COMPARISON OF DENATURING AGENTS USED IN *N*-GLYCAN RELEASE METHODS FOR QUANTITATIVE GLYCOMIC ANALYSIS

Rokia SAKHTA

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Thesis Advisor
Assist. Prof. Dr. Hacı Mehmet KAYILI

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Rokia SAKHTA

T.C.
Karabuk University
Institute of Graduate Programs
Department of Biomedical Engineering
Prepared as
Master's Thesis

Thesis Advisor
Assist. Prof. Dr. Hacı Mehmet KAYILI

KARABÜK
January 2022

I certify that in my opinion the thesis submitted by Rokia SAKHTA titled "COMPARISON OF DENATURING AGENTS USED IN *N*-GLYCAN RELEASE METHODS FOR QUANTITATIVE GLYCOMIC ANALYSIS " 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 Biomedical Engineering

This thesis is accepted by the examining committee with a unanimous vote in the Department of Biomedical Engineering as a Master of Science thesis. 12/01/2022

Examining Committee Members (Institutions)	Signature
Chairman : Assist. Prof. Dr. Mehmet ATAKAY (HÜ)	....
Member : Assist. Prof. Dr. Ammar ALSHEMARY (KBÜ)	....
Member : Assist. Prof. Dr. Hacı Mehmet KAYILI (KBÜ)	....

The degree of Master of Science by the thesis submitted is approved by the Administrative Board of the Institute of Graduate Programs, Karabuk University.

Prof. Dr. Hasan SOLMAZ ....
Director of the Institute of Graduate Programs

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Rokia SAKHTA

ABSTRACT

M.Sc. Thesis

COMPARISON OF DENATURING AGENTS USED IN *N*-GLYCAN RELEASE METHODS FOR QUANTITATIVE GLYCOMIC ANALYSIS

Rokia SAKHTA

Karabük University
Institute of Graduate Programs
Department of Biomedical Engineering

Thesis Advisor:
Assist. Prof. Dr. Hacı Mehmet KAYILI
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Glycosylation is an essential post-translational modification observed in the living proteome. Complex oligosaccharides called glycans are attached to proteins in this process and, the functions of proteins are changed dramatically. In addition, glycosylation profiles can change in commonly observed diseases such as cancer. Identifying these changes is crucial in discovering new biomarkers for the early detection of cancer and the development of new targeted drugs. Therefore, *N*-glycan profiling of glycoproteins extracted from various biological samples is crucial. One of the main steps of *N*-glycan analysis is to remove *N*-glycans from glycoproteins by specific enzymes. The study aims to compare detergent combinations used in *N*-glycan release methods. In the study, human plasma was used to test the release methods of *N*-glycans containing different detergent combinations. The released *N*-glycans were labeled with the procainamide tag and purified using cellulose-containing solid-phase extraction cartridges. Analyses were performed with HPLC-HILIC-FLD (high-

performance liquid chromatography-hydrophilic interaction liquid chromatography equipped with fluorescence detection), which is the golden standard method for profiling *N*-glycans. The results showed that the, sodium dodecyl sulfate + sodium deoxycholate detergent combination provided the most efficiency in the *N*-glycan analysis. It was found that the total and average signal areas and intensities of the detected *N*-glycans were reduced when SDS and SDC detergents combined with DTT. In addition, deglycosylation of glycoproteins with various denaturing agents resulted in different human plasma *N*-glycosylation profiles.

**Key Words : ** Glycosylation, sample preparation, glycan release, glycan, hydrophilic interaction chromatography.

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ÖZET

Yüksek Lisans Tezi

KANTİTATİF GLİKOMİK ANALİZLERİ İÇİN *N*-GLİKAN SALINIM YÖNTEMLERİNDE KULLANILAN DENATURASYON AJANLARININ KARŞILAŞTIRILMASI

Rokia SAKHTA

Karabük Üniversitesi
Lisansüstü Eğitim Enstitüsü
Biyomedikal Mühendisliği Bölümü

Tez Danışmanı:
Dr. Öğr. Üyesi Hacı Mehmet KAYILI
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Glikozilasyon, canlı proteomda gözlenen önemli bir translasyon sonrası modifikasyondur. Bu translasyon sonrası süreçte, proteinlere glikan adı verilen kompleks oligosakkaritler bağlanır ve proteinlerin işlevleri bu bağlanma sonrası değişmektedir. Ayrıca kanser gibi önemli hastalıklarda glikozilasyon profilleri değişmektedir. Bu değişikliklerin belirlenmesi, kanserin erken teşhisine yönelik yeni biyobelirteçlerin keşfedilmesinde ve yeni hedeflenmiş ilaçların geliştirilmesinde çok önemlidir. Bu nedenle, çeşitli biyolojik örneklerden ekstrakte edilen glikoproteinlere ait *N*-glikan profillerininin çıkarılması çok önemlidir. Glikan düzeyinde glikozilasyon analizinin ana adımlarından biri, *N*-glikanları glikoproteinlerden uzaklaştırmaktır. Çalışmanın amacı, *N*-glikan salınma yöntemlerinde kullanılan denaturasyon ajanlarını içeren kombinasyonların kıyaslanmasıdır. Çalışmada, farklı deterjan kombinasyonları içeren *N*-glikan salınma yöntemlerini test etmek için insan plazması kullanıldı. Serbest

