ANALYSIS OF HUMAN PLASMA N-GLYCOPEPTIDES BY MASS SPECTROMETRY USING HIGH-PH FRACTIONATION AND HILIC-BASED STRATEGIES

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Nabil TAHHAN

Thesis Advisor
Assoc. Prof. Dr Hacı Mehmet KAYILI

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Nabil TAHHAN

Thesis Advisor
Assoc. Prof. Dr. Hacı Mehmet KAYILI

T.C.
Karabuk University
Institute of Graduate Programs
Department of Biomedical Engineering

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I certify that in my opinion the thesis submitted by Nabil TAHHAN titled "ANALYSIS OF HUMAN PLASMA N-GLYCOPEPTIDES BY MASS SPECTROMETRY USING HIGH-PH FRACTIONATION AND HILIC-BASED STRATEGIES" is fully adequate in scope and in quality as a thesis for the degree of Master of Science.

Assist. Prof. Dr. Hacı Mehmet KAYILI .....
Thesis Advisor, Department of, Department of Biomedical Engineering

This thesis is accepted by the examining committee with a unanimous vote in the Department of Department of Biomedical Engineering as a Master of Science thesis. JULY 13, 2023

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Nabil TAHHAN

ABSTRACT

M.Sc. Thesis

ANALYSIS OF HUMAN PLASMA *N*-GLYCOPEPTIDES BY MASS SPECTROMETRY USING HIGH-PH FRACTIONATION AND HILIC-BASED STRATEGIES

Nabil TAHHAN

Karabuk University
Institute of Graduate Programs
Department of Biomedical Engineering

Thesis Advisor:
Assist. Prof. Dr. Hacı Mehmet KAYILI
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Glycosylation, a prevalent post-translational modification, plays a significant role in diverse biological processes and exhibits associations with various diseases, including cancer. Among the different types of glycosylation, *N*-glycosylation is particularly prominent. The precise detection and characterization of *N*-glycosylation require a series of enrichment steps. On the other hand, peptide fragmentation methods constitute a crucial step in reducing sample complexity. Moreover, enrichment strategies coupled with mass spectrometry are essential for the analysis and identification of N-glycopeptides. This research aims to enhance the detection methods for N-glycopeptides and N-glycoproteins by employing strategies that involve peptide fractionation and glycopeptide enrichment.

This study targeted to assess and compare the efficacy of an integration method and a direct enrichment approach in terms of identifying glycopeptides from the glycoproteome of human plasma. The results demonstrate that integration method detects 228 *N*-glycopeptides and 95 *N*-glycoproteins, while Cotton-HILIC direct enrichment detects 95 *N*-glycopeptides and 62 *N*-glycoproteins. In conclusion, the employment of Cotton-HILIC enrichment with high pH fractionation exhibits greater qualitative abilities in characterizing the structures and functions of glycopeptides.

Key Words : Glycosylation, N-glycosylation, mass spectrometry peptide fractionation methods, Hydrophilic interaction liquid chromatography (HILIC).

Science Code : 92504

ÖZET

Yüksek Lisans Tezi

İNSAN PLAZMA *N*-GLİKOPEPTİTLERİNİN KÜTLE SPEKTROMETRESİ İLE YÜKSEK PH FRAKSİYONLAMA VE HILIC TABANLI STRATEJİLER KULLANILARAK ANALİZİ

Nabil TAHHAN

Karabük Üniversitesi
Lisansüstü Eğitim Enstitüsü
Biyomedikal Mühendisliği Anabilim Dalı

Tez Danışmanı:
Doç. Dr. Hacı Mehmet KAYILI
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Glikozilasyon, yaygın bir post-translasyonel değişiklik olan ve çeşitli biyolojik süreçlerde önemli bir rol oynayan, aynı zamanda kanser gibi çeşitli hastalıklarla ilişkilendirilen bir süreçtir. Farklı glikozilasyon tipleri arasında özellikle N-glikozilasyon önemlidir. N-glikozilasyonun kesin tespiti ve karakterizasyonu için bir dizi zenginleştirme adımı gerekmektedir. Öte yandan, peptit parçalanma yöntemleri örnek karmaşıklığını azaltmada kritik bir adım oluşturur. Ayrıca, kütle spektrometrisi ile birleştirilen zenginleştirme stratejileri N-glikopeptitlerin analizi ve tanımlanması için önemlidir. Bu araştırma, peptit fraksiyonlama ve glikopeptit zenginleştirme stratejilerini içeren yöntemlerin kullanılarak N-glikopeptitlerin ve N-glikoproteinlerin tespit yöntemlerini geliştirmeyi amaçlamaktadır. Bu çalışma, insan

plazmasının glikkoproteomundan glikopeptitlerin tanımlanması açısından entegrasyon yöntemi ve doğrudan zenginleştirme yaklaşımının etkinliğini değerlendirmeyi hedeflemiştir. Sonuçlar, entegrasyon yönteminin 228 N-glikopeptit ve 95 N-glikoprotein tespit ederken, Pamuk-HILIC doğrudan zenginleştirmenin 95 N-glikopeptit ve 62 N-glikoprotein tespit ettiğini göstermektedir. Sonuç olarak, yüksek pH fraksiyonlama ile birlikte Pamuk-HILIC zenginleştirme yönteminin, glikopeptitlerin yapılarını ve fonksiyonlarını daha iyi nitelendirme yetenekleri sergilediği sonucuna varılmıştır.

Anahtar Sözcükler : Glikozilasyon, N-glikozilasyon, Kütle Spektrometrisi, Zenginleştirme Stratejileri, Peptit Fraksiyonlama Yöntemleri, Hidrofilik Etkileşim Sıvı Kromatografisi (HILIC).

Bilim Kodu : 92504

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INDEX SYMBOLS AND ABBREVITIONS

SİMGELER

mg : milligram

ml : milliliter

M : quantity molar mass

mM : milli molar

RPM : Revolutions per minute

µL : microliter

M/S : mass-to-charge ratio

°C : degree Celsius

ABBREVITIONS

MS : Mass spectrometry

ASN :Amino Acid Asparagine

ER : Endoplasmic Reticulum

ESI : Electrospray Ionization

MALDI : Matrix-Assisted laser Desorption/Ionization

MALDI_TOF : Matrix-Assisted Laser Desorption/Ionization Time-of-Flight

EI : Electron Impact Ionization

FAB : Fast Atomic Bombardment

DC : Direct Current

RF : Radio Frequency

HILIC : Hydrophilic Interaction Chromatography

SPE : Solid-Phase Extraction

PGC : Porous Graphitic Carbon

IEF : Isoelectric Focusing

PI : Isoelectric Point

HPLC : a High-Performance Liquid Chromatography

RPC : Reversed-Phase Chromatography

DTT : Dithiothreitol

IAA : indole-3-cetic acid

TFA : trifluoro acetic acid

CAN : acetonitrile)

PART 1

INTRODUCTION

Human plasma, the fluid component of blood, contains a complex mixture of proteins derived from various tissues and cell types [1]. Glycosylation is a widespread post-translational modification (PTM) that involves the addition of sugar molecules (such as glycans) to proteins, lipids, or other molecules [2]. It plays a significant role in several biological processes, including protein folding, cell recognition, and adhesion, receptor activation, and others [3]. Understanding the glycosylation patterns of proteins in human plasma is of great importance, as alterations in glycosylation have been associated with numerous diseases, including cancer [4]. Characterizing the glycoproteome of human plasma can provide insights into disease mechanisms, identify novel biomarkers, and facilitate the development of targeted therapies [5]. N-glycosylation, a specific type of glycosylation, occurs when an oligosaccharide is linked to the nitrogen atom (N) of the amino acid asparagine (N). It plays an important role in biological processes [6]. the analysis of the glycoproteome in human plasma presents unique challenges due to the high complexity and wide dynamic range of protein abundance [7]. To overcome these challenges, a fractionation-based strategy has been developed to enhance the coverage and identification of *N*-glycosylation sites in human plasma. This strategy involves the enrichment of *N*-glycopeptides [8], followed by fractionation of the enriched sample to reduce complexity and improve detection sensitivity [9].

This thesis provides the following information:

- The introduction presents basic preliminary information summarizing the study.
- The second part presents information from the referenced literature.
- The third part provides information about the materials used and methods.

- The fourth part interprets the results observed in the figures.
- The final part discusses the results and conclusions, along with the references.

PART 2

LITERATURE REVIEW

2.1 GLYCOSYLATION

Glycosylation, a highly prevalent and complex post-translational modification (PTM), it is a process that involves the interaction of carbohydrates with proteins or lipids through enzymatic reactions occurring in the endoplasmic reticulum and Golgi apparatus. This process plays crucial functional roles in various biological processes, including protein folding and stability, cell-cell interactions, immune recognition and response, and others [10].

Glycosylation is influenced by several factors, including:

Enzymatic Machinery: The process of glycosylation involves a complex enzymatic machinery consisting of glycosyltransferases, glycosidases, and other enzymes. The expression and activity of these enzymes can vary among different cell types, tissues, and developmental stages, thereby leading to variations in glycosylation patterns.

Substrate Availability: The availability of glycan precursors, specifically nucleotide sugars, is a crucial factor influencing glycosylation [11].

Genetic Factors: Genetic variations can have an impact on glycosylation patterns. Polymorphisms or mutations in genes encoding glycosylation enzymes or transporters can affect the activity or specificity of these enzymes, resulting in differences in glycan structures [12].

2.1.1. Types of Glycosylation

Glycosylation types are classified based on the amino acid atom to which the sugar chain is attached. The two most important types are N-Linked Glycosylation and O-linked Glycosylation

2.1.1.1. N-linked Glycosylation

N-linked glycosylation involves the attachment of an oligosaccharide carbohydrate to the nitrogen (N) atom of an asparagine (ASN) amino acid residue. It occurs in the endoplasmic reticulum (ER) of eukaryotic cells and is the most prevalent form of glycosylation in mammals. The initial step in N-linked glycosylation involves the synthesis of dolichol phosphate. lipid-linked oligosaccharide (LLO) is assembled on the cytoplasmic side of the ER membrane. it is flipped across the ER membrane to the luminal side, where protein glycosylation occurs. The LLO on the luminal side is the donor for the oligosaccharide chain that will be attached to the target protein. The Golgi apparatus contains specific enzymes that determine the final composition of the N-linked glycan structure by adding or removing sugar residues. (Figure 2.1) [13,14].

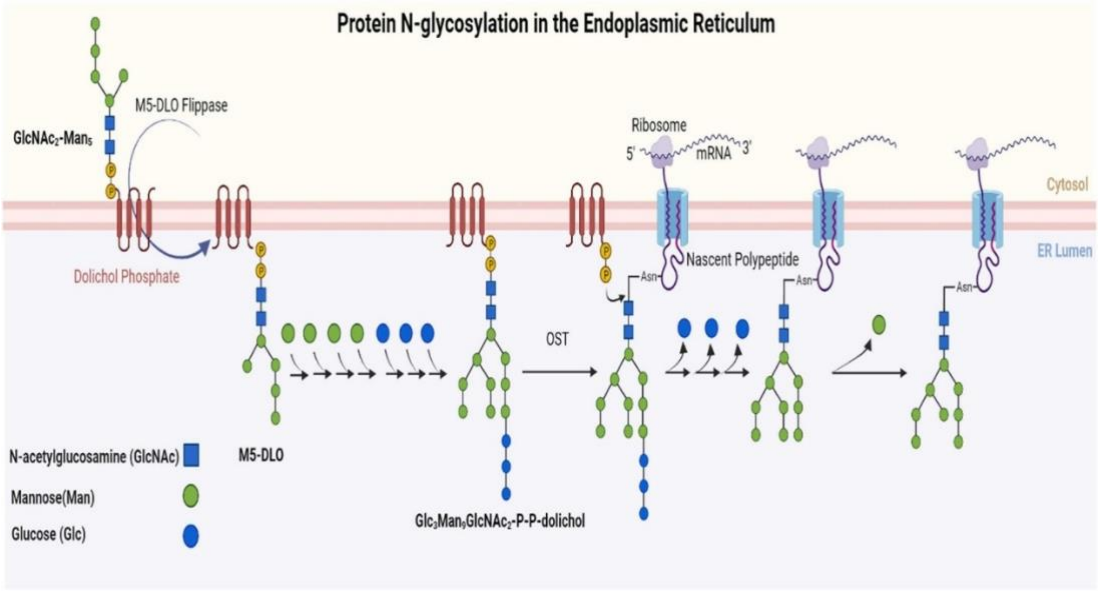

Figure 2.1. N-linked Glycosylation pathway [15].

2.1.1.2. O-linked Glycosylation

O-Linked glycosylation is another common type of glycosylation where a glycan is attached to the hydroxyl group of serine or threonine amino acid residues. This attachment occurs in the Golgi apparatus through the O-glycosyltransferase enzyme

and is known as O-linked glycosylation, particularly with mucins. Subsequently, elongation takes place by adding other sugars to form O-glycans [10]. Types of O-linked glycans include O-mannose, O-fucose, O-glucose, and O-xylose [16]. O-glycosylation plays a crucial role in various biological processes within the cell, and alterations in O-glycans are associated with numerous diseases, including cancer and diabetes [17].

2.2. GLYCOSYLATION AND CANCER

Glycosylation is a vital and intricate process that serves numerous biological functions within the cell. Any alterations in cellular processes or glycosylation can lead to risks and diseases, including cancer. Changes in glycosylation differ based on the types of glycans and various cancer types. Studying these changes aids in understanding cancer mechanisms, its developmental stages, and potential treatment methods [18].

Cancer cells can produce glycoproteins that modulate immune responses throughout cancer development. These glycoproteins manipulate immune system activity through various mechanisms, including:

- The formation of a glycoprotein shields surrounding cancer cells that prevents the formation of immune synapses.
- Interaction with receptors on the surface of immune cells.
- Production of autoantibodies against abnormal endogenous glycoproteins.
- Induction of T cell apoptosis through lectin expression.

In advanced stages of cancer development, cancer cell-produced glycoproteins can alter the function of immune cells, leading to a loss of tumor control mechanisms. Notable glycosylation changes associated with cancer include increased N-glycosylation branching, incomplete glycan formation, increased O-glycosylation, increased sialylation, and increased fucosylation [19].

2.3. MASS SPECTROMETRY

Mass spectrometry (MS) is an analytical technique used to identify and quantify the chemical composition of a sample based on the mass-to-charge ratio (m/z) of ions. It is a highly sensitive and versatile technique applicable in various scientific fields, including chemistry, biochemistry, biology, physics, and engineering. MS is employed to analyze chemical and biological compounds and their products, such as proteins, nucleic acids, lipids, and glycans [20].

