf, D. V., "O-linked protein glycosylation structure and function", ***Glycoconj. J.,*** 13(1): 19–26 (1996).

5. Lithgow, K. V., Scott, N. E., Iwashkiw, J. A., Thomson, E. L., Foster, L. J., Feldman, M. F., & Dennis, J. J., "A general protein O-glycosylation system within the B urkholderia cepacia complex is involved in motility and virulence", ***Molecular Microbiology,*** *92*(1): 116-137 (2014).

6. Vik, Å., Aas, F. E., Anonsen, J. H., Bilsborough, S., Schneider, A., Egge-Jacobsen, W.,&Koomey, M., "Broad spectrum O-linked protein glycosylation in the human pathogen Neisseria gonorrhoeae", ***Proceedings of the National Academy of Sciences,*** 106(11): 4447-4452 (2009).

7. Iwashkiw, J. A., Seper, A., Weber, B. S., Scott, N. E., Vinogradov, E., Stratilo, C., & Feldman, M. F., "Identification of a general O-linked protein glycosylation system in Acinetobacter baumannii and its role in virulence and biofilm formation", ***PLoS Pathogens,*** 8(6): 102-758 (2012).

8. Sawyers, C. L., "The cancer biomarker problem", ***Nature,*** 452(7187): 548-552 (2008).

9. Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D., "Global cancer statistics", ***CA: A Cancer Journal for Clinicians***, 61(2): 69-90 (2011).

10. Siegel, R. L., Miller, K. D., & Jemal, A., "Cancer statistics, 2016", ***CA: A Cancer Journal for Clinicians,*** 66(1): 7-30 (2016).

11. Kailemia, M. J., Park, D., & Lebrilla, C. B., "Glycans and glycoproteins as specific biomarkers for cancer", ***Analytical and Bioanalytical Chemistry,*** 409(2): 395-410 (2017).

12. Liu, L., "Antibody glycosylation and its impact on the pharmacokinetics and pharmacodynamics of monoclonal antibodies and Fc-fusion proteins", ***Journal of Pharmaceutical Sciences,*** 104(6): 1866-1884 (2015).

13. Lechner, A., Giorgetti, J., Gahoual, R., Beck, A., Leize-Wagner, E., & François, Y. N., "Insights from capillary electrophoresis approaches for characterization of monoclonal antibodies and antibody drug conjugates in the period 2016–2018", ***Journal of Chromatography B,*** 11(22): 1-17 (2019).

14. Zhang, L., Luo, S., & Zhang, B., "Glycan analysis of therapeutic glycoproteins", ***Taylor & Francis,*** 8(2): 205-215 (2016).

15. Patterson. M.C., "Metabolic imitators: N-bound glycosyl binding disorders", ***Semin Pediatr Neurol,*** 12(3): 44–51 (2005).

16. Dell. A., Galadari. A., Hitchen. P., "Similarities and differences in the mechanisms of binding to glycosyl in prokaryotes and eukaryotes", ***Int. J. Microbiol.,*** 3(2): 1–14 (2010).

17. Drickamer, T. M. K, "An Introduction to Glycology", ***Oxford University Press,*** UK, 89- 102 (2006).

18. Bovin, N. V., "Carbohydrate-carbohydrate interaction", ***Biokhimiia,*** 6(61): 968-983(1996).

19. Adam W., Caulfield J. & Nancy L., "Division of Clinical Microbiology", ***J. Clin. Tuberc.Other Mycobact. Dis.,*** 55(5): 200–300 (2016).

20. Ho et al., "Electrospray ionisation mass spectrometry: principles and clinical applications", ***Clin. Biochem. Rev.,*** 24(1):3-9 (2003).

21. Hillenkamp, F., Karas, M., Beavis, R. C., & Chait, B. T., "Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers", ***Analytical Chemistry,*** 63(24): 1193-1203 (1991).

22. Zhigilei, L. V., Leveugle, E., Garrison, B. J., Yingling, Y. G., & Zeifman, M. I., "Computer simulations of laser ablation of molecular substrates", ***Chemical Reviews,*** 103(2): 321-348 (2003).

23. Buckingham, A. D., "Molecular quadrupole moments", ***Quarterly Reviews, Chemical Society,*** 13(3): 183-214 (1959).

24. Buckingham, A. D., Disch, R. L., & Dunmur, D. A., "Quadrupole moments of some simple molecules", ***Journal of the American Chemical Society,*** 90(12): 3104-3107 (1968).

25. March, R. E., & Todd, J. F., "Quadrupole ion trap mass spectrometry", ***John Wiley & Sons,*** Texas, USA, 67-78 (2005).