bırakılan *N*-glikanlar, proka inamid etiketi ile etiketlendi ve selüloz içeren katı faz kartuşları kullanılarak saflaştırıldı. Analizler, *N*-glikanların profillenmesi için altın standart yöntem olan HPLC-HILIC-FLD (hidrofilik etkileşimli sıvı kromatografisi ve floresan algılama ile donatılmış yüksek performanslı sıvı kromatografisi) ile gerçekleştirilmiştir. Sonuçlar, SDS + SDC deterjan kombinasyonunun *N*-glikan analizlerinde en fazla verimi sağladığını gösterdi. SDS ve SDC deterjanları DTT ile içerdiğinde, tespit edilen *N*-glikanların toplam sinyal alanlarının ve yoğunluklarının düştüğü belirlendi. Ek olarak, glikoproteinlerin çeşitli deterjan kombinasyonları ile deglikosilasyonu, farklı insan plazma *N*-glikozilasyon profilleri ile sonuçlanmıştır.

Anahtar Sözcükler : Glikozilasyon, örnek hazırlama, glikan salınımı, glikan, hidrofilik etkileşim kromatografisi.

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SYMBOLS AND ABBREVITIONS INDEX

SYMBOLS

µL : microliter

m/z : mass-to-charge ratio

M : quantity molar mass

mg : milligram

mL : milliliter

rpm : Revolutions per minute

ABBREVITIONS

Asn : Asparagine

CDG : Congenital Disorders of Glycosylation

ER : Endoplasmic Reticulum

ESI : Electrospray Ionization

FLD : Fluorescence Detection

HILIC : Hydrophilic Interaction Liquid Chromatography

HPLC : High-performance Liquid Chromatography

LC : Liquid Chromatography

MALDI : Matrix-Assisted Laser Desorption Ionization

MS : Mass Spectrometry

PTM : Post Transitional Modifications

Ser : Serine

SUMO : Small Ubiquitin-related Modifier

Thr : Threonine

TOF : Time-Of-Flight

PART 1

INTRODUCTION

The ribosome uses the genetic code to translate the mRNA molecule into polypeptides in the living cells. The polypeptide is often not ready to use directly after the translation process; that is why it undergoes multiple steps and modifications to become a functional, mature, and active protein. These modifications are known as post-translational modifications (PTM) [1,2]. PTM refers to the polypeptide chain changes by adding or removing special functional groups to amino acid residues [3]. Altering the cellular sites of proteins is one of the functional regulations induced by PTM [4]. Thanks to these modifications, the 20 natural amino acids found in the body are converted into a more diverse group. There are two classifications of post-translation modification, which are reversible and irreversible [5].

Glycosylation is one of the most numerous types of post-transitional modifications [6]. Adding carbohydrates molecules (oligosaccharides) to the proteins is covalently known as glycosylation [7]. Glycosylation includes the reaction of a glycosyl donor and a glycosyl acceptor with the aid of a promoter [8,9]. Glycosylation is a process that mostly depends on enzymes. The enzymes controlling the glycosylation process are glycosidase and glycosyltransferases [10]. These enzymes are responsible for the formation of glycan structures [11].

N-glycans bind to glycoproteins after the translation step. The required sequence for an N-glycan binding is Asn-X-Ser/Thr (X: any amino acid except proline). N-glycans have many different functions. These functions may be different than the glycoprotein that they are located. Factors leading optimal N-glycan binding are controlled by ~300 genes. Mutations in these genes give rise to congenital disorders of glycosylation (CDG), which are rare diseases caused by mutations in genes [12]. There are many types of N-linked oligosaccharides that play a role in a wide variety of biological

functions [13]. Because N-linked glycan is associated with many diseases, it is needed to find the best way to analyze them.

The first step in sample preparation for N-glycan analysis includes the extraction of proteins from biological samples. Since most of the glycoproteins are integral membrane proteins, the use of detergents is required for maximizing the extraction of proteins. Many detergents have been introduced and compared regarding the efficient protein extraction for mass spectrometry-based proteomics. However, commonly used detergents in glycan analysis have not been examined yet.

In the thesis, different denaturing agents and their combinations were investigated for quantitative glycomics performed by HPLC-HILIC-FLD (high-performance liquid chromatography-hydrophilic interaction liquid chromatography equipped with fluorescence detection). The N-glycans were released from human plasma glycoproteins with different detergents and detergent combinations, labeled with procainamide tag, and purified by cellulose-containing solid-phase extraction cartridges. The obtained data were evaluated based on the total areas, intensities, and relative abundances of the detected N-glycan peaks.

PART 2

LITERATURE REVIEW

2.1. POST-TRANSLATIONAL MODIFICATIONS

PTMs are modification catalyzed by the enzymes, which cause significant heterogeneity in the final protein structures. Enzymes play an essential role in controlling these modifications [14]. Abnormality of PTMs may cause diseases. Understanding the PTM changes is vital to treat some diseases such as cancer [15]. PTMs have a lot of variations like methylation, phosphorylation, ubiquitination, and glycosylation [16].