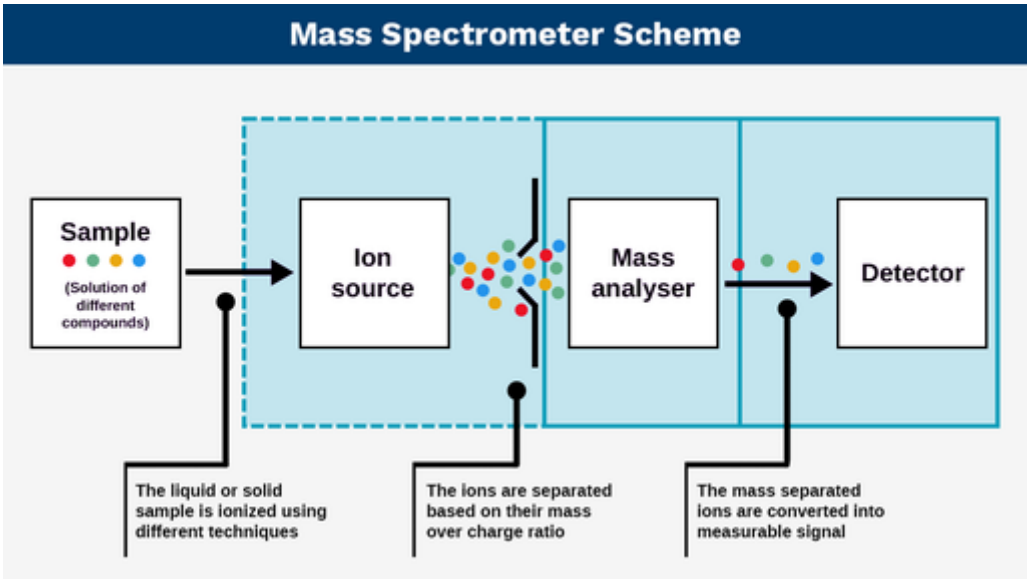

Figure 1.2. Mass Spectrometer scheme [15].

The fundamental principle of mass spectrometry is the ionization of molecules in the sample, followed by the separation of ions based on their mass-to-charge ratio (m/z), and detection of the ions by a detector. Various techniques can be used for the ionization step, including electron ionization (EI), electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI), and others. Each technique has its own advantages and limitations, depending on the sample nature and analytes of interest. Once ionized, the ions are separated based on their m/z ratio using a mass analyzer, which can be a magnetic sector, quadrupole, or ion trap. Each type of mass analyzer has unique characteristics such as resolution, mass range, and sensitivity.

Finally, the ions are detected by a detector, such as an electron multiplier (Figure 2.2) [21].

2.3.1. Ionization Techniques

In mass spectrometry, various ionization methods are employed, including classic techniques like electron impact ionization (EI) and fast atomic bombardment (FAB), as well as modern techniques such as atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), and matrix-assisted laser desorption/ionization (MALDI). APCI and ESI are compatible with liquid chromatography and are commonly used for measuring large molecular weights [22]. The selection of ionization techniques depends on the nature and type of analysis required. Two main categories of ionization techniques are hard ionization techniques that produce radical ions leading to fragmentation, and soft ionization techniques used for large molecular masses that produce no radical ions, converting large molecular mass ions into small droplets [23].

2.3.1.1. Electrospray Ionization Mass Spectrometry (ESI)

ESI is a mass spectrometry technique that ionizes a sample using an electrospray. It is particularly useful for ionizing large molecules, as it helps to prevent their fragmentation, earning it the name "soft ionization technology" [24]. The principle of electrospray ionization involves electric spraying to convert samples into ions in the gas phase through an ion source. High voltage is applied to generate charged droplets from the sample solution in the electric spray. Subsequently, the charged droplets evaporate, resulting in gas-phase ions. (Figure 2.3) Initially, the sample is dissolved using water or methanol, and then the solution is pumped into the electrospray, leading to the formation of charged droplets, which are subsequently dried using a dry gas stream like nitrogen. Electrospray ionization is highly useful for analyzing large and complex samples such as proteins, for determining glycopeptides after protein digestion to describe the structure of glycans, and it has applications in various fields, including drug discovery [24,25].

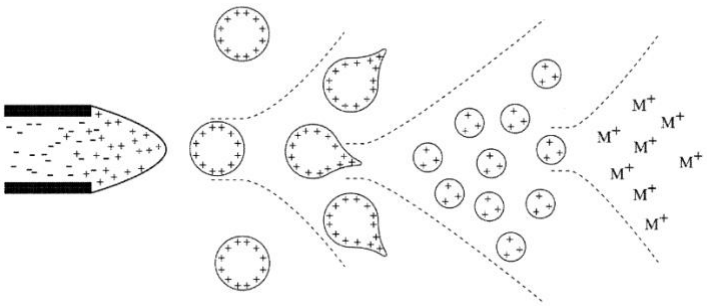

Figure 2.3. Mechanism of Electrospray Ionization [22].

2.3.1.2. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI_TOF)

MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight) is an analytical technique that ionizes the sample based on its mass-to-charge ratio and measures the time it takes for ions to reach the detector at the end of the flight tube. The sample is mixed with an acidic matrix, which has high adsorption properties, resulting in the formation of crystals in the sample-matrix mixture [26]. The ionization process involves preparing a matrix solution by mixing a basic material with water and organic substances, which are mixed with the analyte. This mixture is deposited on a MALDI plate and allowed to dry before being analyzed within a MALDI analyzer. Next, a laser is used to ionize the sample particles in the matrix, resulting in the generation of positively charged particles. A strong electric field is applied in the flight tube to accelerate the movement of the ions toward the detector. An analyzer is used to measure specific ions by multiplying the detector. The time required for each ion to reach the detector is related to its mass-to-charge ratio, with smaller ions requiring less time than larger ions. Finally, the mass spectrometry data is generated by detecting the ionic fractions at the end of the flight tube for each ion group and providing the data based on the time measurement (time of flight of ion in m/z) (Figure 2.4) [27].

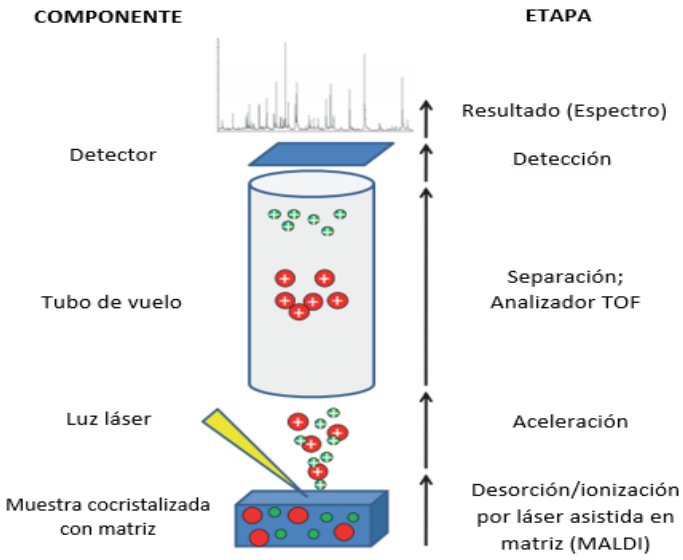

Figure 2.4. Technical description of MALDI_TOF [25].

2.3.2. Mass Analyzers

The mass analyzer is a crucial component of mass spectrometry as it determines the masses of ions and collects them in the gas phase region. There are various types of mass analyzers that utilize different methods, such as magnetic or electric fields, but they all rely on measuring ions based on their mass-to-charge ratio (m/z). The selection of an appropriate analyzer depends on its specific advantages and disadvantages. Important properties of the analyzer include its ability to resolve different ion peaks at different m/z values (power of resolution), the accuracy of the m/z measurement (mass precision), the range of masses that can be analyzed (mass range), and the range of concentrations that can be detected (linear dynamic range) [28]. We have a lot of kind form mass analyzers:

- Quadrupole Mass Analyzer.
- Time of Flight Mass Analyzer.
- Magnetic Sector Mass Analyzer.
- Electrostatic Sector Mass Analyzer.
- Quadrupole Ion Trap Mass Analyzer.
- Ion Cyclotron Resonance

2.3.2.1. Quadrupole Mass Analyzer

The quadrupole mass analyzer is a commonly used type of mass analyzer in mass spectrometry. It utilizes an array of four parallel metal rods, typically made of stainless steel, to create a radiofrequency (RF) and direct current (DC) electric field. When ions enter the analyzer, the RF and DC voltages are applied to the rods, creating a potential gradient that selectively filters ions based on their mass-to-charge ratio (m/z) (Figure 2.5) [29]. Ions within a certain range of m/z values follow a stable trajectory through the analyzer, while ions outside of this range collide with the rods and are lost. By adjusting the RF and DC voltages applied to the rods, the range of m/z values that can pass through the quadrupole can be controlled, enabling the selective isolation and detection of specific ions.

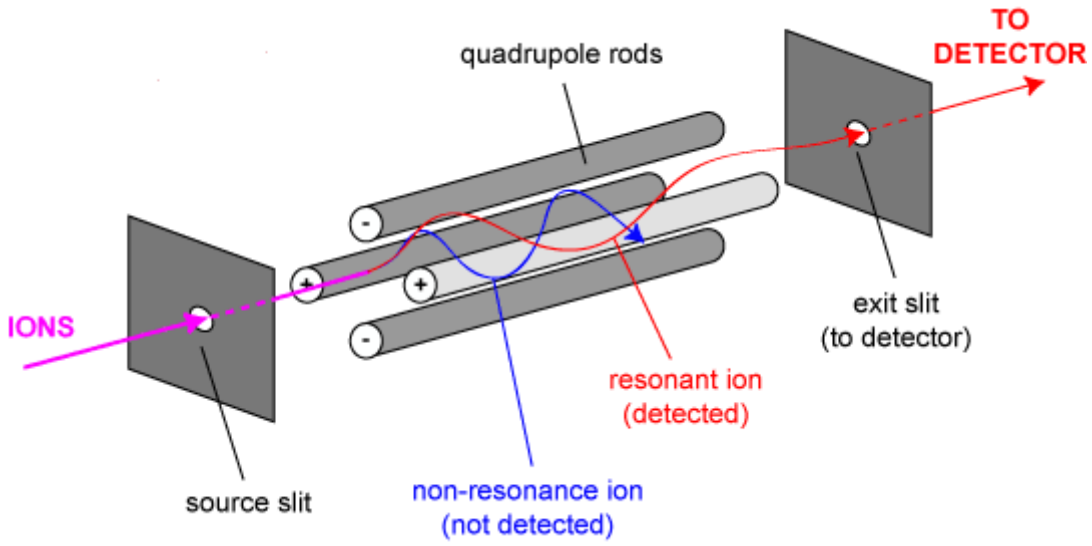

Figure 2.5. Quadrupole Mass Analyzer [28].

Quadrupole mass analyzers are frequently utilized in conjunction with other ionization techniques, such as electron impact ionization or electrospray ionization. They find applications in various fields such as chemistry, biology, and environmental science. Additionally, they are commonly employed as the primary stage of a tandem mass spectrometry (MS/MS) system, where ions are selected and fragmented within the quadrupole before further analysis by a second mass analyzer [30].

2.3.2.2. Quadrupole Ion Trap Mass Analyzers

Quadrupole ion trap mass analyzers trap ions in a three-dimensional chamber, where they are confined in stable orbits and sequentially ejected. The analyzer consists of three electrodes: an inlet electrode, an outlet electrode, and a circular pole. The poles have a central hole through which ions can move, while the annular electrode is positioned between the two poles. These electrodes create a gap where ions are trapped and analyzed (Figure 2.6) [15].

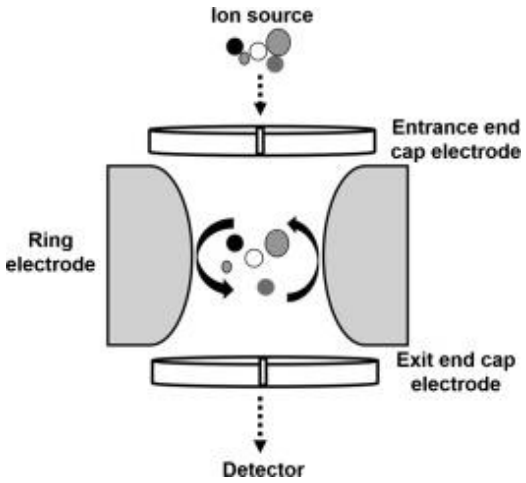

Figure 2.6. Quadrupole Ion trap Mass Analyzer [30].

The quadrupole ion trap operates by using an electric field to trap ions and measure their m/z ratio. An additional electric field is employed to excite a specific mass, causing the ions to escape and be transferred to the detector, where they are converted into a signal. The analysis involves scanning the additional electric field to excite each ion's m/z. The quadrupole ion trap, known for its ion retention capabilities, exhibits high sensitivity, enabling the analysis of all captured ions in the detector [31].

2.3.3. Proteomics Analysis by Mass Spectrometry

Mass spectrometers play a crucial role in protein characterization due to their accuracy, ease of analysis, and availability of analysis tools at a reasonable cost [32].

Proteomics analysis by mass spectrometry involves identifying and quantifying proteins in a biological sample using mass spectrometry-based techniques. The process typically includes multiple steps, such as protein extraction, digestion, peptide separation, mass spectrometry analysis, and data interpretation. Protein extraction involves breaking down cells or tissues to isolate the proteins of interest. then, the extracted proteins are digested into smaller peptides using proteolytic enzymes like trypsin. The resulting mixture of peptides is separated through liquid chromatography, either offline or online with the mass spectrometer, to enhance detection sensitivity and resolution [33].

Proteomics analysis by mass spectrometry provides a powerful tool for comprehensive proteome characterization and finds broad applications in basic and clinical research, including biomarker discovery, drug development, and disease diagnosis [34].

2.4. SAMPLE PREPARATION METHODS FOR GLYCOPROTEOMIC

Sample preparation methods play a crucial role in obtaining target compounds and ensuring reliable results in glycoproteomic analysis. The preparation process for glycoproteomics involves a series of steps to enrich and analyze glycoproteins and glycopeptides from complex biological samples. Here are some commonly used samples preparation methods for glycoproteomic analysis:

Protein extraction: Proteins are extracted from the biological sample using an appropriate buffer system.

Protein digestion: Proteins are digested into peptides using specific proteases like trypsin, chymotrypsin, or Glu-C, resulting in a complex mixture of peptides, including glycopeptides.

Glycan release: N-glycans can be enzymatically released from glycoproteins using PNGase F, while O-glycans can be released using β-elimination or hydrazinolysis. This step is important for analyzing the glycan structures attached to glycopeptides.

Glycopeptide enrichment: Various methods can be used to enrich glycopeptides from the digested peptide mixture, such as hydrazide chemistry, lectin affinity chromatography, and hydrophilic interaction chromatography (HILIC) [35].