26. Mertz, C., Vellidis, C. E., Alarcon, R., Barkhuff, D. H., Bernstein, A. M., Bertozzi, W., ... & Young, A., "Search for Quadrupole Strength in the Electroexcitation of the Δ+(1232)", ***Physical Review Letters,*** 86(14): 2963 (2001).

27. Xian, F., Hendrickson, C. L., & Marshall, A. G., "High resolution mass spectrometry", ***Analytical Chemistry,*** 84(2): 708-719 (2012).

28. Paša-Tolić, L., Huang, Y., Guan, S., Kim, H. S., & Marshall, A. G., "Ultrahigh-resolution matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectra of peptides", ***Journal of Mass Spectrometry,*** 30(6): 825-833 (1995).

29. Ruotolo, B. T., Verbeck IV, G. F., Thomson, L. M., Woods, A. S., Gillig, K. J., & Russell, D. H., "Distinguishing between phosphorylated and nonphosphorylated peptides with ion mobility− mass spectrometry", ***Journal of Proteome Research,*** 1(4): 303-306 (2002).

30. Hanić, M., Lauc, G., & Trbojević-Akmačić, I., "N-Glycan Analysis by Ultra-Performance Liquid Chromatography and Capillary Gel Electrophoresis with Fluorescent Labeling", ***Current Protocols in Protein Science,*** 97(1): 95 (2019).

31. Snyder, L. R., Kirkland, J. J., & Dolan, J. W., "*Introduction to modern liquid chromatography"*, ***John Wiley & Sons,*** Texas, USA, 23-35 (2011).

32. Niessen, W. M., "Liquid chromatography-mass spectrometry", ***Crc Press,*** New York, 56- 67 (2006).

33. Meyer, V. R., "Practical high-performance liquid chromatography", ***John Wiley & Sons,*** Texas, USA, 12-22 (2013).

34. Covey, T. R., Lee, E. D., Bruins, A. P., & Henion, J. D., "Liquid chromatography/mass spectrometry", ***Analytical Chemistry,*** 58(14): 1451-1461 (1986).

35. Lee, Y. H., & Lin, T. I., "Determination of carbohydrates by high-performance capillary electrophoresis with indirect absorbance detection", ***Journal of Chromatography B: Biomedical Sciences and Applications,*** 681(1): 87-97 (1996).

36. Bouche, L., "Glycoproteomic research using mass spectrometry", ***Imperial College London Department of Life Sciences,*** UK, 67-99 (2017).

37. Mokhtar, M. N., "Biocatalytic Production, Preparation and Characterization of Large-ring Cyclodextrins", ***Faculty of Mechanical Engineering, Chemnitz University of Technology,*** Chemnitz, German, 31-39 (2009).

38. Bermingham, M. L., Colombo, M., McGurnaghan, S. J., Blackbourn, L. A., Vučković, F., Baković, M. P. & Colhoun, H. M., "N-glycan profile and kidney disease in type 1 diabetes", ***Diabetes Care,*** 41(1): 79-87 (2018).

39. Halbach, K., Wagner, S., Scholz, S., Luckenbach, T., & Reemtsma, T., "Elemental imaging (LA-ICP-MS) of zebrafish embryos to study the toxicokinetics of the acetylcholinesterase inhibitor naled", ***Analytical and Bioanalytical Chemistry,*** 411(3):617-627 (2019)

40. Lü, Y., Yang, H., Hu, H., Wang, Y., Rao, Z., & Jin, C., "Mutation of Trp137 to glutamate completely removes transglycosyl activity associated with the Aspergillus fumigatus AfChiB1", ***Glycoconjugate Journal,*** 26(5): 525-534 (2009).

41. Tokuyasu, K., Ono, H., Hayashi, K., & Mori, Y., "Reverse hydrolysis reaction of chitin deacetylase and enzymatic synthesis of β-d-GlcNAc-(1→4)-GlcN from chitobiose", ***Carbohydrate Research,*** 322(2): 26-31 (1999).

42. Mekasha, S., Toupalová, H., Linggadjaja, E., Tolani, H. A., Anděra, L., Arntzen, M. Ø., & Agger, J. W., "A novel analytical method for d-glucosamine quantification and its application in the analysis of chitosan degradation by a minimal enzyme cocktail", ***Carbohydrate Research,*** 43(3): 18-24 (2016).

43. Zhang, Z., Li, C., Wang, Q., & Zhao, Z. K., "Efficient hydrolysis of chitosan in ionic liquids", ***Carbohydrate Polymers,*** *78*(4): 685-689 (2009).

44. Xiong, C., Wu, H., Wei, P., Pan, M., Tuo, Y., Kusakabe, I., & Du, Y., "Potent angiogenic inhibition effects of deacetylated chitohexaose separated from chitooligosaccharides and its mechanism of action in vitro", ***Carbohydrate Research,*** 344(15): 1975-1983 (2009).