One example of a post-transitional modification type is ubiquitination, in which a ubiquitin protein is linked to a protein [17]. Another type of PTM is lipidation. In lipidation, the lipid is attached to the proteins covalently and mainly helps to stay the protein in the cell membrane [18]. Also, phosphorylation is one PTM in which phosphate groups are attached to serine or threonine amino acid residues. The phosphorylation and dephosphorylation process works with two critical enzymes named protein kinases and protein phosphatases [19]. Methylation is a type of modification that occurs to proteins, which causes peptides to encode extra data by changing their initial sequence [20]. Another type of PTM is acetylation in this process an acetyl group binds to the ε-amino group of lysine residues, the α amino group of the N-terminus of proteins by the action of acetyl-coenzyme A.

Acetylation as a PTM has diverse impacts on the protein and metabolome [21]. Also, sumoylation is a small protein called (small ubiquitin-related modifier, SUMO) that binds to the proteins. This binding is dependent on a group of enzymes [22]. Hydroxylation is a type of PTM. The hydroxylation process starts in the endoplasmic reticulum, and dioxygen-dependent enzymes are responsible [23].

Disulfide bonding in proteins is also found among PTM's, which is highly effective in stabilizing proteins. The proteins are not being broken down when they are outside cells. This modification is achieved by adding covalent crosslink binding of cysteine residues between the sulfhydryl groups [24].

2.2. GLYCOSYLATION

Glycosylation is an important process that plays a significant role in the cellular events such as cell-cell adhesion, endocytosis, protein folding, the transit of molecules, and activation of receptors, plus signal transduction [25]. The biosynthesis of glycan does not contain any template [26]. Glycosylation of proteins and lipids starts in the endoplasmic reticulum (ER) and continues with the Golgi apparatus. The heterogeneity of protein glycosylation comes from the production of these polymers within the living Cell [26]. Protein glycosylation in the individual is considered stable, but it has a lot of variations at the population level [27].

2.3. GLYCOSYLATION TYPES

In eukaryotes, there are two primary glycosylation forms: *N*-linked and *O*-linked [28].

2.3.1. *N*-linked Glycosylation

This type of modification is present in eukaryotic and prokaryotic cells and archaea (Figure 2.1) [29]. *N*-linked glycosylation is the most abundant type of glycosylation that is present in mammals [30]. The synthesis of N-glycan occurs in the ER membrane on the cytosolic face and then flipped to the lumen face. Diverse glycosyltransferase enzymes gradually incorporate the monosaccharides to form glycans on a lipid base (dolichol phosphate) in the membrane of the endoplasmic reticulum in the cell (Figure 2.2). This combination of lipids bound to sugars changes their place from the cytoplasmic direction to the lumen by turning itself [29]. And, the entire oligosaccharide Glc3Man9GlcNAc2 binds to the nitrogen atom of chosen asparagine residues by oligosaccharide transferase [31]. Then, an enzyme called glycosidase cut

the glucose residues from the entire oligosaccharide. The N-linked protein moves freely to the Golgi apparatus, and several mannose residues are cut by another enzyme. The *N*-acetylglucosamine is added by N-acetylglucosamine transferase. The protein then moves to the trans part where other modifications occur like adding N-acetylmuramic acid or sialic acid, or galactose.

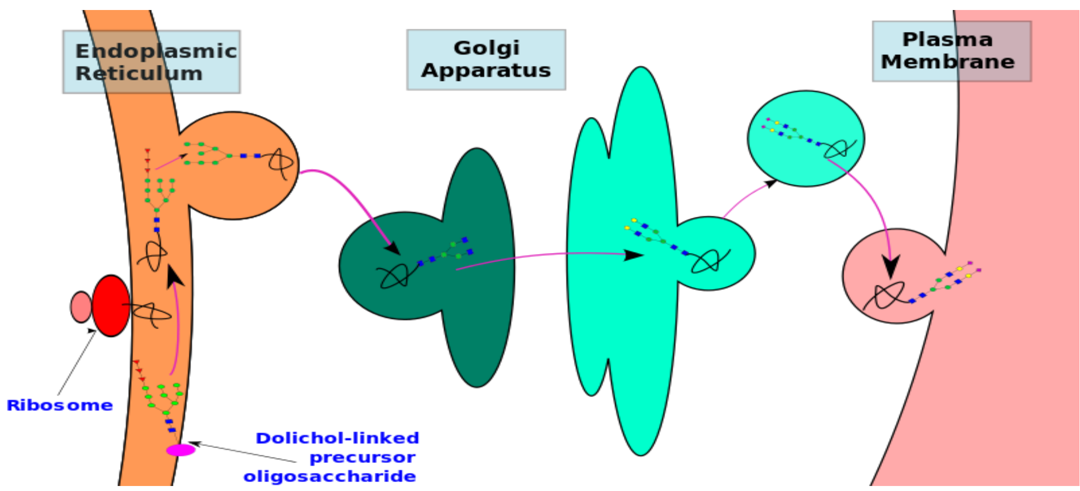

Figure 2.1. *N*-linked glycosylation pathway in eukaryotic cells.