Fractionation: Fractionation techniques like hydrophilic interaction liquid chromatography (HILIC) can be employed to separate glycopeptides based on their physicochemical properties, reducing sample complexity and increasing downstream analysis sensitivity.

Mass spectrometry: Enriched glycopeptides are analyzed using mass spectrometry to determine glycosylation sites, glycan composition, and linkage nature. Different mass spectrometry methods like MALDI-TOF and ESI-MS can be utilized for glycoproteomic analysis (Figure 2.7) [36].

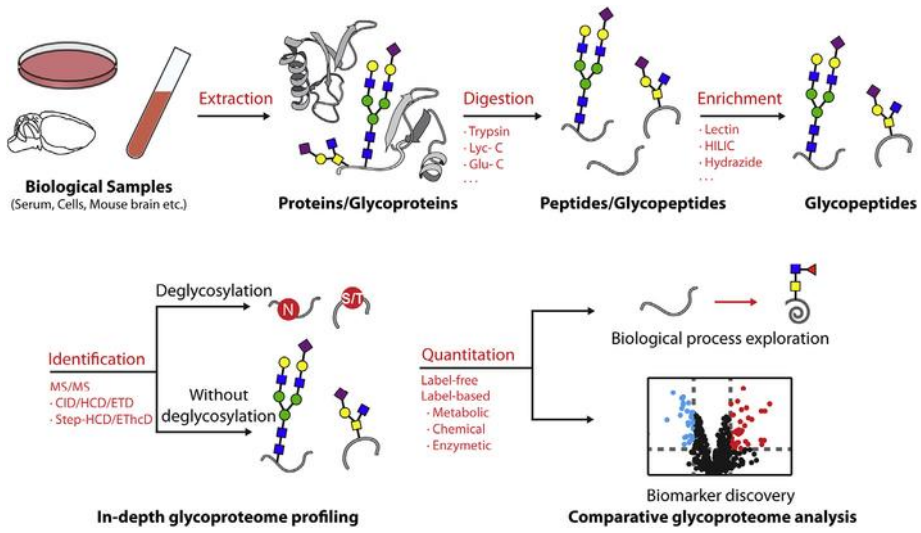

Figure 2.7. Sample preparation methods for Glycoproteomes [36].

2.4.1. Site-specific Glycosylation Analysis

Site-specific glycosylation analysis involves identifying glycosylation sites on specific proteins. Glycosylation is a complex and heterogeneous modification occurring at various protein sites, making it challenging to identify and characterize glycosylation patterns. Here are some common methods used for site-specific glycosylation analysis:

Mass spectrometry (MS): Glycoproteomics based on MS combines proteomics and glycan analysis to identify and quantify glycopeptides, which are peptides with attached glycans. MS can provide information on the mass, composition, and structure of glycans, enabling site-specific glycosylation analysis.

Liquid chromatography (LC): Techniques such as hydrophilic interaction liquid chromatography (HILIC) or reverse-phase chromatography are employed to separate glycopeptides from complex biological samples. This separation step is often coupled with mass spectrometry for subsequent glycan analysis.

Glycan release: Glycan release can be achieved using enzymatic or chemical methods. Enzymes such as peptide-N-glycosidase F (PNGase F) or endoglycosidases are utilized to specifically cleave N-linked glycans from proteins.

Glycan analysis: Released glycans can be further characterized using various techniques, including MS, nuclear magnetic resonance (NMR) spectroscopy, and glycan microarray analysis. These methods provide insights into glycan composition, linkage, branching patterns, and anomeric configurations.

Bioinformatics analysis: Computational tools and databases are essential for interpreting data obtained from glycoproteomics experiments. Bioinformatics tools assist in the identification and annotation of glycopeptides, glycan structures, and glycosylation sites in proteins [36,37].

2.4.2. Stationary Phases for The Enrichment of Glycopeptides

The enrichment of glycopeptides is vital for detecting glycoproteins and identifying glycosylation binding sites. After the sample preparation process, glycopeptide enrichment must be performed before mass spectrometry analysis to enhance detection capacity and reduce sample complexity. Various methods and materials based on stationary phases are available for enriching glycopeptides. Each method has specific physical and chemical properties for the stable enrichment of glycopeptides, but none of these methods fulfill all enrichment requirements [38].

2.4.2.1. Hydrophilic Interaction Liquid Chromatography (HILIC)

Hydrophilic Interaction Liquid Chromatography (HILIC) is a chromatographic technique that separates molecules based on their hydrophilicity and hydrophobicity properties. In HILIC, the stationary phase is a polar and hydrophilic material, while the mobile phase is a less polar and more hydrophobic solvent (Figure 2.8) [38]. Analyte retention in HILIC is dependent on its hydrophilicity. The hydrophilic

stationary phase can interact with the glycan moiety of glycopeptides, while the hydrophobic mobile phase can interact with the peptide portion of the glycopeptide. This leads to the retention of glycopeptides on the HILIC column, while non-glycosylated peptides elute early. Detection is performed using a mass spectrometer due to its high accuracy and sensitivity. Although the use of HILIC in glycopeptide separation is limited due to the presence of high organic solvents in the mobile phase, it is considered the most efficient method for glycopeptide enrichment [39,40].

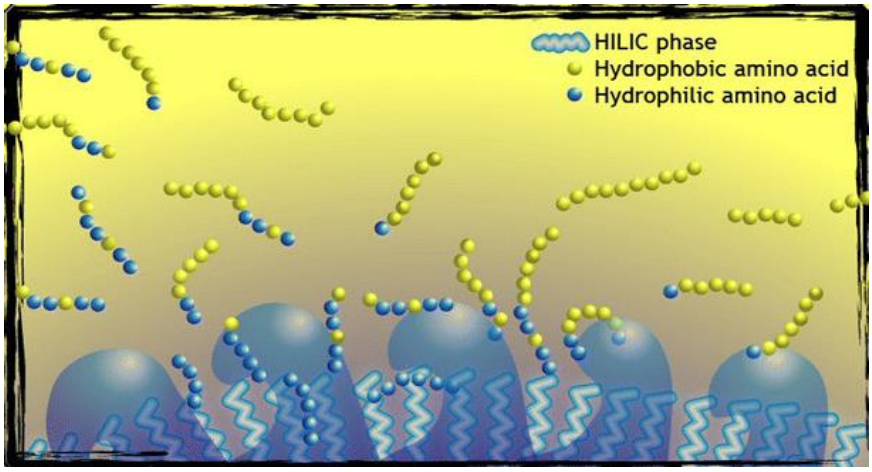

Figure 2.8. Work principle of Hydrophilic Interaction Liquid Chromatography [41].

2.4.2.2. Porous Graphitic Carbon (PGC)

Porous Graphitic Carbon (PGC) is a commonly used stationary phase for enriching glycopeptides in proteomics studies. PGC consists of highly hydrophobic graphitic carbon layers with micropores and mesopores, providing a large surface area for analyte interaction. Its hydrophobic nature enables the retention of hydrophobic and neutral glycopeptides [41]. The principle behind using PGC as a stationary phase for glycopeptide enrichment is the interactions between the graphitic carbon layers and the glycan moiety of glycopeptides. However, PGC may be susceptible to non-specific binding, which can compromise the specificity of glycopeptide enrichment. To minimize non-specific binding, several methods have been developed, including using high concentrations of organic solvent in the mobile phase, adding salts to the mobile phase, and implementing specific washing steps [42].

2.4.3. Peptide Fractionation Methods

Peptide fractionation involves separating a complex mixture of peptides into smaller, more homogeneous groups based on specific physical and chemical properties. The most common method for peptide fractionation is liquid chromatography, which employs various separation mechanisms such as reverse-phase chromatography, ion exchange chromatography, and hydrophilic interaction chromatography. Peptide fractionation serves several purposes. Firstly, it enhances sensitivity and resolution in mass spectrometry-based proteomics by increasing the number of detectable peptides. By dividing a complex peptide mixture into smaller, more homogeneous groups, the number of identified peptides can be increased. Additionally, peptide fractionation reduces sample complexity [43]. Peptide fractionation is also valuable for identifying post-translational modifications (PTMs) of proteins. Fractionation enables the enrichment of peptides with specific PTMs, aiding in the identification and quantification of modified peptides [44].

2.4.3.1. Isoelectric Focusing (IEF)

isoelectric focusing (IEF) is a separation technique used to purify proteins based on their isoelectric point (pI), which is the pH at which a protein has no net charge. In IEF, a protein mixture is applied to a gel with a pH gradient generated by incorporating ampholytes or buffering agents with a range of pI values. (Figure 2.9) then, an electric field is applied to the gel, causing the proteins to migrate toward the region where the gel's pH matches their pI. As the proteins traverse the gel, they become focused into distinct bands according to their pI. IEF achieves high-resolution separation of proteins with similar molecular weights but different pIs. It is often used in conjunction with other separation techniques like SDS-PAGE or mass spectrometry to achieve optimal separation and identification of complex protein mixtures [43,45].

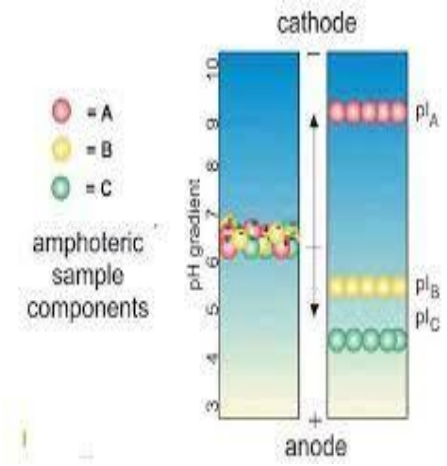

Figure 2.9. Representation of Isoelectric Focusing method [46].

2.4.3.2. Reverse-Phase Chromatography (RPC)

Reverse-Phase Chromatography (RP-HPLC) is a widely employed method for peptide fractionation. It is a high-performance liquid chromatography (HPLC) technique that separates peptides based on their hydrophobicity. RP-HPLC employs a hydrophobic stationary phase, typically made of C18 (silica) or C8 material, and a mobile phase composed of a solvent mixture, usually a blend of water (or buffer) and organic solvent, such as acetonitrile [43,47]. The separation mechanism in RP-HPLC is based on the interaction between peptides and the hydrophobic stationary phase. Hydrophobic peptides interact with the stationary phase, adhering to it, while hydrophilic peptides exhibit weak interaction and pass through the column more swiftly. As a result, hydrophobic peptides are separated from hydrophilic peptides based on their hydrophobicity (Figure 2.10) [43].

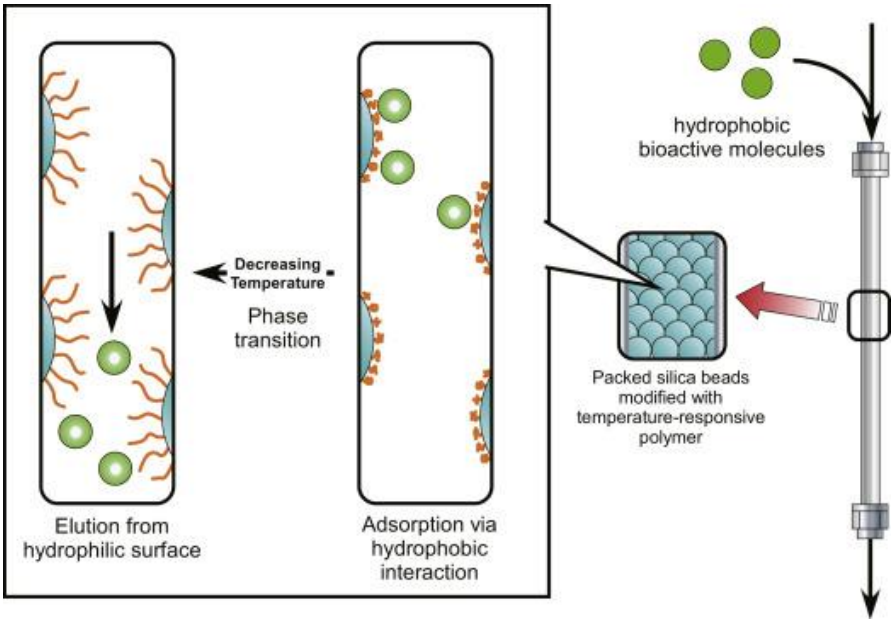

Figure 2.10. Principle of reverse phase chromatography [47].

PART 3

MATERIAL AND METHODS

3.1. MATERIALS

Human plasma, DTT (1,4-Dithiothreitol), IAA (iodo acetic acid), C18 membrane type filter, trypsin enzyme, TFA (trifluoro acetic acid), Deionized water, methanol, ACN (acetonitrile) were bought from Sigma Aldrich (St Louis, MO, USA). Cotton was bought from local market. Acetic acid (LC/MS grade) was bought from Carlo Erba Reagents.

3.2. METHODS

3.2.1. Digestion of Human Plasma Glycoproteins

One mg of human plasma (lyophilized) was taken and transferred into a tube. Subsequently, 100 µl of a 10 mM DTT solution prepared in 25 µM ABC solution was added and the resulting mixture was then subjected to incubation at a temperature of 56 °C for a duration of 40 minutes. After this step, the proteins were alkylated with 20 mM of IAA and the entire mixture was then placed in a dark environment and maintained at room temperature. The samples were incubated with trypsin enzyme with a ratio of 1/30, enzyme/protein, by weight overnight. In the following day, the digested samples (100 µg) was taken and dissolved with 0.1% TFA for the fractionation.

3.2.2. Fractionation of Peptides Using High-pH C18 Fractionation Method

In the fractionation process at high pH, a 10 mM ammonium formate solution was used as the loading solvent. The pH of the loading solution was adjusted to 10.0

using ammonia. The dried samples were dissolved in 300 µL of the loading solution. Yellow pipette tips containing C18 membrane were added with 5 mg of C18 solid sorbent. The pipette tips were washed once with 300 µL of 100% methanol and once with 300 µL of 100% ACN, centrifuged at 1500 × g for 2 minutes. The samples were loaded and centrifuged at 1500 × g for 2 minutes. Fractionation of the samples for analysis was performed using the elution solutions (300 µL) specified in Table 3.1. After each elution solution was added, the samples were centrifuged at 1500 × g for 2 minutes. The elutions were dried at 45°C. The dried samples were dissolved in 15 µL of deionized water and stored for analysis. The fractions are indicated below, and after fractionation, they were combined in three separate microcentrifuge tubes. The fractions were collected and pooled in three different microcentrifuge tubes for further analysis as indicated in Table 3.1 before HILIC enrichment of glycopeptides.

Table 3.1. The used elution solvents for the fractionation of the samples.