45. Nakagawa et al., "Screening of modified N-glycan profiles in complete serum from rheumatoid arthritis patients", ***Chromatogr. J.,*** 853(2): 7–133 (2007).

46. Maverakis, E., Kim, K., Shimoda, M., Gershwin, M. E., Patel, F., Wilken, R. & Lebrilla, C. B., "Glycans in the immune system and The Altered Glycan Theory of Autoimmunity:a critical review", ***Journal of Autoimmunity,*** 5(7):1-13 (2015).

47. Sinclair, A. M., & Elliott, S., "Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins", ***Journal of Pharmaceutical Sciences,*** 94(8):1626- 1635 (2005).

48. Knott, S. R., Wagenblast, E., Khan, S., Kim, S. Y., Soto, M., Wagner, M. & Hannon, G.J., "Asparagine bioavailability governs metastasis in a model of breast cancer", ***Nature,*** *554*(76):378-381 (2018).

49. Huang, H., Vandekeere, S., Kalucka, J., Bierhansl, L., Zecchin, A., Brüning, U. & Carmeliet, P., "Role of glutamine and interlinked asparagine metabolism in vessel formation", ***The EMBO Journal,*** *36*(16): 2334-2352 (2017).

50. Lomelino, C. L., Andring, J. T., McKenna, R., & Kilberg, M. S., "Asparagine synthetase: function, structure, and role in disease", ***Journal of Biological Chemistry,*** *292*(49): 19952-19958 (2017).

51. Chiu, M., Taurino, G., Bianchi, M. G., Kilberg, M. S., & Bussolati, O., "Asparagine synthetase in cancer: beyond acute lymphoblastic leukemia", ***Frontiers in Oncology,*** *9(2):* 14-80 (2020).

52. Luo, M., Brooks, M., & Wicha, M. S., "Asparagine and glutamine: co-conspirators fueling metastasis", ***Cell Metabolism,*** *27*(5): 947-949 (2018).

53. Kayılı, H. M., "Devekuşu yumurta akı ve sarısı glikoproteomlarından izole edilmiş n-glikanların HPLC-HILIC-FLD-MS/MS ile derinlemesine profillenmesi", ***Phys. Rev.***, 4(7): 777-780 (2021).

54. Kayili, H. M., "In-depth Profiling of N-glycans Isolated from Ostrich Egg White and Yolk Glycoproteomes by HPLC-HILIC-FLD-MS/MS", ***Adıyaman University Journal ofScience,*** 11(1): 191-204 (2021).

55. Segu, Z., Stone, T., Berdugo, C., Roberts, A., Doud, E., & Li, Y., "A rapid method for relative quantification of N-glycans from a therapeutic monoclonal antibody during trastuzumab biosimilar development", ***Taylor & Francis,*** 12(2): 1750-794 (2020).

56. Alpert, A. J., "Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds", ***Journal of Chromatography A,*** 49(9): 177- 196 (1990).

57. Huddleston, M. J., Bean, M. F., and Carr, S. A., "Collisional fragmentation of glycopeptides by electrospray ionization LC/MS and LC/MS/MS: methods for selective detection of glycopeptides in protein digests", ***Analytical Chemistry***, 65 (7): 877–884 (1993).

58. Carr, S. A., Huddleston, M. J., and Bean, M. F., "Selective identification and differentiation of N-and O-linked oligosaccharides in glycoproteins by liquid chromatography-mass spectrometry", ***Protein Science***, 2 (2): 183–196 (1993).

59. Settineri, C. A., Medzihradszky, K. F., Masiarz, F. R., Burlingame, A. L., Chu, C., and George-Nascimento, C., "Characterization of O-glycosylation sites in recombinant B-chain of platelet-derived growth factor expressed in yeast using liquid secondary ion mass spectrometry, tandem mass spectrometry and edman sequence analysis", ***Biomedical & Environmental Mass Spectrometry***, 19 (11): 665–676 (1990).

60. Wuhrer, M., Catalina, M. I., Deelder, A. M., and Hokke, C. H., "Glycoproteomics based on tandem mass spectrometry of glycopeptides Cite this paper Glycoproteomics based on tandem mass spectrometry of glycopeptides", ***Journal Of Chromatography B***, 849: 115–128 (2007).

61. Hanisch, F., Green, B. N., Bateman, R., and Peter-Katalinic, J., "Localization of o-glycosylation sites of MUC1 tandem repeats by QTOF ESI mass spectrometry", ***Journal Of Mass Spectrometry***, 33 (4): 358–362 (1998).

62. Grass, J., Pabst, M., Chang, M., Wozny, M., & Altmann, F., "Analysis of

recombinant human follicle-stimulating hormone (FSH) by mass spectrometric approaches", ***Analytical and Bioanalytical Chemistry,*** 400(8): 2427-2438 (2011).