The basic glycan structure is mainly composed of two *N*-acetyl glucosamine and three mannose residues, then other modifications are added to this core structure, giving it a greater variety [32]. There are three main types of *N*-linked glycans based on their sugars structures: oligomannose, complex- and hybrid [33]. The *N*-linked glycan has many important functions related to the immunity in our bodies, including controlling the migration of the immune cells.

For the different immunoglobulin types, the glycosylation style gives them unrivaled functions by changing their affinities for Fc and other immune receptors. In addition, they are played an essential role in "self" and "nonself" recognition [34]. Glycosylation alterations may change the biological actions of the proteins [35].

Figure 2.2. The formation of an *N*-linked glycan by the action of oligosaccharide transferase.

2.3.2. *O*-linked Glycosylation

The O-linked glycosylation process starts at the Golgi complex by attaching a GalNAc-residue to the oxygen atom in the alcohol group of serine or threonine of the protein; this binding is done by the N-acetyl galactosamine transferase enzyme [33,36]. The oligosaccharide sequences in *O*-linked glycoprotein can form over 50% of its mass, such as mucins. *O*-linked glycoproteins that play a critical role in the body. For example, they are the component of the zona pellucida and mucins that is a component of saliva [1]. Types of *O*-linked glycan include *O*-GalNAc glycan, *O*-fucose, *O*-mannose, reversible cytoplasmic and nuclear *O*-GlcNAc, initiator *O*-xylose[36].

2.4. MASS SPECTROMETRY

Mass spectrometry is a valuable analytical tool for identifying the component elements of a substance or molecule, like peptides and other chemical compounds. The mass spectrometer includes an ion source, mass analyzers, and a detector (Figure 2.3). Ion sources convert the sample particles into ions. The mass analyzer function is to apply electromagnetic fields to sort the ions according to their mass/charge ratio. A detector measures the m/z ratio of ions. It gives information for calculating the abundances of the current ions [37]. It has been found that mass spectrometry has both quantitative and qualitative uses. It is used to determine the exact or relative amount of a compound

in a sample besides investigation the chemistry of ions in the gas phase. Also, the isotopic composition of a molecule can be defined to identify the structure of unknown compounds [37,38].

Because the ions have an electric charge, their location in space can be controlled with electric and magnetic fields. The individual can be analyzed in a vacuum. This means that the ions must be in the gas phase, which is helpful for separating them by mass analyzers [39]. One of the advantages of mass spectrometry is giving information about the material to be analyzed even its amount is in nanograms, which makes mass spectrometry the best for analyzing the material since its consumption of the sample is very low [40].

The ion source is one of the critical parts of the mass spectrometer, and there are several types of it. Electrospray and matrix-assisted laser desorption/ionization (MALDI) are the most commonly used ionization techniques for biological liquid and solid samples [37] .

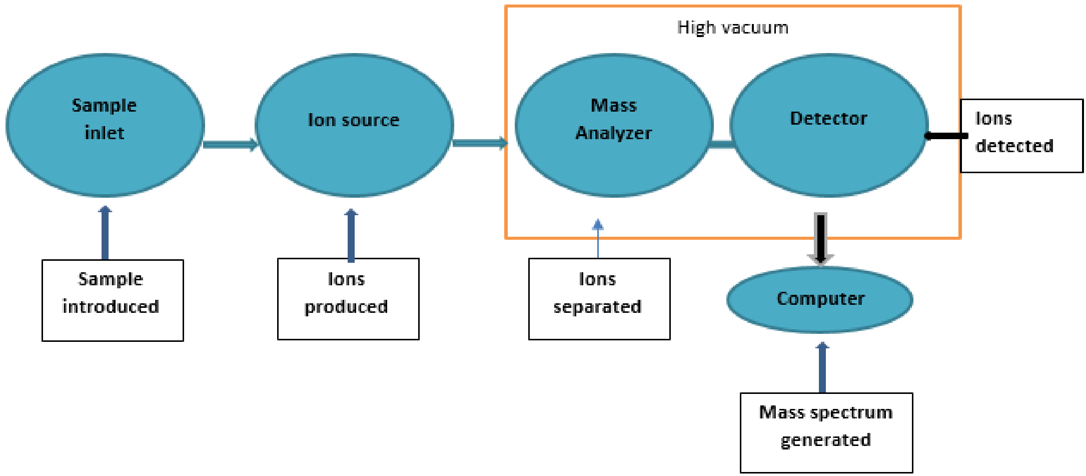

Figure 2.3. The essential parts of a mass spectrometer.

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2.4.1. Electrospray Ionization (ESI)

ESI is an ionization technique used in mass spectrometry to produce ions for the compound to be analyzed. Advantages of electrostatic spray ionization (ESI) can produce intact charged ions for biologically important species [41]. It is one of the

soft ionization methods because it does not cause a significant fragmentation of the compound. It is widely used to produce ions from large molecules with polar properties such as proteins and nucleic acids.

The process involves transferring sample ions from the solution to the gas phase by subjecting them to a strong electric field at atmospheric pressure before the ions gradually move to the high-vacuum regions of the mass spectrometer (Figure 2.4). It is included in the techniques of ionization under atmospheric pressure [42,43].