Fraction Number	**Elution Solutions**	**Combination Before HILIC Enrichment**
1	100 µL 5/95; ACN/10 mM Ammonium Formate (pH:10); 100 µL 7,5/92,5; ACN/10 mM Amonyum Format (pH:5).	Combination 1
2	100 µL %10/90; ACN/10 mM Ammonium Formate (pH:10); 100 µL 12,5/87,5; ACN/10 mM Amonyum Format (pH:10)	
3	100 µL 15/85; ACN/10 mM Ammonium Formate (pH:10); 100 µL 17,5/82,5 ACN/10	

	mM Ammonium Formate (pH:10).	
4	100 µL 20/80; ACN/10 mM Ammonium Formate (pH:10); 100 µL 22,5/77,5; ACN/10 mM Ammonium Formate (pH:10).	Combination 2
5	100 µL 25/75; ACN/10 mM Ammonium Formate (pH:10); 100 µL 27,5/72,5; ACN/10 mM Ammonium Formate (pH:10).	Combination 3
6	100 µL 30/70; ACN/10 mM Amonyum Format (pH:10); 100 µL 32,5/67,5; ACN/10 mM Ammonium Formate (pH:10); 100 µL 35/65; ACN/10 mM Ammonium Formate (pH:10); 100 µL 37,5/62,5; ACN/10 mM Ammonium Formate (pH:10).	
7	100 µL 40/60; ACN/10 mM Ammonium Formate (pH:10); 100 µL 50/50; ACN/10 mM Ammonium Formate (pH:10); 100 µL 60/40; ACN/10 mM	

	Ammonium Formate (pH:10); 100 µL70/30; ACN/10 mM Ammonium Formate (pH:10); 100 µL 80/20; ACN/10 mM A Ammonium Formate (pH:10).	

3.2.3. Enrichment of Glycopeptides using Cotton-HILIC

For the direct enrichment of glycopeptides, 100 µg of glycopeptides were taken and dissolved with 100 % ACN to bring the final concentration of ACN to the solution as 85%. A small piece of cotton was added to the pipettes (20-200 µL). The cotton was washed three times with 250 µL of water and 250 µL of 85/14/1 ACN/water/TFA (v/v/v) using a draw-dispense method. The samples were subjected to 20 draw-dispense cycles using the cotton pipettes. The cotton pipettes were then washed six times with 250 µL of 85/14/1 ACN/water/TFA (v/v/v) using the draw-dispense method. The samples were eluted from the cotton pipettes using 250 µL of water using the draw-dispense method. The samples were dried at 45°C.

The fractionated samples were combined with certain fraction numbers. The dried samples were dissolved with 85% of ACN. Subsequently, the glycopeptides found in each fraction was enriched with Cotton-HILIC as described above. The dried samples were then dissolved with 75 µL of water including 0.1% of formic acid for their mass spectrometric analysis.

3.2.4. Mass Spectrometric Analysis

The mass spectrometric analyses were performed using the Thermo Q-Exactive Plus instrument, integrated with the Ultimate 3000 RSLC nano liquid chromatography system (Dionex/Thermo Scientific). For glycoproteomic analyses, 5 µL of elution were injected into the instrument. The chromatographic separation system utilized an

Acclaim PepMap 100 trap column (100 μm × 5 cm, particle size 5 μm, Dionex/Thermo Scientific) and an Acclaim PepMap RSLC C18 nano separation column (75 μm × 50 cm, particle size 3 μm, Dionex/Thermo Scientific). The mobile phases A) (0.1% formic acid solution containing 2% ACN) and B) (80% ACN containing 0.1% formic acid solution) were used as the gradient elution solvents. The gradient elution was programmed for solvent B to reach 30% from 5% in 90 minutes.

3.2.5. Data Analysis

The MS/MS data were processed using the Byonic software (Protein Metrics Inc., USA). The data were analyzed using a sequence file (FASTA file) corresponding to the human proteome. The common N-glycan database specified in the software (the default 309 common mammalian N-glycans in Byonic) was included in the study. The mass tolerance for precursor ions was set to 20 ppm, and for MS/MS ions, it was set to 0.05 Da. Oxidation was selected as a variable modification. The mass range for the search was set from 350 to 5000 Da. Glycopeptide signals with a score of 30 or higher were considered significant.

PART 4

RESULT AND DISCUSSION

4.1. APPLIED STRATEGY IN THE STUDY

In this research study, the isolation of peptides from plasma was conducted using the widely employed enzymatic digestion method with trypsin. The enzymatic digestion process was carefully executed to ensure efficient cleavage of proteins into peptides. Subsequently, to specifically target and enrich glycopeptides, two distinct methodologies were employed (Figure 4.1).

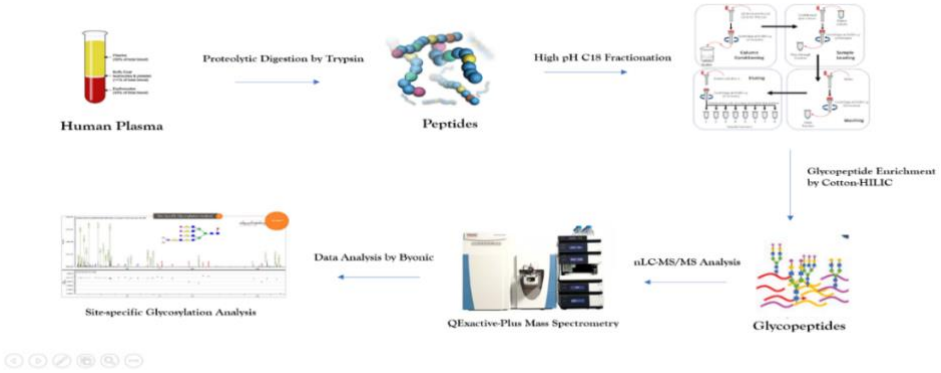

Figure 4.1. Study workflow.

The first methodology involved high pH-C18 fractionation, which aimed to separate the peptide mixture into different fractions based on their hydrophobicity. This fractionation step facilitated the enrichment of glycopeptides by isolating them from other peptide species present in the sample. The fractions obtained from the high pH-C18 fractionation were further subjected to Cotton-HILIC enrichment, which exploits the unique interaction between glycopeptides and the hydrophilic stationary phase of the Cotton-HILIC column. This selective enrichment process allowed for the enrichment of glycopeptides and their subsequent analysis. In contrast, the

second methodology focused on direct enrichment of glycopeptides using Cotton-HILIC without fractionation. This approach aimed to simplify the workflow by bypassing the fractionation step, potentially reducing sample handling and processing time. The direct enrichment process involved the direct application of the plasma-derived peptide mixture onto the Cotton-HILIC column, allowing for the specific retention and enrichment of glycopeptides.

Following the enrichment procedures, the samples were subjected to analysis using an nLC MS/MS mass spectrometry platform. This powerful analytical technique enabled the identification and characterization of the enriched glycopeptides. The acquired data were then processed and analyzed using GraphPad Prism software, which facilitated statistical analysis and visualization of the results.

The primary objective of this research was to evaluate the impact of the different fractionation methods on enhancing the detection of glycoproteins and glycopeptides. By comparing the results obtained from the high pH-C18 fractionation followed by Cotton-HILIC enrichment and the direct enrichment using Cotton-HILIC, the performances of these two approaches were assessed. This comprehensive investigation aimed to provide valuable insights into the effectiveness and efficiency of the fractionation-based and direct enrichment strategies for the targeted analysis of glycopeptides.

4.2. THE DETECTED N-GLYCOPEPTIDES AND N-GLYCOPROTEIN AND AVERAGE SCORES

The analysis of the obtained results, as illustrated in Figure 4.2, was performed. By applying the Cotton-HILIC enrichment technique to the fractionated samples, a significantly higher number of glycopeptides (228) and glycoproteins (95) were detected compared to the relatively lower numbers of glycopeptides (95) and glycoproteins (62) identified through direct enrichment using Cotton-HILIC.

The application of Cotton-HILIC enrichment of fractionated samples proved to be advantageous in terms of enhancing the detection of N-glycopeptides and N-

glycoproteins. The fractionation step enabled the isolation and enrichment of a larger pool of glycopeptides, which subsequently contributed to the detection of a greater number of glycoproteins. This observed disparity in the number of identified glycopeptides and glycoproteins between the two enrichment approaches underscores the importance of fractionation in improving the sensitivity and efficiency of glycoprotein analysis.

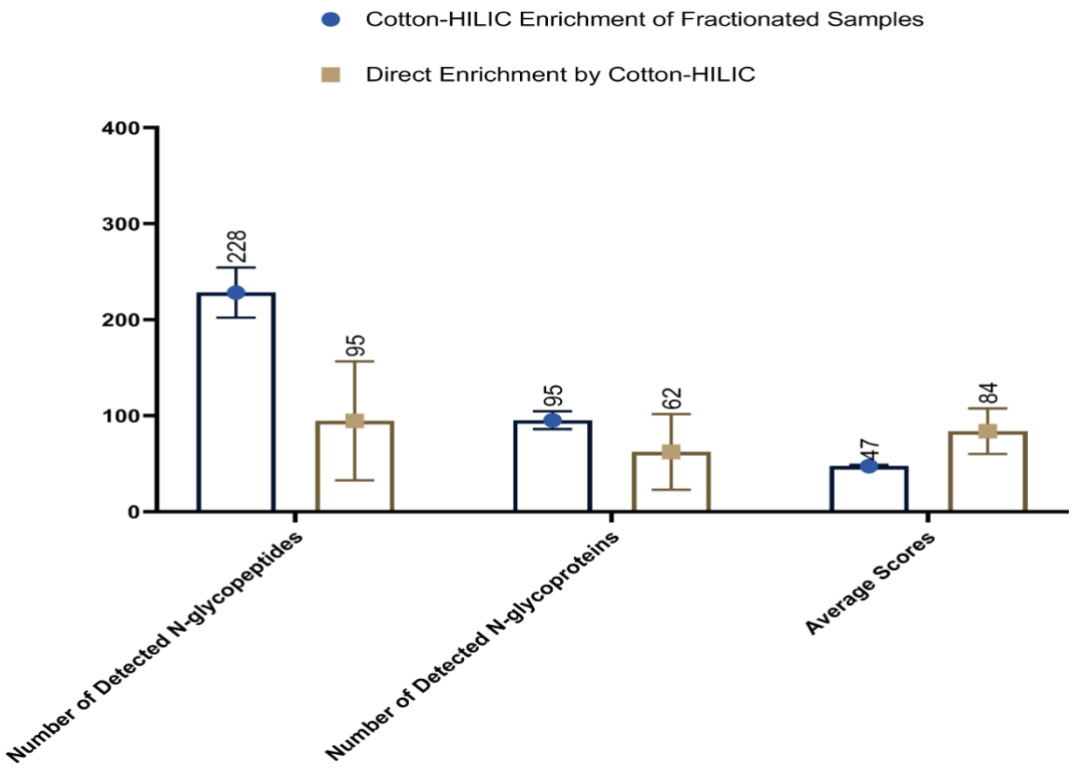

Figure 4.2. The graph showing the number of detected N-Glycopeptides and N-Glycoproteins and average scores in each of the Cotton-HILIC enrichment of fractionated samples and direct enrichment by Cotton-HILIC.

These findings provide valuable insights into the effectiveness of the Cotton-HILIC enrichment technique, particularly when combined with fractionation, in enabling the comprehensive profiling and characterization of N-glycopeptides and N-glycoproteins. The higher yield of identified glycopeptides and glycoproteins achieved through the fractionation-based Cotton-HILIC enrichment strategy further emphasizes its potential utility in glycoproteomic studies and biomarker discovery efforts.

4.3. THE DETECTED N-GLYCOPROTEINS AND N-GLYCOPEPTIDES BETWEEN EXPERIMENTS IN EACH REPLICATE AND APPLIED STRATEGIES

The data analysis results presented in Figure 4.3 were obtained through the utilization of sophisticated software tools. When employing the Cotton-HILIC enrichment technique on fractionated samples, the experimental outcomes revealed the detection of 98 glycoproteins in the first experiment, 102 in the second experiment, and 85 in the third experiment. In contrast, the direct enrichment approach using Cotton-HILIC identified a relatively lower number of glycoproteins, with 72 detected in the first experiment, 19 in the second experiment, and 50 in the third experiment.

Moreover, upon closer examination of the data, it was observed that the Cotton-HILIC enrichment of fractionated samples technique yielded a set of 12 glycoproteins that were consistently detected across all three experiments. Additionally, 24 glycoproteins were found to be common between the first and second experiments, while 21 glycoproteins were shared between the second and third experiments. Furthermore, a total of 22 glycoproteins were identified as being shared between the first and third experiments. In contrast, the direct enrichment method using Cotton-HILIC showed the presence of 7 glycoproteins that were consistently detected across all three experiments. Additionally, 18 glycoproteins were found to be common between the first and second experiments, while 50 glycoproteins were shared between the first and third experiments. Interestingly, only 7 glycoproteins were identified as being shared between the second and third experiments.

Furthermore, when considering the glycoproteins that were not shared across the experiments, it was observed that the Cotton-HILIC enrichment of fractionated samples technique detected 64 unshared glycoproteins in the first experiment, 69 in the second experiment, and 54 in the third experiment. In contrast, the direct enrichment approach using Cotton-HILIC only identified 11 unshared glycoproteins

in the first experiment, one in the second experiment, and none in the third experiment.

In summary, the data analysis results highlight the superior effectiveness of the Cotton-HILIC enrichment of fractionated samples technique in detecting a larger number of glycoproteins compared to the direct enrichment approach. The observed variations in the number of identified glycoproteins and the patterns of overlap between experiments underscore the influence of the enrichment method on the detection capabilities in glycoproteomic studies. These findings contribute to a deeper understanding of the strengths and limitations of different enrichment strategies, ultimately facilitating the optimization of experimental approaches for comprehensive glycoprotein analysis.

Cotton-HILIC enrichment of fractionated samples **Direct enrichment by Cotton-HILIC**

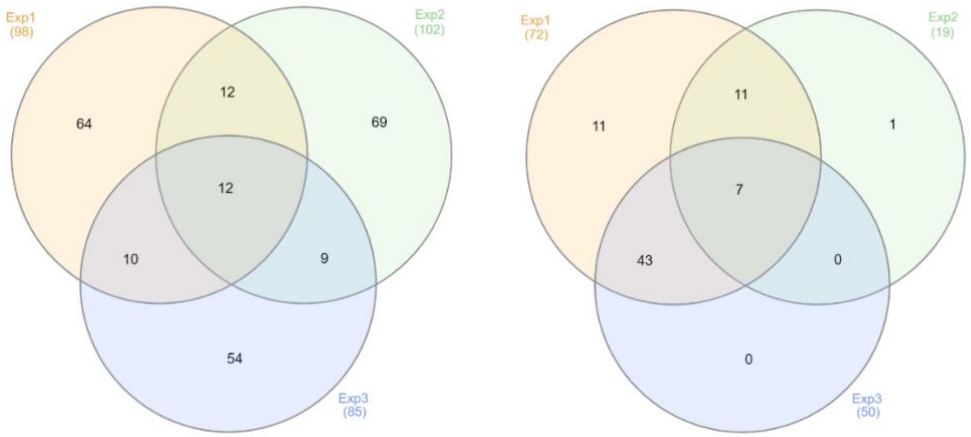

Figure 4.3. Shared and unshared glycoproteins in the three experiments for each of the enrichment techniques used.