63. Desaire, H., "Glycopeptide analysis, recent developments and applications", ***Molecular& Cellular Proteomics,*** 12(4): 893-901 (2013).

64. Devabhaktuni, A., Lin, S., Zhang, L., Swaminathan, K., Gonzalez, C. G., Olsson, N. & Elias, J. E., "TagGraph reveals vast protein modification landscapes from large tandem mass spectrometry datasets", ***Nature Biotechnology,*** 37(4): 469-479 (2019).

65. Gouveia, D., Grenga, L., Gaillard, J. C., Gallais, F., Bellanger, L., Pible, O., & Armengaud, J., "Shortlisting SARS-CoV-2 peptides for targeted studies from experimental data-dependent acquisition tandem mass spectrometrydata", ***Proteomics,*** 20(14): 20-107 (2020).

66. Chen, D., Yang, Z., Shen, X., & Sun, L., "Capillary Zone Electrophoresis-Tandem Mass Spectrometry as an Alternative to Liquid Chromatography-Tandem Mass Spectrometryfor Top-down Proteomics of Histones", ***Analytical Chemistry,*** 93(10): 4417-4424 (2021).

67. McNaught, A. D., "Compendium of chemical terminology", ***Blackwell Science,*** Oxford***,*** 45-78 (1997).

68. Boune, S., Hu, P., Epstein, A. L., & Khawli, L. A., "Principles of N-linked glycosylation variations of IgG-based therapeutics: pharmacokinetic and functional considerations", ***Antibodies,*** 9(2): 22 (2020).

69. Fernández de la Ossa, M. Á., Ortega-Ojeda, F., & García-Ruiz, C., "Analysis and differentiation of paper samples by capillary electrophoresis and multivariate analysis", ***Electrophoresis,*** 35(22): 3264-3271 (2014).

70. Kipanga, Z. H., "Factors associated with noncompliance to tuberculosis treatment among patients attending Municipal Hospitals", ***Doctoral Dissertation, Hubert Kairuki Memorial University,*** Tanzania, 32-38 (2017).

71. Kayili, H. M., "Identification of bisecting N-glycans in tandem mass spectra using a procainamide labeling approach for in-depth N-glycan profiling of biological samples", ***International Journal of Mass Spectrometry,*** 45(7): 116-412 (2020).

72. Kozak, R. P., Tortosa, C. B., Fernandes, D. L., & Spencer, D. I., "Comparison of procainamide and 2-aminobenzamide labeling for profiling and identification of glycans by liquid chromatography with fluorescence detection coupled to electrospray ionization– mass spectrometry", ***Analytical Biochemistry,*** 48(6): 38-40 (2015).

73. Keser, T., Pavić, T., Lauc, G., & Gornik, O., "Comparison of 2-aminobenzamide,

procainamide and RapiFluor-MS as derivatizing agents for high-throughput HILIC-UPLC-FLR-MS N-glycan analysis", ***Frontiers in Chemistry,*** 6(1): 324 (2018).

74. Nwosu, C., Yau, H. K., & Becht, S., "Assignment of core versus antenna fucosylation types in protein N-glycosylation via procainamide labeling and tandem mass spectrometry", ***Analytical Chemistry,*** 87(12): 5905-5913 (2015).

75. Kayili, H.M., "Identification of bisecting N-glycans in tandem mass spectra using a procainamide labeling approach for in-depth N-glycan profiling of biological samples", ***International Journal of Mass Spectrometry***, 45(7): 116-412 (2020).

76. Huhn, M.H. Selman, L.R. Ruhaak, A.M. Deelder, M., "IgG glycosylation analysis", ***Proteomics*** 9(2): 882-913 (2009).

77. Hinneburg, H., Stavenhagen, K., Schweiger-Hufnagel, U., Pengelley, S., Jabs, W., Seeberger, P. H. & Kolarich, D., "The art of destruction: optimizing collision energies in quadrupole-time of flight (Q-TOF) instruments for glycopeptide-based glycoproteomics", ***Journal of The American Society for Mass Spectrometry,*** 27(3): 507-519 (2016).

78. Huhn, C., Selman, M.H., Ruhaak, L.R., Deelder, A.M., Wuhrer, M. "IgG glycosylation analysis", ***Proteomics,*** 9 (2): 882-913 (2009).

79. Li, J., Wang, F., Liu, J., Xiong, Z., Huang, G., Wan, H. & Zou, H., "Functionalizing with glycopeptide dendrimers significantly enhances the hydrophilicity of the magnetic nanoparticles", ***Chemical Communications,*** *51*(19): 4093-4096 (2015).

RESUME

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