In ESI, the sample in the solution begins to pass through a stainless-steel capillary tube towards a strong electric field with the help of a stream containing nitrogen gas heated to a temperature up to 350 ° C. This helps to evaporate the solvent containing the sample. Droplets with an electric charge ion then reach the mass analyzer [43]. Since the particles have a total positive charge, the mass/charge ratio gets to be sufficiently small to allow the analysis of materials in mass spectrometry. The same molecule causes a series of peaks with varying numbers of charges that offer details that simplify the identification [44].

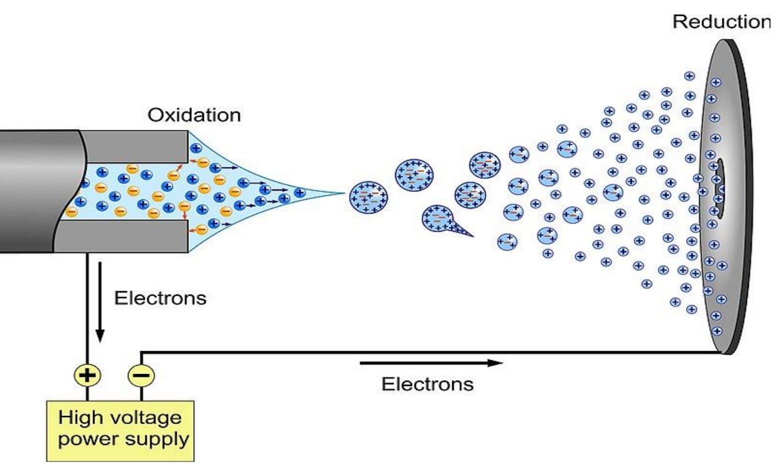

Figure 2.4. Mechanism of ESI technique.

2.4.2. Matrix-Assisted Laser Desorption Ionization (MALDI)

MALDI is another soft ionization method based on using laser power. The matrix absorbs laser to generate ions for large molecules with minimal fractionation [45].

MALDI–MS can be used in detecting the mass/charge ratio of molecules varying from small peptides to large proteins [46]. MALDI process starts by mixing the sample with an appropriate matrix substance. This mixture is then dropped on a metal sheet. After applying the laser, the mixture heats by this energy and converts it to gas. The ions are then formed by undergoing protonation or deprotonation (Figure 2.5). The resulting ions enter the mass analyzer [47]. The mass analyzer determines the mass to charge ratio (m/z) of ions of the analytes. Choosing the type of the mass analyzer is dependent on the properties of the ionized substance and the protocol that will be performed.

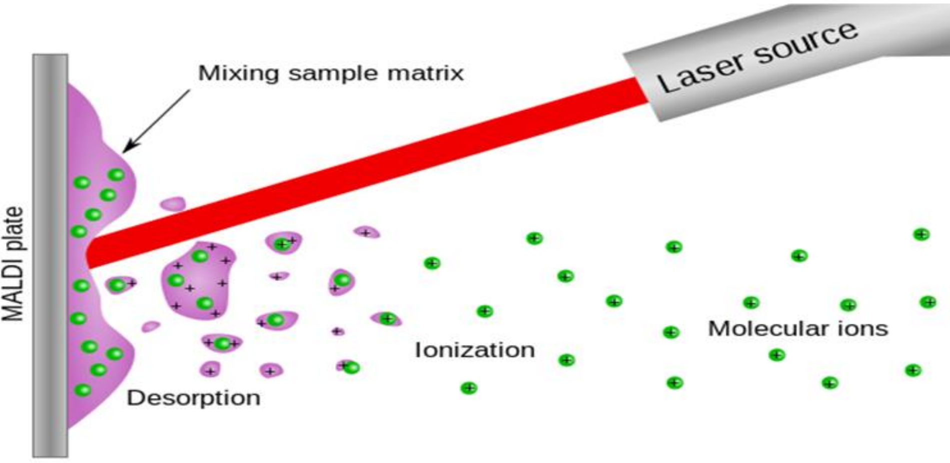

Figure 2.5. The principle of matrix-assisted laser absorption/desorption ionization (MALDI).

2.4.3. Mass Analyzers

Mass analyzers play many roles in addition to their ability to resolve ions of diverse m/z. It can also be able to trap and store ions. The most common analyzers include ion trap, time-of-flight (TOF), quadrupole, fourier transform-ion cyclotron [FT-ICR], and orbitrap [48].

2.4.3.1. Time-of-Flight

Measuring the ions with the time of flight mass analyzers allows us to determine its mass to charge ratio with the high scan velocity, provide almost infinity mass range and the high resolution. In TOF-MS, ions with the same energies but different masses

move at different speeds in drift tubes. The electrostatic field accelerates ions created due to ionization, which acquire the same kinetic energy. Ions are then transferred along with the drift path and arrive in the detector at different time points. Lighter ions are separated from heavier ones, and the mass spectrum is recorded [49].