4.4. THE DIFFERENCE IN THE DETECTED N-GLYCAN TYPES

Figure 4.4 provides valuable insights into the diversity of glycan structures analyzed in the study. Notably, the application of the Cotton-HILIC enrichment technique on fractionated samples revealed the detection of a remarkable number of over 100 distinct glycan types. In contrast, the direct enrichment approach using Cotton-

HILIC resulted in the identification of a comparatively limited set of less than 40 glycan types.

This disparity in the number of detected glycan structures clearly indicates the superior efficacy of the Cotton-HILIC enrichment of fractionated samples technique in capturing a broader range of glycans. The comprehensive fractionation approach employed in this method likely contributes to the enhanced detection and profiling of a larger pool of glycans present in the samples. The fractionation step prior to enrichment enables a more selective targeting of glycopeptides and subsequently allows for a more comprehensive coverage of glycan structures.

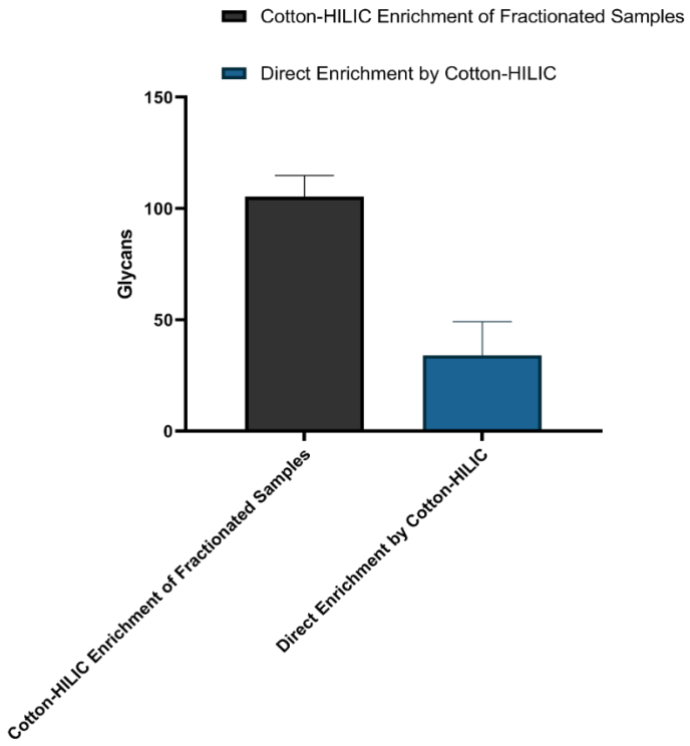

Figure 4.4. Number of different Glycan structures.

4.5. THE NUMBER OF N-GLYCANS DETECTED IN EXPERIMENTS FOR EACH TECHNIQUE AND BETWEEN ENRICHMENT TECHNIQUES

As depicted in Figure 4.5, the Cotton-HILIC enrichment of fractionated samples technique revealed 96 glycans in the first experiment, 105 in the second experiment,

and 115 in the third experiment. In contrast, direct enrichment by Cotton-HILIC only detected 46 glycans in the first experiment, 17 in the second experiment, and 39 in the third experiment. Among the three experiments, the Cotton-HILIC enrichment of fractionated samples technique exhibited 54 glycans common to all three, 65 shared between the first and second experiments, 74 shared between the first and third experiments, and 77 shared between the second and third experiments. In contrast, direct enrichment by Cotton-HILIC showed 12 common glycans between all three experiments, 17 between the first and second experiments, 39 between the first and third experiments, and 12 between the second and third experiments. Furthermore, the Cotton-HILIC enrichment of fractionated samples technique identified 11 unshared glycans in the first experiment, 17 in the second experiment, and 18 in the third experiment. On the other hand, direct enrichment by Cotton-HILIC only detected 2 unshared glycans in the first experiment, with no unshared glycans in the second and third experiments. In summary, the Cotton-HILIC enrichment of fractionated samples technique demonstrated a higher number of glycans detected in each experiment and a greater number of shared glycans across the three experiments.

Cotton-HILIC enrichment of fractionated samples **direct enrichment by Cotton-HILIC**

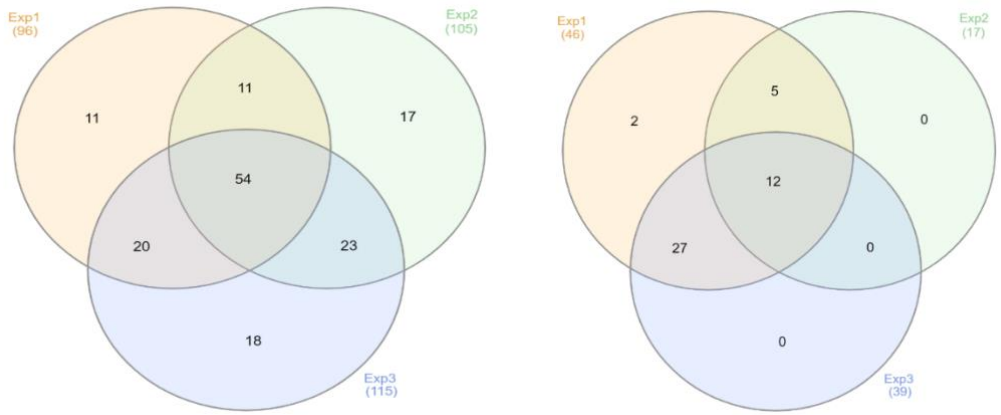

Figure 4.5. Shared and unshared Glycans in the three experiments for each of the enrichment techniques used.

4.6. HEAT MAP OF GLYCANS

To assess the prevalence and abundance of specific glycans within the analyzed samples, we constructed a heat map representing the frequency distribution of glycan types. The heat map, as depicted in Figure 4.6, visually portrays the relative abundance of each glycan based on its frequency across the samples. The colour gradient employed in the heat map provides a clear representation of the varying degrees of glycan frequency, with darker hues indicating higher occurrence.

Upon careful examination of the heat map, it was evident that the glycan species (HexNAc(4)Hex(5)NeuAc(2)) exhibited the highest prevalence among the identified glycans. This particular glycan was consistently detected across the samples, demonstrating its prominence in the glycan repertoire. Following closely, (HexNAc(4)Hex(5)NeuAc(1), (HexNAc(4)Hex(5)Fuc(1), and (HexNAc(4)Hex(4)Fuc(1) were identified as the subsequent most abundant glycan types.

Remarkably, the prevalence of the (HexNAc(4)Hex(5)NeuAc(2)) glycan aligns with previous studies on human plasma, where it has been consistently reported as the most abundant glycan species[48]. The consistency between our findings and the established literature further reinforces the reliability and accuracy of our analysis. The identification of this prominent glycan species, along with the other highly abundant glycan types, contributes valuable insights into the glycan profile of the analyzed samples.

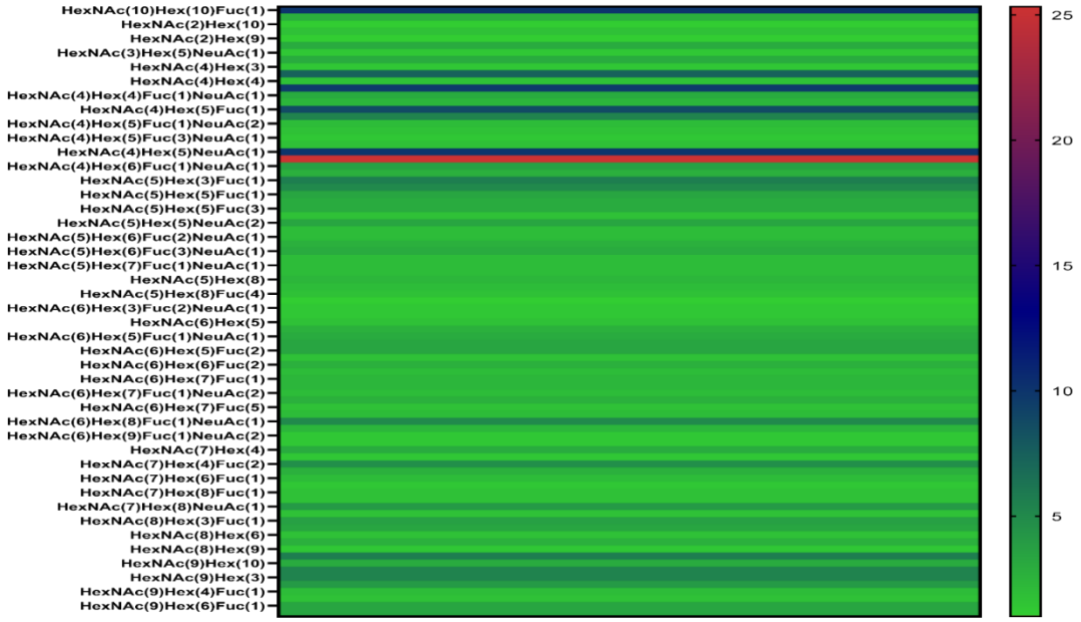

Figure 4.6. Heat Map of Glycans.

4.7. COMPARİNG THE RESULTS WİTH RESULTS OF LİTERATURE REVİEW USİNG HILIC METHOD FOR ENRİCHMENT OF GLYCOPEPTİDES

In a referenced literature, the workflow involved the extraction of N-glycosylation from cancer cells. The methods applied were HILIC enrichment and HILIC-ERLIC enrichment to isolate glycopeptides, followed by analysis of the results using a mass spectrometer. By applying only HILIC enrichment, a total of 355 glycopeptides were detected. By comparing the research results with the reference results, a significant difference in the number of identified glycopeptides is observed, which is dependent on the sample type. However, it can be concluded that HILIC technology is more effective in detecting a larger quantity of glycopeptides [49].

In a cited literature, a novel type of polymer hybrid graphene oxide (GO) was developed for the enrichment of N-glycopeptides. The workflow encompassed the extraction of N-glycosylation from 10 µL of human plasma, followed by N-glycopeptide enrichment through a combination of hydrazide capturing and HILIC interaction. Mass spectrometry analysis was then performed to identify the enriched

N-glycopeptides. Relevant data analysis software and databases were utilized for the interpretation and identification of the glycopeptides. As a result, a total of 480 glycopeptides and 232 glycoproteins were detected. By comparing the research results with the reference results, a significant increase in the number of detected glycopeptides was observed when using 10 μL of human plasma and the hybrid polymer. It is important to note that variations in the results can arise from changes in the sample size and enrichment methods utilized. [50].

PART 5

CONCLUSION

In conclusion, the utilization of Cotton-HILIC enrichment in conjunction with High-pH C18 fractionation proved to be more effective in detecting a greater number of N-glycoproteins and N-glycopeptides compared to the direct enrichment approach using Cotton-HILIC. The application of Cotton-HILIC enrichment to fractionated samples demonstrated a significant improvement in the detection of these glycosylated compounds. This combined strategy exhibited higher efficiency and consistency in identifying a considerable number of common and unshared glycoproteins and glycans when compared to the direct enrichment method. Moreover, the Cotton-HILIC enrichment of fractionated samples played a crucial role in qualitatively characterizing glycan structures, offering more comprehensive insights into their structures and functions. It is worth noting that both enrichment strategies yielded comparable outcomes regarding the identification of the most abundant types of glycans.

When comparing the results obtained in this study using HILIC-based enrichment of glycopeptides with the findings reported in the literature, notable differences were observed in the number of detected glycopeptides through HILIC enrichment. These differences can be attributed to variations in sample types, variations in the enrichment methods employed, and disparities in sample sizes. However, despite these variations, the HILIC technique consistently demonstrated significant efficacy in detecting a higher number of glycopeptides.