2.5. CHROMATOGRAPHIC TECHNIQUES

Mass spectrometry is an important technique and compatible with liquid chromatography for the identification of separated components [50]. The chromatography technique is known as one of the physical separation methods. The separated components are selectively dispersed between two immiscible phases: the mobile phase flows through a fixed phase layer (stationary phase) [51]. Due to the physical situation of those phases, various types of chromatography are classified.

Liquid chromatography is a method used to separate the compounds present in the mixture depending on the interactivity of the sample with the mobile and stationary phases. Liquid column chromatography has been used widely for low-resolution separation of sample mixtures in the past [50]. Liquid-solid column chromatography is one of the famous chromatography techniques for the properties of the liquid mobile phase that is suitable for mass spectrometric analysis. Hydrophilic interaction liquid chromatography (HILIC) has lately shown valid potential for characterizing glycoforms of intact proteins as a type of liquid chromatography [52].

2.5.1. High-performance Liquid Chromatography (HPLC)

HPLC is a technique used to separate and detect and quantify components in a mixture (Figure 2.6). Its mechanism is to push a pressurized liquid solvent with the sample mixture onto a column loaded with a solid adsorbent. The components of all samples interact with the adsorbent material in various ways, resulting in different retention times for each component. [53]. In glycobiology, HPLC is commonly used for the separation of the glycans of complex bio-samples.

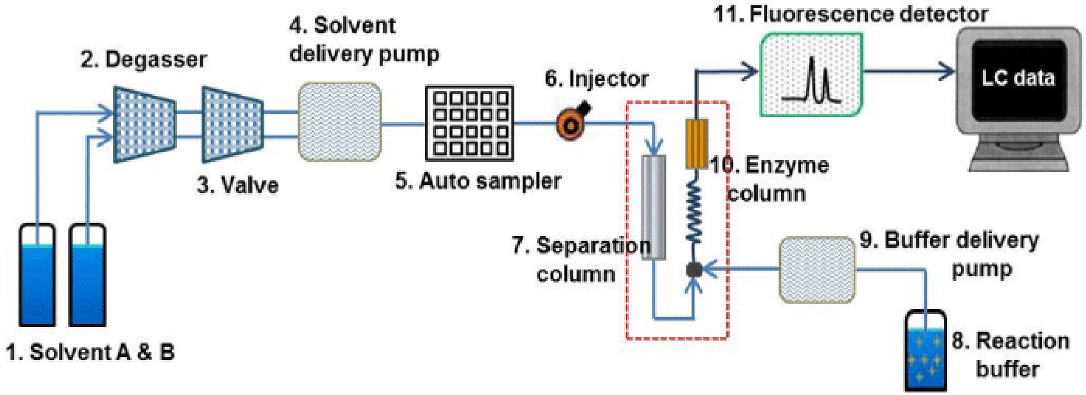

Figure 2.6. High performance liquid chromatography with a fluorescence detector [54].

2.5.2. Hydrophilic Interaction Liquid Chromatography

HILIC is commonly used to separate polar substances. This technique began to emerge in the early 1990s, and the method has been called hydrophilic interaction chromatography (HILIC). Polar compounds have gained importance in recent years due to the need to study in increasingly complex mixtures. Another factor contributing to its increase in importance is the widespread use of MS with LC [55].

2.6. PROFILING APPROACHES FOR *N*-GLYCANS

Profiling of N-glycans can be done using either mass spectrometric or chromatographic techniques. It is frequently used in methods where both methods are used together. N-glycans can be analyzed directly or after derivatization after being released with the PNGase F enzyme. *N*-glycan release methods can be performed in solution or on a membrane such as PVDF. Sometimes chemical methods such as hydrazine can also be used for this purpose. Chemical methods are faster than enzymatic methods regarding *N*-glycan release [56].

2.6.1. Mass Spectrometry-based Methods

Mass spectrometry is used as a very sensitive and selective method for glycan characterization. MALDI-MS is a powerful tool for analyzing glycans in simple or complex samples. The glycan profile is determined by MALDI-TOF analysis. The conformations of N-glycans are determined according to their molecular weights [57]. MALDI-MS has also been used for glycan profiling in tissues in addition to allow identification and localization of *N*-glycans [58]. However, while this method is useful for profiling glycoforms, it cannot provide structural information such as sugar anomericity or glycan site specificity. The combination of a MALDI-TOF analysis with tandem mass spectrometry or post-source decay (PSD) approaches is often required to obtain such information [57].

2.6.2. Chromatographic Techniques-based Methods

Liquid chromatography combined with electrospray ionization is a powerful analytical approach to identify and characterize N-glycan structural isomers. This technique is extensively combined with reversed phase (RP) chromatography in many bioanalytical laboratories and is widely used in carbohydrate analysis. Derivatization of N-glycans is necessary to increase their hydrophobicity so that they can be analyzed in reversed phase liquid chromatography [59]. This system usually requires a mass spectrometric detection. On the other hand, graphitized carbon chromatography is also frequently used in N-glycan analysis with mass spectrometric detection [60]. Here, reduction of N-glycans is generally applied, as it makes analyzes easier. Hydrophilic interaction liquid chromatography (HILIC) is one of the most ideal techniques for analyzing glycans [61]. The ability of glycans to present a hydrophilic physical structure and to be labeled at their reducing ends with fluorophores has made the HILIC-FLD technique the gold standard. Along with these techniques, anion exchange chromatography (HPAEC) is also used as a suitable alternative for glycan analysis due to its solubility properties [62].