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APPENDIX

Table 2. Glycoprotein detected by Direct enrichment by Cotton-HILIC

	A	B	C	D	E
1	EXPERIMENT 1	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	EXPERIMENT 3
2	>sp|A0A1B0GVY4|SIM31_HUMAN	>sp|Q53FE4|CD017_HUMAN	>sp|O75179|ANR17_HUMAN	>sp|Q92540|SMG7_HUMAN	>sp|A2VEC9|SSPO_HUMAN
3	>sp|A2VEC9|SSPO_HUMAN	>sp|Q5CZC0|FSIP2_HUMAN	>sp|O75326|SEM7A_HUMAN	>sp|Q96J94|PIWL1_HUMAN	>sp|A6NK06|IRG1_HUMAN
4	>sp|A6NK06|IRG1_HUMAN	>sp|Q5TCY1|TTBK1_HUMAN	>sp|P01008|ANT3_HUMAN	>sp|Q96QD5|DEPD7_HUMAN	>sp|A7E2F4|GOG8A_HUMAN
5	>sp|A7E2F4|GOG8A_HUMAN	>sp|Q68DH5|LMBD2_HUMAN	>sp|P01859|IGHG2_HUMAN	>sp|Q96RV3|PCX1_HUMAN	>sp|K9M1U5|IFNL4_HUMAN
6	>sp|K9M1U5|IFNL4_HUMAN	>sp|Q6UN15|FIP1_HUMAN	>sp|P01871|IGHM_HUMAN	>sp|Q99460|PSMD1_HUMAN	>sp|O00533|NCHL1_HUMAN
7	>sp|O00533|NCHL1_HUMAN	>sp|Q7Z418|KCNKI_HUMAN	>sp|P02647|APOA1_HUMAN	>sp|Q9H8G2|CAAP1_HUMAN	>sp|O15014|ZN609_HUMAN
8	>sp|O15014|ZN609_HUMAN	>sp|Q86VV8|RTTN_HUMAN	>sp|P02763|A1AG1_HUMAN	>sp|Q9HA82|CERS4_HUMAN	>sp|O60551|NMT2_HUMAN
9	>sp|O60551|NMT2_HUMAN	>sp|Q8IYD8|FANCM_HUMAN	>sp|P16066|ANPRA_HUMAN	>sp|Q9NR11|ZN302_HUMAN	>sp|P00734|THRB_HUMAN
10	>sp|O75179|ANR17_HUMAN	>sp|Q8IYT1|GAR4_HUMAN	>sp|Q86VV8|RTTN_HUMAN	>sp|Q9UKN1|MUC12_HUMAN	>sp|P00738|HPT_HUMAN
11	>sp|O75326|SEM7A_HUMAN	>sp|Q8TAU3|ZN417_HUMAN	>sp|Q8TAU3|ZN417_HUMAN	>sp|Q9UQ52|CNTN6_HUMAN	>sp|P01008|ANT3_HUMAN
12	>sp|O95544|NADK_HUMAN	>sp|Q8TF72|SHRM3_HUMAN	>sp|Q8WV22|NSE1_HUMAN	>sp|Q9Y272|RASD1_HUMAN	>sp|P01591|IGJ_HUMAN
13	>sp|P00734|THRB_HUMAN	>sp|Q8WUA7|TB22A_HUMAN	>sp|Q96QD5|DEPD7_HUMAN	>sp|Q9Y572|RIPK3_HUMAN	>sp|P01857|IGHG1_HUMAN
14	>sp|P00738|HPT_HUMAN	>sp|Q8WV22|NSE1_HUMAN	>sp|Q99453|PHX2B_HUMAN	>tr|A0A4P8J2C9|A0A4P8J2C9_HUMAN	>sp|P01859|IGHG2_HUMAN
15	>sp|P01008|ANT3_HUMAN	>sp|Q8WVC0|LEO1_HUMAN	>sp|Q9H5U6|ZCHC4_HUMAN	>tr|H7C505|H7C505_HUMAN	>sp|P01861|IGHG4_HUMAN
16	>sp|P01591|IGJ_HUMAN	>sp|Q92540|SMG7_HUMAN	>sp|Q9HC35|EMAL4_HUMAN	>sp|P01871|IGHM_HUMAN	
17	>sp|P01857|IGHG1_HUMAN	>sp|Q96J94|PIWL1_HUMAN	>sp|Q9UPZ6|THS7A_HUMAN	>sp|P01877|IGHA2_HUMAN	
18	>sp|P01859|IGHG2_HUMAN	>sp|Q96QD5|DEPD7_HUMAN	>sp|Q9Y485|DMXL1_HUMAN	>sp|P02763|A1AG1_HUMAN	
19	>sp|P01861|IGHG4_HUMAN	>sp|Q96RV3|PCX1_HUMAN	>sp|Q9Y572|RIPK3_HUMAN	>sp|P05155|IC1_HUMAN	
20	>sp|P01871|IGHM_HUMAN	>sp|Q99453|PHX2B_HUMAN	>tr|A0A1B0GUS7|A0A1B0GUS7_HUMAN	>sp|P05546|HEP2_HUMAN	
21	>sp|P01877|IGHA2_HUMAN	>sp|Q99460|PSMD1_HUMAN		>sp|P05556|ITB1_HUMAN	
22	>sp|P02675|FIBB_HUMAN	>sp|Q9H5U6|ZCHC4_HUMAN		>sp|P13535|MYH8_HUMAN	
23	>sp|P02763|A1AG1_HUMAN	>sp|Q9H8G2|CAAP1_HUMAN		>sp|P35968|VGFR2_HUMAN	
24	>sp|P02790|HEMO_HUMAN	>sp|Q9HA82|CERS4_HUMAN		>sp|P41440|S19A1_HUMAN	
25	>sp|P05155|IC1_HUMAN	>sp|Q9HC35|EMAL4_HUMAN		>sp|Q13291|SLAF1_HUMAN	
26	>sp|P05546|HEP2_HUMAN	>sp|Q9NR11|ZN302_HUMAN		>sp|Q14573|ITPR3_HUMAN	
27	>sp|P05556|ITB1_HUMAN	>sp|Q9UKN1|MUC12_HUMAN		>sp|Q16576|RBBP7_HUMAN	
28	>sp|P13535|MYH8_HUMAN	>sp|Q9ULW0|TPX2_HUMAN		>sp|Q53FE4|CD017_HUMAN	
29	>sp|P16066|ANPRA_HUMAN	>sp|Q9UPZ6|THS7A_HUMAN		>sp|Q5CZC0|FSIP2_HUMAN	
30	>sp|P16278|BGAL_HUMAN	>sp|Q9UQ52|CNTN6_HUMAN		>sp|Q68DH5|LMBD2_HUMAN	
31	>sp|P35968|VGFR2_HUMAN	>sp|Q9UQB3|CTND2_HUMAN		>sp|Q6UN15|FIP1_HUMAN	
32	>sp|P41440|S19A1_HUMAN	>sp|Q9Y272|RASD1_HUMAN		>sp|Q7Z418|KCNKI_HUMAN	
33	>sp|Q13291|SLAF1_HUMAN	>sp|Q9Y485|DMXL1_HUMAN		>sp|Q8IYT1|GAR4_HUMAN	
34	>sp|Q14573|ITPR3_HUMAN	>sp|Q9Y572|RIPK3_HUMAN		>sp|Q8TAU3|ZN417_HUMAN	
35	>sp|Q16576|RBBP7_HUMAN	>tr|A0A1B0GUS7|A0A1B0GUS7_HUMAN		>sp|Q8TF72|SHRM3_HUMAN	
36	>sp|Q16610|ECM1_HUMAN	>tr|A0A1W2PQ58|A0A1W2PQ58_HUMAN		>sp|Q8WUA7|TB22A_HUMAN	
37	>tr|H7C505|H7C505_HUMAN	>tr|A0A4P8J2C9|A0A4P8J2C9_HUMAN		>sp|Q8WVC0|LEO1_HUMAN	

Table 3. Glycoprotein detected by Cotton-HILIC enrichment of fractionated samples

	A	B	C	D	E	F	G	H	I
1	EXPERIMENT 1	EXPERIMENT 1	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 2	EXPERIMENT 2	EXPERIMENT 3	EXPERIMENT 3	EXPERIMENT 3
2	>sp|A1Z1Q3|MACD2_HUMAN	>sp|Q7Z6E9|RBBP6_HUMAN	>sp|P48169|GBRA4_HUMAN	>sp|P53804|TTC3_HUMAN	>sp|A2AJT9|BCLA3_HUMAN	(Ctrl) 7|CAPS2_HUMAN	>sp|A2VEC9|SSPO_HUMAN	>sp|Q68E01|INT3_HUMAN	>sp|P78536|ADA17_HUMAN
3	>sp|A2AJT9|BCLA3_HUMAN	>sp|Q86UW7|CAPS2_HUMAN	>sp|P53621|COPA_HUMAN	>sp|Q13075|BIRC1_HUMAN	>sp|A2PYH4|HFM1_HUMAN	LRP11_HUMAN	>sp|A6NL46|YF016_HUMAN	>sp|Q05209|PTN12_HUMAN	
4	>sp|A2VEC9|SSPO_HUMAN	>sp|Q8IY18|SMC5_HUMAN	>sp|P53804|TTC3_HUMAN	>sp|Q13418|ILK_HUMAN	>sp|A2VEC9|SSPO_HUMAN	>sp|Q8IYK2|CC105_HUMAN	>sp|O00750|P3C2B_HUMAN	>sp|Q7Z7J9|CK2N1_HUMAN	>sp|Q08999|RBL2_HUMAN
5	>sp|B1AL88|NALF1_HUMAN	>sp|Q8IZP9|AGRG2_HUMAN	>sp|P98161|PKD1_HUMAN	>sp|Q13535|ATR_HUMAN	>sp|A8TX70|CO6A5_HUMAN	>sp|Q8IZP9|AGRG2_HUMAN	>sp|O14646|CHD1_HUMAN	>sp|Q8NEY1|NAV1_HUMAN	>sp|Q13418|ILK_HUMAN
6	>sp|M0R2J8|DCDC1_HUMAN	>sp|Q8NCN4|RN169_HUMAN	>sp|Q02763|TIE2_HUMAN	>sp|Q14D04|MELT_HUMAN	>sp|O00533|NCHL1_HUMAN	>sp|Q8NE09|RGS22_HUMAN	>sp|O43166|SI1L1_HUMAN	>sp|Q8NFP9|NBEA_HUMAN	>sp|Q13635|PTC1_HUMAN
7	>sp|O00273|DFFA_HUMAN	>sp|Q8NF50|DOCK8_HUMAN	>sp|Q08462|ADCY2_HUMAN	>sp|Q15119|PDK2_HUMAN	>sp|O00750|P3C2B_HUMAN	>sp|Q8TBF8|FA81A_HUMAN	>sp|O43390|HNRPR_HUMAN	>sp|Q8WXG9|AGRV1_HUMAN	>sp|Q14CB8|RHG19_HUMAN
8	>sp|O43166|SI1L1_HUMAN	>sp|Q8NHU6|TDRD7_HUMAN	>sp|Q13308|PTK7_HUMAN	>sp|Q2LD37|K1109_HUMAN	>sp|O15265|ATX7_HUMAN	>sp|Q8TDI7|TMC2_HUMAN	>sp|O75362|ZN217_HUMAN	>sp|Q92828|COR2A_HUMAN	>sp|Q460N5|PAR14_HUMAN
9	>sp|O60671|RAD1_HUMAN	>sp|Q8NHZ8|CDC26_HUMAN	>sp|Q13322|GRB10_HUMAN	>sp|Q3SY69|AL1L2_HUMAN	>sp|O60469|DSCAM_HUMAN	>sp|Q8WTR7|ZN473_HUMAN	>sp|O94782|UBP1_HUMAN	>sp|Q96RT1|ERBIN_HUMAN	>sp|P35555|FBN1_HUMAN
10	>sp|O75167|PHAR2_HUMAN	>sp|Q8TBF8|FA81A_HUMAN	>sp|Q14139|UBE4A_HUMAN	>sp|Q53TS8|CTSRT_HUMAN	>sp|O60486|PLXC1_HUMAN	>sp|Q8WX94|NALP7_HUMAN	>sp|O95900|TRUB2_HUMAN	>sp|Q9BWV1|BOC_HUMAN	>sp|P43652|AFAM_HUMAN
11	>sp|O94782|UBP1_HUMAN	>sp|Q8TF63|DCNP1_HUMAN	>sp|Q149N8|SHPRH_HUMAN	>sp|Q5CZC0|FSIP2_HUMAN	>sp|P00533|EGFR_HUMAN	>sp|Q8WXD9|CSKI1_HUMAN	>sp|P00450|CERU_HUMAN	>sp|Q9BYG0|B3GN5_HUMAN	>sp|P51679|CCR4_HUMAN
12	>sp|O95831|AIFM1_HUMAN	>sp|Q8WX94|NALP7_HUMAN	>sp|Q14CB8|RHG19_HUMAN	>sp|Q5H913|AR13A_HUMAN	>sp|P00738|HPT_HUMAN	>sp|Q8WZ42|TITIN_HUMAN	>sp|P00488|F13A_HUMAN	>sp|Q9BZW8|CD244_HUMAN	>sp|P55107|GDF10_HUMAN
13	>sp|P00533|EGFR_HUMAN	>sp|Q92922|SMRC1_HUMAN	>sp|Q15700|DLG2_HUMAN	>sp|Q5SVQ8|ZBT41_HUMAN	>sp|P00748|FA12_HUMAN	>sp|Q92772|CDKL2_HUMAN	>sp|P00738|HPT_HUMAN	>sp|Q9H2U9|ADAM7_HUMAN	>sp|P55210|CASP7_HUMAN
14	>sp|P00738|HPT_HUMAN	>sp|Q92995|UBP13_HUMAN	>sp|Q16787|LAMA3_HUMAN	>sp|Q5VV63|ATRN1_HUMAN	>sp|P01009|A1AT_HUMAN	>sp|Q92995|UBP13_HUMAN	>sp|P01008|ANT3_HUMAN	>sp|Q9H6Z9|EGLN3_HUMAN	>sp|P78504|JAG1_HUMAN
15	>sp|P01859|IGHG2_HUMAN	>sp|Q93008|USP9X_HUMAN	>sp|Q460N5|PAR14_HUMAN	>sp|Q6UXL0|I20RB_HUMAN	>sp|P01591|IGJ_HUMAN	>sp|Q96FN5|KIF12_HUMAN	>sp|P01591|IGJ_HUMAN	>sp|Q9HCG8|CWC22_HUMAN	>sp|Q4G163|FBX43_HUMAN
16	>sp|P01860|IGHG3_HUMAN	>sp|Q96GC5|RM48_HUMAN	>sp|Q5KU26|COL12_HUMAN	>sp|Q6ZMT9|DTHD1_HUMAN	>sp|P01857|IGHG1_HUMAN	>sp|Q96JC4|ZN479_HUMAN	>sp|P01859|IGHG2_HUMAN	>sp|Q9P0J6|RM36_HUMAN	>sp|Q5VTT5|MYOM3_HUMAN
17	>sp|P01871|IGHM_HUMAN	>sp|Q96MR9|ZN560_HUMAN	>sp|Q6DHV5|C2D2B_HUMAN	>sp|Q6ZSY5|PPR3F_HUMAN	>sp|P01859|IGHG2_HUMAN	>sp|Q96JL9|ZN333_HUMAN	>sp|P01860|IGHG3_HUMAN	>sp|Q9P272|TRM9B_HUMAN	>sp|Q5VV63|ATRN1_HUMAN
18	>sp|P01877|IGHA2_HUMAN	>sp|Q96PD2|DCBD2_HUMAN	>sp|Q6PKH6|DR4L2_HUMAN	>sp|Q86UQ4|ABCAD_HUMAN	>sp|P01860|IGHG3_HUMAN	>sp|Q96LD1|SGCZ_HUMAN	>sp|P01871|IGHM_HUMAN	>sp|Q9UKV5|AMFR_HUMAN	>sp|Q68D10|SPT2_HUMAN
19	>sp|P02765|FETUA_HUMAN	>sp|Q9BXU3|TX13A_HUMAN	>sp|Q6UB99|ANR11_HUMAN	>sp|Q9ULS5|TMCC3_HUMAN	>sp|P01861|IGHG4_HUMAN	>sp|Q96M93|ADAD1_HUMAN	>sp|P02675|FIBB_HUMAN	>sp|Q9UKY4|POMT2_HUMAN	
20	>sp|P02787|TRFE_HUMAN	>sp|Q9BY15|AGRE3_HUMAN	>sp|Q6UXL0|I20RB_HUMAN	>sp|Q9UM73|ALK_HUMAN	>sp|P01871|IGHM_HUMAN	>sp|Q96MW7|TIGD1_HUMAN	>sp|P02679|FIBG_HUMAN	>sp|Q9ULD9|ZN608_HUMAN	
21	>sp|P02790|HEMO_HUMAN	>sp|Q9BZW8|CD244_HUMAN	>sp|Q6ZT07|TBCD9_HUMAN	>sp|Q9Y5I0|PCDAD_HUMAN	>sp|P01877|IGHA2_HUMAN	>sp|Q96RT1|ERBIN_HUMAN	>sp|P02763|A1AG1_HUMAN	>sp|Q9ULG6|CCPG1_HUMAN	
22	>sp|P05496|AT5G1_HUMAN	>sp|Q9GZY0|NXF2_HUMAN	>sp|Q7Z333|SETX_HUMAN	>sp|Q9Y5P3|RAI2_HUMAN	>sp|P02763|A1AG1_HUMAN	>sp|Q99758|ABCA3_HUMAN	>sp|P02765|FETUA_HUMAN	>sp|Q9UM73|ALK_HUMAN	
23	>sp|P08069|IGF1R_HUMAN	>sp|Q9H2U9|ADAM7_HUMAN	>sp|Q9UL59|ZN214_HUMAN	>sp|Q9Y5X3|SNX5_HUMAN	>sp|P02765|FETUA_HUMAN	>sp|Q9BVS4|RIOK2_HUMAN	>sp|P02787|TRFE_HUMAN	>sp|Q9Y2Z4|SYYM_HUMAN	
24	>sp|P0DOX5|IGG1_HUMAN	>sp|Q9H6Z9|EGLN3_HUMAN	>sp|Q9UM73|ALK_HUMAN	>sp|Q9Y653|AGRG1_HUMAN	>sp|P02787|TRFE_HUMAN	>sp|Q9BYG0|B3GN5_HUMAN	>sp|P02790|HEMO_HUMAN	>sp|Q9Y320|TMX2_HUMAN	
25	>sp|P10909|CLUS_HUMAN	>sp|Q9HCH5|SYTL2_HUMAN	>sp|Q9Y2H8|ZN510_HUMAN	>tr|A0A1B0GVU9|A0A1B0GVU9_H	>sp|P02790|HEMO_HUMAN	>sp|Q9GZY0|NXF2_HUMAN	>sp|P04196|HRG_HUMAN	>sp|Q9Y512|SAM50_HUMAN	
26	>sp|P11047|LAMC1_HUMAN	>sp|Q9HCK4|ROBO2_HUMAN	>sp|Q9Y4D7|PLXD1_HUMAN	>tr|A0A1W1B6M6|A0A1W1B6M6_	>sp|P0DKX4|SIM18_HUMAN	>sp|Q9H3P2|NELFA_HUMAN	>sp|P08069|IGF1R_HUMAN	>tr|A0A0S2Z4G9|A0A0S2Z4G9_HUMAN	
27	>sp|P26378|ELAV4_HUMAN	>sp|Q9NVH1|DJC11_HUMAN	>sp|Q9Y512|SAM50_HUMAN	>tr|A0A6I8PRU0|A0A6I8PRU0_HU	>sp|P0DOX5|IGG1_HUMAN	>sp|Q9H4L7|SMRCD_HUMAN	>sp|P08603|CFAH_HUMAN	>tr|A0A2P9DU05|A0A2P9DU05_HUMAN	
28	>sp|P29375|KDM5A_HUMAN	>sp|Q9UGJ0|AAKG2_HUMAN	>sp|Q9Y5X3|SNX5_HUMAN	>tr|C9J4J5|C9J4J5_HUMAN	>sp|P10909|CLUS_HUMAN	>sp|Q9H5U6|ZCHC4_HUMAN	>sp|P0DOX5|IGG1_HUMAN	>tr|A0A494C0I9|A0A494C0I9_HUMAN	
29	>sp|P35637|FUS_HUMAN	>sp|Q9UKV5|AMFR_HUMAN	>tr|G1FM86|G1FM86_HUMAN	>tr|D3DRN8|D3DRN8_HUMAN	>sp|P41180|CASR_HUMAN	>sp|Q9H756|LRC19_HUMAN	>sp|P10909|CLUS_HUMAN	>tr|A0A8I5KVU2|A0A8I5KVU2_HUMAN	
30	>sp|P41440|S19A1_HUMAN	>sp|Q9UKX5|ITA11_HUMAN	>tr|G3V3A5|G3V3A5_HUMAN	>tr|H0YHE2|H0YHE2_HUMAN	>sp|P42785|PCP_HUMAN	>sp|Q9HBW9|AGRL4_HUMAN	>sp|P13473|LAMP2_HUMAN	>tr|A8MXW3|A8MXW3_HUMAN	
31	>sp|P42702|LIFR_HUMAN	>sp|Q9UKY4|POMT2_HUMAN	>tr|Q13739|Q13739_HUMAN	>tr|Q6P514|Q6P514_HUMAN	>sp|P43119|PI2R_HUMAN	>sp|Q9P0J6|RM36_HUMAN	>sp|P13497|BMP1_HUMAN	>tr|B7Z407|B7Z407_HUMAN	
32	>tr|A0A7S5EX36|A0A7S5EX36_HUMAN	>tr|C6GLZ2|C6GLZ2_HUMAN		>tr|Q6VIH9|Q6VIH9_HUMAN	>sp|P43652|AFAM_HUMAN	>sp|Q9UFH2|DYH17_HUMAN	>sp|P14222|PERF_HUMAN	>tr|G9B020|G9B020_HUMAN	
33	>tr|A8K8W7|A8K8W7_HUMAN	>tr|A0A0S2RRN6|A0A0S2RRN6_HUMAN		>tr|Q7Z344|Q7Z344_HUMAN	>sp|P49221|TGM4_HUMAN	>sp|Q9UK96|FBX10_HUMAN	>sp|P14923|PLAK_HUMAN	>tr|Q8NF78|Q8NF78_HUMAN	
34	>tr|B4DUS8|B4DUS8_HUMAN	>tr|A0A2R8Y4P2|A0A2R8Y4P2_HUMAN		>tr|Q8IXB8|Q8IXB8_HUMAN	>sp|P49619|DGKG_HUMAN	>sp|Q9UKA1|FBXL5_HUMAN	>sp|P26374|RAE2_HUMAN	>tr|Q8NHB6|Q8NHB6_HUMAN	
35	>tr|B7Z884|B7Z884_HUMAN	>tr|A0A4Y5R233|A0A4Y5R233_HUMAN		>tr|Q9UG08|Q9UG08_HUMAN	>sp|P50454|SERPH_HUMAN	>sp|Q9UKY4|POMT2_HUMAN	>sp|P28289|TMOD1_HUMAN	>tr|Q8NHB6|Q8NHB6_HUMAN	