2.6.3. Glycan Labeling Approaches

Procainamide tag can be applied as a standard technique for glycan characterization by HPLC-FLD assays, as it provides more sensitive results than the two fluorescent labels 2-aminobenzamide and 2-aminobenzoic acid. Procainamide derivatization provides good chromatographic separation for relative quantification. It also offers good ESI-MS analysis sensitivity because it has more functional ionization (Figure 2.7) [63].

A

B

Figure 2.7. Chemical structure of procainamide and a procainamide labeled N-glycan.

PART 3

MATERIALS AND METHOD

3.1. MATERIALS

Human plasma (lyophilized), cellulose, Igepal Ca-630, sodium dodecyl sulfate (SDS), sodium deoxycholate (SDC), 1,4-Dithiothreitol (DTT), phosphate buffer saline (PBS), dimethyl sulfoxide (DMSO), de-ionized H_2O, acetonitrile (ACN), trifluoroacetic acid (TFA), sodium cyanoborohydride were bought from Sigma Aldrich: (St Louis, MO, USA). Acetic acid (LC/MS grade) was bought from Carlo Erba Reagents. PNGase F enzyme was obtained from Promega (Madison, WI, USA). Procainamide HCl was purchased from Abcam (Cambridge, UK). Deionized water (dH_2O) was obtained using an Expe-Ultrapure Water System (Mirae St., Korea).

3.2. METHODS

3.2.1. Glycan Release of Human Plasma Glycoproteins with Different Detergents

First, human plasma was prepared with a concentration of a 70 µg μL^{-1}. The following detergents were prepared: 2% SDS (w/v) (1), 2% SDC (w/v) (2), 2 % SDS including 0.5 M DTT (3), 2% SDC including 0.5 M DTT (4), 2% SDS + 2% SDC mixture, 1/1, v/v, (5), 2% SDS+ 2% SDC mixture, 1/1, v/v, including 0.5M DTT (6). Then, 20 µL of human plasma was mixed with 20 µL of each detergent. Subsequently, the samples were incubated at 60 ^oC for 10 min to denature the glycoproteins. Twenty µL of 4 % Igepal-CA630 and 20 µL of 5X PBS were added into the samples, respectively. Finally, 1 U of PNGase F enzyme was inserted into the samples and, all samples were incubated at 37 ^oC for 16 h.

3.2.2. Procainamide Labeling of Released *N*-glycans

The glycan release samples were mixed with 100 µL of a labeling solution including 50 µL of procainamide hydrochloric acid (110 mg mL^{-1} in DMSO/ Acetic acid, 7/3, v/v) , and 50 µL of sodium cyanoborohydride (65 mg mL^{-1} in DMSO/ Acetic acid 7/3, v/v). Then, the samples were incubated at 65 ^{o}C for 2 h.

3.2.3. Purification of Procainamide Labeled *N*-glycans

Purification of procainamide labeled *N*-glycans was achieved by using SPE cartridges. A solution of microcrystalline cellulose (100 mg mL^{-1}) in dH_2O was first prepared. A 300 µL microcrystalline cellulose was inserted into the microcentrifuge tubes. The microcrystalline cellulose containing SPE cartridges was washed with 1 mL of dH_2O and 1 mL of ACN/dH_2O, 85/15, v/v for three times, respectively. Then, the glycan release samples (120 µL) was mixed with 680 µL of ACN to obtain proper loading conditions (85/15, v/v, ACN/sample). The microcrystalline cellulose was mixed with the loading samples and the samples were incubated at room temperature in a thermomixer by shaking at 500 rpm for 15 min. Then, the slurry was transferred to a SPE cartridges and the loading samples discarded by applying a vacuum. The microcrystalline cellulose containing SPE cartridges were washed with 1 mL of ACN/ dH_2O/TFA mixture (85/14/1, v/v/v) and 1 mL of ACN/dH_2O mixture (85/15, v/v) three times, respectively. The procainamide labeled *N*-glycans were eluted with 0.75 mL of water. The elution solutions were dried with a speed vacuum concentrator overnight. The dried samples were dissolved in a mixture of 100 µL of ACN/MQ, 75/25, v/v, and transferred to the vials for HPLC-HILIC-FLD analysis.

3.2.4. HPLC-HILIC-FLD Analysis

An Agilent 1200 series HPLC system with Agilent 1260 FLD detector was employed for procainamide labeled *N*-glycan analysis. The analysis was achieved using a Waters Glycan BEH Amide 2.5 µm (2.1 mm ID x 15 cm L) column. The wavelengths of the FLD detector for excitation and emission were set to 310 and 370, respectively. 100 % ACN and 50 mM ammonium formate pH:4.4 were used as mobile phase A and

mobile phase B, respectively. Mobile phase A was changed from 75% to 53 % in 60 minutes for the analytical separations. The flow rate was 0.25 mL min^{-1}. The injection volume was set to 10 μL.