Table 4. Glycan detected by Direct enrichment by Cotton-HILIC

	A	B	C	D	E
1	EXPERIMENT 1	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	EXPERIMENT 3
2	HexNAc(4)Hex(4)Fuc(1)	HexNAc(5)Hex(5)	HexNAc(6)Hex(6)Fuc(1)	HexNAc(5)Hex(6)Fuc(2)	HexNAc(2)Hex(5)
3	HexNAc(5)Hex(6)Fuc(2)	HexNAc(5)Hex(5)NeuAc(2)	HexNAc(2)Hex(7)	HexNAc(6)Hex(5)Fuc(2)	HexNAc(2)Hex(6)
4	HexNAc(6)Hex(5)Fuc(2)	HexNAc(5)Hex(6)Fuc(3)	HexNAc(4)Hex(5)NeuAc(2)	HexNAc(5)Hex(5)Fuc(1)NeuAc(2)	HexNAc(3)Hex(6)
5	HexNAc(5)Hex(5)Fuc(1)NeuAc(2)	HexNAc(3)Hex(6)Fuc(1)NeuAc(1)	HexNAc(4)Hex(4)Fuc(1)	HexNAc(5)Hex(6)Fuc(2)NeuAc(1)	HexNAc(4)Hex(4)NeuAc(1)
6	HexNAc(5)Hex(6)Fuc(2)NeuAc(1)	HexNAc(3)Hex(3)Fuc(1)	HexNAc(3)Hex(6)NeuAc(1)	HexNAc(5)Hex(4)Fuc(2)	
7	HexNAc(5)Hex(4)Fuc(2)	HexNAc(3)Hex(5)Fuc(1)NeuAc(1)	HexNAc(3)Hex(4)Fuc(1)NeuAc(1)	HexNAc(6)Hex(6)	
8	HexNAc(6)Hex(6)	HexNAc(5)Hex(6)Fuc(1)	HexNAc(5)Hex(6)NeuAc(3)	HexNAc(4)Hex(5)NeuAc(1)	
9	HexNAc(4)Hex(5)NeuAc(1)	HexNAc(2)Hex(5)	HexNAc(5)Hex(4)Fuc(1)	HexNAc(4)Hex(5)NeuAc(2)	
10	HexNAc(4)Hex(5)NeuAc(2)	HexNAc(2)Hex(6)	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	HexNAc(5)Hex(4)Fuc(1)	
11	HexNAc(5)Hex(4)Fuc(1)	HexNAc(3)Hex(6)	HexNAc(5)Hex(5)	HexNAc(6)Hex(4)Fuc(1)	
12	HexNAc(6)Hex(4)Fuc(1)	HexNAc(4)Hex(4)NeuAc(1)	HexNAc(5)Hex(6)Fuc(3)	HexNAc(6)Hex(5)Fuc(1)	
13	HexNAc(6)Hex(5)Fuc(1)		HexNAc(4)Hex(3)Fuc(1)	HexNAc(4)Hex(4)Fuc(1)	
14	HexNAc(4)Hex(5)Fuc(1)		HexNAc(5)Hex(6)	HexNAc(4)Hex(5)Fuc(1)	
15	HexNAc(5)Hex(6)Fuc(1)NeuAc(2)		HexNAc(4)Hex(5)NeuAc(1)	HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	
16	HexNAc(6)Hex(6)Fuc(1)		HexNAc(5)Hex(5)Fuc(1)	HexNAc(5)Hex(6)NeuAc(2)	
17	HexNAc(2)Hex(7)		HexNAc(5)Hex(6)Fuc(1)	HexNAc(3)Hex(4)Fuc(1)NeuAc(1)	
18	HexNAc(5)Hex(5)NeuAc(1)		HexNAc(5)Hex(3)Fuc(1)	HexNAc(3)Hex(5)NeuAc(1)	
19	HexNAc(5)Hex(6)NeuAc(2)			HexNAc(4)Hex(3)Fuc(1)	
20	HexNAc(3)Hex(4)Fuc(1)NeuAc(1)			HexNAc(4)Hex(5)	
21	HexNAc(3)Hex(5)NeuAc(1)			HexNAc(5)Hex(3)	
22	HexNAc(4)Hex(3)Fuc(1)			HexNAc(5)Hex(3)Fuc(1)	
23	HexNAc(4)Hex(5)			HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	
24	HexNAc(5)Hex(3)			HexNAc(3)Hex(4)NeuAc(1)	
25	HexNAc(5)Hex(3)Fuc(1)			HexNAc(3)Hex(6)NeuAc(1)	
26	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)			HexNAc(5)Hex(5)Fuc(1)NeuAc(1)	
27	HexNAc(3)Hex(4)NeuAc(1)			HexNAc(4)Hex(5)Fuc(2)NeuAc(1)	
28	HexNAc(3)Hex(6)NeuAc(1)			HexNAc(5)Hex(6)	
29	HexNAc(5)Hex(5)Fuc(1)NeuAc(1)			HexNAc(5)Hex(5)Fuc(1)	
30	HexNAc(5)Hex(6)NeuAc(3)			HexNAc(6)Hex(3)Fuc(2)	
31	HexNAc(6)Hex(5)			HexNAc(5)Hex(5)NeuAc(1)	
32	HexNAc(4)Hex(5)Fuc(2)NeuAc(1)			HexNAc(5)Hex(5)NeuAc(2)	
33	HexNAc(5)Hex(6)			HexNAc(5)Hex(4)	
34	HexNAc(5)Hex(5)Fuc(1)			HexNAc(5)Hex(5)	
35	HexNAc(6)Hex(3)Fuc(2)			HexNAc(3)Hex(5)Fuc(1)NeuAc(1)	
36	HexNAc(5)Hex(4)			HexNAc(3)Hex(6)Fuc(1)NeuAc(1)	

Table 5. Glycan detected by Cotton-HILIC enrichment of fractionated samples

	A	B	C	D	E	F	G	H	I
1	EXPERIMENT 1	EXPERIMENT 1	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 2	EXPERIMENT 2	EXPERIMENT 3	EXPERIMENT 3	EXPERIMENT 3
2	HexNAc(7)Hex(4)Fuc(2)	HexNAc(10)Hex(7)	HexNAc(5)Hex(5)Fuc(1)	HexNAc(4)Hex(4)Fuc(1)	HexNAc(4)Hex(5)NeuAc(1)	HexNAc(5)Hex(5)Fuc(1)NeuAc(1)	HexNAc(9)Hex(3)	HexNAc(4)Hex(3)	HexNAc(5)Hex(4)NeuAc(2)
3	HexNAc(4)Hex(3)Fuc(1)	HexNAc(3)Hex(3)Fuc(1)	HexNAc(4)Hex(6)Fuc(1)NeuAc(1)	HexNAc(5)Hex(3)Fuc(1)	HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	HexNAc(5)Hex(5)Fuc(2)	HexNAc(5)Hex(5)Fuc(3)	HexNAc(4)Hex(4)	HexNAc(5)Hex(7)Fuc(1)NeuAc(2)
4	HexNAc(4)Hex(5)Fuc(1)	HexNAc(4)Hex(5)	HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	HexNAc(4)Hex(5)Fuc(1)	HexNAc(4)Hex(3)	HexNAc(6)Hex(5)Fuc(1)	HexNAc(8)Hex(4)	HexNAc(8)Hex(5)Fuc(1)	HexNAc(7)Hex(7)
5	HexNAc(8)Hex(4)	HexNAc(7)Hex(8)Fuc(1)	HexNAc(5)Hex(8)	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	HexNAc(4)Hex(3)Fuc(1)	HexNAc(4)Hex(6)Fuc(1)NeuAc(1)	HexNAc(2)Hex(12)	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	HexNAc(7)Hex(6)Fuc(1)NeuAc(4)
6	HexNAc(5)Hex(6)Fuc(2)	HexNAc(2)Hex(10)	HexNAc(6)Hex(5)Fuc(1)NeuAc(1)	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	HexNAc(4)Hex(4)	HexNAc(4)Hex(5)Fuc(2)NeuAc(1)	HexNAc(5)Hex(6)Fuc(2)	HexNAc(5)Hex(6)NeuAc(2)	HexNAc(4)Hex(6)NeuAc(1)
7	HexNAc(6)Hex(6)Fuc(2)	HexNAc(4)Hex(5)Fuc(2)	HexNAc(9)Hex(4)Fuc(1)	HexNAc(10)Hex(7)	HexNAc(4)Hex(5)	HexNAc(5)Hex(7)Fuc(1)NeuAc(1)	HexNAc(5)Hex(7)Fuc(1)	HexNAc(6)Hex(3)Fuc(3)	HexNAc(9)Hex(10)
8	HexNAc(5)Hex(5)Fuc(1)NeuAc(1)	HexNAc(8)Hex(9)	HexNAc(6)Hex(8)Fuc(1)NeuAc(1)	HexNAc(9)Hex(3)	HexNAc(5)Hex(4)	HexNAc(7)Hex(8)Fuc(1)	HexNAc(9)Hex(4)	HexNAc(5)Hex(5)NeuAc(2)	HexNAc(7)Hex(7)Fuc(1)NeuAc(3)
9	HexNAc(2)Hex(12)	HexNAc(7)Hex(8)Fuc(1)NeuAc(2)	HexNAc(7)Hex(8)NeuAc(1)	HexNAc(5)Hex(5)Fuc(3)	HexNAc(5)Hex(4)Fuc(1)	HexNAc(6)Hex(7)Fuc(4)	HexNAc(5)Hex(6)Fuc(3)	HexNAc(4)Hex(4)Fuc(2)NeuAc(1)	HexNAc(10)Hex(7)
10	HexNAc(6)Hex(7)Fuc(1)	HexNAc(7)Hex(8)Fuc(1)NeuAc(3)	HexNAc(6)Hex(7)Fuc(1)NeuAc(2)	HexNAc(5)Hex(6)Fuc(2)	HexNAc(3)Hex(6)NeuAc(1)	HexNAc(7)Hex(8)Fuc(1)NeuAc(1)	HexNAc(7)Hex(6)Fuc(1)	HexNAc(7)Hex(4)Fuc(2)	HexNAc(7)Hex(8)Fuc(1)NeuAc(1)
11	HexNAc(7)Hex(6)Fuc(1)	HexNAc(9)Hex(6)Fuc(1)	HexNAc(7)Hex(8)Fuc(1)NeuAc(1)	HexNAc(5)Hex(7)Fuc(1)	HexNAc(5)Hex(5)NeuAc(2)	HexNAc(4)Hex(5)Fuc(3)NeuAc(1)	HexNAc(6)Hex(3)Fuc(1)NeuAc(2)	HexNAc(9)Hex(6)	HexNAc(5)Hex(4)Fuc(1)NeuAc(1)
12	HexNAc(5)Hex(6)Fuc(2)NeuAc(1)	HexNAc(3)Hex(4)NeuAc(1)	HexNAc(6)Hex(4)NeuAc(1)	HexNAc(6)Hex(7)Fuc(1)	HexNAc(6)Hex(7)NeuAc(2)	HexNAc(3)Hex(6)Fuc(1)NeuAc(1)	HexNAc(6)Hex(6)Fuc(2)	HexNAc(6)Hex(7)Fuc(1)NeuAc(1)	HexNAc(7)Hex(4)
13	HexNAc(6)Hex(5)Fuc(1)NeuAc(2)	HexNAc(3)Hex(5)NeuAc(1)	HexNAc(6)Hex(7)Fuc(5)	HexNAc(5)Hex(8)Fuc(1)	HexNAc(5)Hex(4)Fuc(2)	HexNAc(5)Hex(5)Fuc(1)	HexNAc(4)Hex(5)Fuc(3)NeuAc(2)	HexNAc(6)Hex(9)Fuc(1)NeuAc(2)	HexNAc(6)Hex(4)NeuAc(1)
14	HexNAc(6)Hex(7)Fuc(4)	HexNAc(6)Hex(6)NeuAc(2)	HexNAc(4)Hex(7)Fuc(1)	HexNAc(5)Hex(6)NeuAc(1)	HexNAc(2)Hex(10)	HexNAc(6)Hex(6)Fuc(1)	HexNAc(6)Hex(3)Fuc(1)NeuAc(1)	HexNAc(7)Hex(6)	HexNAc(2)Hex(10)
15	HexNAc(8)Hex(9)Fuc(1)	HexNAc(5)Hex(7)Fuc(1)NeuAc(2)	HexNAc(8)Hex(6)	HexNAc(7)Hex(6)Fuc(1)	HexNAc(4)Hex(5)Fuc(3)	HexNAc(6)Hex(7)Fuc(4)NeuAc(1)	HexNAc(6)Hex(3)Fuc(2)	HexNAc(8)Hex(8)	HexNAc(8)Hex(6)
16	HexNAc(6)Hex(7)Fuc(1)NeuAc(3)	HexNAc(5)Hex(5)NeuAc(2)		HexNAc(9)Hex(4)	HexNAc(8)Hex(8)Fuc(1)	HexNAc(6)Hex(6)Fuc(2)	HexNAc(6)Hex(5)	HexNAc(9)Hex(9)Fuc(1)	HexNAc(4)Hex(5)
17	HexNAc(4)Hex(6)Fuc(3)	HexNAc(6)Hex(7)NeuAc(1)		HexNAc(5)Hex(6)Fuc(2)NeuAc(1)	HexNAc(7)Hex(4)Fuc(1)	HexNAc(7)Hex(7)Fuc(1)	HexNAc(7)Hex(3)Fuc(1)	HexNAc(9)Hex(10)Fuc(1)	HexNAc(8)Hex(7)
18	HexNAc(9)Hex(4)	HexNAc(6)Hex(6)		HexNAc(6)Hex(3)Fuc(1)NeuAc(2)	HexNAc(4)Hex(4)Fuc(2)NeuAc(1)	HexNAc(10)Hex(10)Fuc(1)	HexNAc(8)Hex(3)Fuc(1)	HexNAc(6)Hex(6)Fuc(3)	HexNAc(5)Hex(6)Fuc(3)NeuAc(1)
19	HexNAc(8)Hex(3)Fuc(1)	HexNAc(5)Hex(7)Fuc(1)NeuAc(1)		HexNAc(5)Hex(6)Fuc(3)NeuAc(1)	HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	HexNAc(4)Hex(4)NeuAc(1)	HexNAc(4)Hex(5)Fuc(3)NeuAc(1)	HexNAc(7)Hex(7)Fuc(1)NeuAc(2)	HexNAc(6)Hex(7)NeuAc(4)
20	HexNAc(3)Hex(6)NeuAc(1)	HexNAc(3)Hex(6)		HexNAc(6)Hex(9)Fuc(1)NeuAc(2)	HexNAc(6)Hex(6)NeuAc(2)	HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	HexNAc(6)Hex(5)Fuc(1)NeuAc(3)	HexNAc(5)Hex(6)Fuc(1)	HexNAc(6)Hex(8)Fuc(1)NeuAc(1)
21	HexNAc(6)Hex(6)Fuc(1)NeuAc(3)	HexNAc(7)Hex(3)Fuc(1)		HexNAc(5)Hex(3)	HexNAc(6)Hex(10)Fuc(1)NeuAc(1)	HexNAc(6)Hex(6)Fuc(1)NeuAc(2)	HexNAc(8)Hex(9)Fuc(1)	HexNAc(8)Hex(3)	HexNAc(6)Hex(5)Fuc(2)
22	HexNAc(9)Hex(10)Fuc(1)	HexNAc(4)Hex(6)Fuc(1)		HexNAc(8)Hex(8)	HexNAc(7)Hex(8)	HexNAc(3)Hex(5)Fuc(1)	HexNAc(5)Hex(5)NeuAc(1)	HexNAc(6)Hex(6)Fuc(1)	HexNAc(4)Hex(5)Fuc(2)
23	HexNAc(10)Hex(10)Fuc(1)	HexNAc(6)Hex(7)Fuc(1)NeuAc(1)		HexNAc(6)Hex(4)Fuc(1)	HexNAc(6)Hex(6)NeuAc(1)	HexNAc(6)Hex(7)Fuc(5)	HexNAc(6)Hex(3)Fuc(2)NeuAc(1)	HexNAc(4)Hex(6)Fuc(2)	HexNAc(4)Hex(6)Fuc(1)NeuAc(1)
24	HexNAc(6)Hex(3)Fuc(2)NeuAc(1)	HexNAc(5)Hex(6)Fuc(3)		HexNAc(6)Hex(6)	HexNAc(3)Hex(3)Fuc(1)	HexNAc(4)Hex(6)Fuc(3)	HexNAc(6)Hex(5)Fuc(1)NeuAc(2)	HexNAc(6)Hex(5)Fuc(1)	HexNAc(4)Hex(5)Fuc(1)NeuAc(2)
25	HexNAc(6)Hex(7)Fuc(4)NeuAc(1)	HexNAc(2)Hex(9)		HexNAc(8)Hex(3)Fuc(1)	HexNAc(2)Hex(4)	HexNAc(3)Hex(5)NeuAc(1)	HexNAc(6)Hex(7)NeuAc(2)	HexNAc(6)Hex(7)	HexNAc(5)Hex(8)
26	HexNAc(6)Hex(5)Fuc(1)NeuAc(3)	HexNAc(6)Hex(6)Fuc(1)NeuAc(1)		HexNAc(6)Hex(7)	HexNAc(6)Hex(8)NeuAc(1)	HexNAc(5)Hex(5)	HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	HexNAc(6)Hex(8)NeuAc(1)	HexNAc(6)Hex(5)Fuc(1)NeuAc(1)
27	HexNAc(8)Hex(5)	HexNAc(4)Hex(5)Fuc(3)NeuAc(2)		HexNAc(7)Hex(6)	HexNAc(7)Hex(8)NeuAc(1)		HexNAc(6)Hex(6)Fuc(1)NeuAc(3)	HexNAc(2)Hex(11)	HexNAc(9)Hex(4)Fuc(1)
28	HexNAc(3)Hex(6)Fuc(1)NeuAc(1)	HexNAc(9)Hex(10)		HexNAc(8)Hex(5)	HexNAc(8)Hex(9)Fuc(1)		HexNAc(6)Hex(7)Fuc(4)NeuAc(1)	HexNAc(4)Hex(5)Fuc(4)	HexNAc(6)Hex(7)NeuAc(1)
29	HexNAc(4)Hex(5)NeuAc(2)	HexNAc(4)Hex(7)Fuc(2)		HexNAc(6)Hex(3)Fuc(2)	HexNAc(9)Hex(10)Fuc(1)		HexNAc(7)Hex(8)Fuc(1)	HexNAc(5)Hex(8)Fuc(1)	HexNAc(6)Hex(6)NeuAc(2)
30	HexNAc(6)Hex(5)Fuc(2)	HexNAc(5)Hex(5)NeuAc(1)		HexNAc(6)Hex(5)	HexNAc(4)Hex(6)Fuc(1)		HexNAc(6)Hex(10)Fuc(1)NeuAc(1)	HexNAc(6)Hex(7)Fuc(1)	HexNAc(7)Hex(8)NeuAc(1)
31	HexNAc(4)Hex(5)NeuAc(1)	HexNAc(7)Hex(4)		HexNAc(7)Hex(3)Fuc(1)	HexNAc(6)Hex(8)Fuc(1)NeuAc(1)		HexNAc(7)Hex(8)Fuc(1)NeuAc(2)	HexNAc(5)Hex(9)Fuc(1)	HexNAc(6)Hex(7)Fuc(1)NeuAc(2)
32	HexNAc(5)Hex(6)NeuAc(3)	HexNAc(5)Hex(6)Fuc(3)NeuAc(1)		HexNAc(5)Hex(4)Fuc(1)NeuAc(1)	HexNAc(6)Hex(7)Fuc(1)NeuAc(1)		HexNAc(3)Hex(6)Fuc(1)NeuAc(1)	HexNAc(9)Hex(6)Fuc(1)	HexNAc(10)Hex(10)Fuc(1)
33	HexNAc(4)Hex(4)Fuc(1)	HexNAc(3)Hex(5)		HexNAc(5)Hex(5)NeuAc(1)	HexNAc(6)Hex(7)Fuc(1)NeuAc(2)		HexNAc(4)Hex(5)NeuAc(2)	HexNAc(4)Hex(6)Fuc(3)	HexNAc(4)Hex(5)Fuc(3)
34	HexNAc(5)Hex(3)Fuc(1)	HexNAc(6)Hex(9)Fuc(1)NeuAc(2)		HexNAc(7)Hex(4)Fuc(2)	HexNAc(5)Hex(6)Fuc(1)NeuAc(3)		HexNAc(4)Hex(5)Fuc(2)NeuAc(1)	HexNAc(8)Hex(5)	HexNAc(6)Hex(7)Fuc(5)
35	HexNAc(5)Hex(4)Fuc(1)	HexNAc(8)Hex(8)		HexNAc(5)Hex(6)Fuc(3)	HexNAc(5)Hex(4)NeuAc(2)		HexNAc(3)Hex(4)Fuc(1)NeuAc(1)	HexNAc(9)Hex(3)Fuc(1)	
36	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	HexNAc(9)Hex(9)Fuc(1)		HexNAc(9)Hex(9)Fuc(1)	HexNAc(5)Hex(6)NeuAc(2)		HexNAc(4)Hex(5)NeuAc(1)	HexNAc(5)Hex(8)Fuc(4)	
37	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	HexNAc(4)Hex(6)Fuc(2)		HexNAc(6)Hex(5)Fuc(1)NeuAc(3)	HexNAc(5)Hex(8)Fuc(4)		HexNAc(4)Hex(3)Fuc(1)	HexNAc(5)Hex(6)NeuAc(3)	
38	HexNAc(3)Hex(4)Fuc(1)NeuAc(1)	HexNAc(6)Hex(7)NeuAc(2)		HexNAc(9)Hex(10)Fuc(1)NeuAc(4)	HexNAc(2)Hex(8)		HexNAc(4)Hex(4)Fuc(1)	HexNAc(7)Hex(7)Fuc(1)	
39	HexNAc(4)Hex(5)Fuc(3)	HexNAc(9)Hex(3)		HexNAc(6)Hex(5)Fuc(2)	HexNAc(6)Hex(11)Fuc(1)		HexNAc(5)Hex(3)Fuc(1)	HexNAc(5)Hex(5)Fuc(1)	
40	HexNAc(5)Hex(5)Fuc(2)	HexNAc(4)Hex(4)NeuAc(1)		HexNAc(4)Hex(5)NeuAc(2)	HexNAc(2)Hex(12)		HexNAc(4)Hex(5)Fuc(1)	HexNAc(5)Hex(5)Fuc(1)NeuAc(2)	
41	HexNAc(6)Hex(4)Fuc(2)	HexNAc(7)Hex(6)		HexNAc(3)Hex(4)Fuc(1)NeuAc(1)	HexNAc(8)Hex(5)Fuc(1)		HexNAc(5)Hex(4)Fuc(1)	HexNAc(9)Hex(10)Fuc(1)NeuAc(4)	
42	HexNAc(5)Hex(5)Fuc(3)	HexNAc(7)Hex(7)					HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	HexNAc(4)Hex(7)	

RESUME

Nabil Tahhan started his undergraduate studies at Aleppo University, Faculty of Engineering Technology, Department of Biotechnology Engineering, in 2013, and graduated with a good degree in 2018.then, he began his Master's studies at the Higher Institute of Education at Karabuk University, Department of Biomedical Engineering, in 2020, and completed his thesis in 2023 by defending his research titled 'Analysis of human plasma N-glycopeptides by mass spectrometry using high-ph fractionation and HILIC-based strategies".