3.2.5. Identification of Procainamide Labeled *N*-glycans

Identification of *N*-glycan peaks in the FLD chromatogram was achieved by HPLC-HILIC-FLD-MS/MS analysis performed previously with our group. The area and intensity values of procainamide labeling *N*-glycans was achieved using the OpenLAb software. The relative intensities and areas of each detected *N*-glycans were calculated applying total area normalization approach.

PART4

RESULT AND DISCUSSION

4.1. APPLIED STRATEGY IN THE STUDY

In this thesis, the *N*-glycans were released with different glycan release methods using various detergents, labeled by the procainamide tag, and purified via cellulose-containing SPE cartridges. Then, *N*-glycan analysis of human plasma was performed by an HPLC-HILIC-FLD**.** The data was processed by Agilent OpenLab software and evaluated using Graphpad Prism. The experiments were achieved on two different days with three replicates.

The main goal of this work was to compare the detergent combinations used in *N*-glycan release methods and determine which will be the most efficient method for extracting *N*-glycans from complex samples. Therefore, we compared the six detergent combinations commonly used in the *N*-glycan release methods (Table 4.1).

Table 4.1. The detergent combinations used in *N*-glycan release methods.

	Detergent Combinations	First Concentration	Final Concentration Before PNGase F treatment
1	SDS	2%	0.5%
2	SDC	2%	0.5%
3	SDS + DTT	2% + 0.5 M	0.5% + 0.125M
4	SDC + DTT	2% + 0.5 M	0.5% + 0.125M
5	SDS + SDC	1% + 1%	0.25% + 0.25%
6	SDS + SDC + DTT	1% + 1% + 0.5M	0.25% + 0.25% + 0.125M

Figure 4.1 presents an example FLD chromatogram obtained from the analysis of the procainamide labeled *N*-glycans by the HPLC-HILIC-FLD. We detected 22 *N*-glycan peaks belonging to human plasma *N*-glycome in the research. The peaks were annotated based on the literature knowledge, and our previous works applied previously using mass spectrometric detection [64,65].

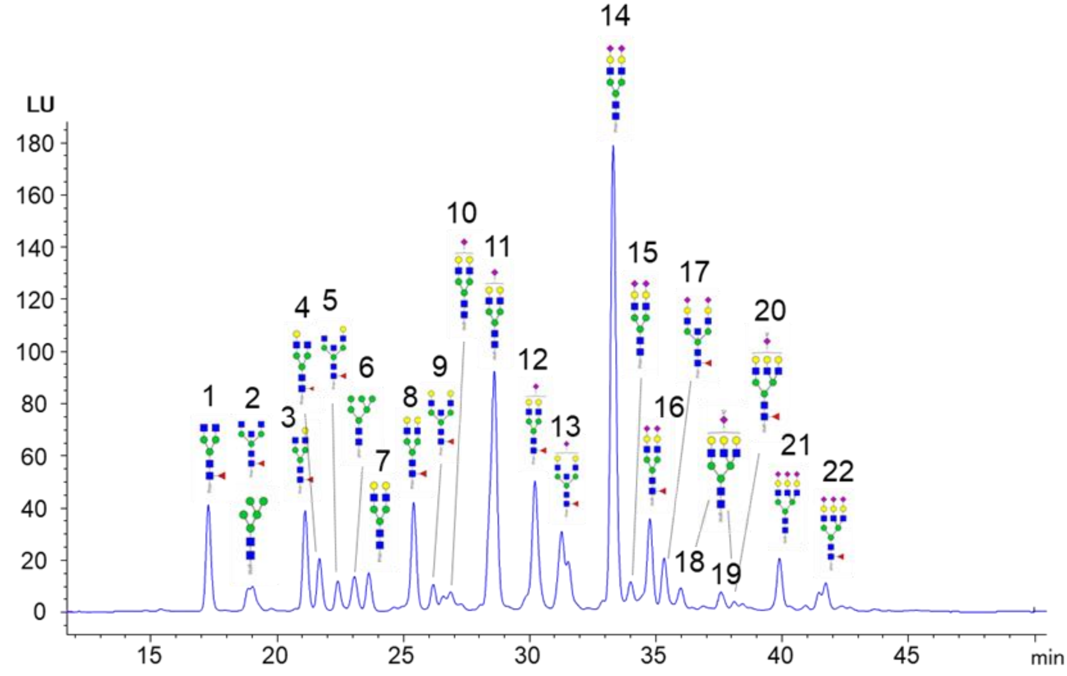

Figure 4.1. A typical chromatogram of procainamide labeled *N*-glycans of human plasma profiled by HPLC-HILIC-FLD.

4.2. COMPARISON OF THE *N*-GLYCAN RELEASE METHODS INCLUDING DIFFERENT DETERGENT COMBINATIONS

In the study, the detergents given in Table 4.2 were used as a single or a combined form. These detergents were commonly used in *N*-glycan release methods. The most used detergent is SDS having the following chemical formula $CH_3(CH_2)_{11}SO_4Na$. It is a common substance that uses in biochemistry, and it has an impact on the protein by denaturing its structure at high temperatures [66]. the other chemical named sodium deoxycholate has the following chemical formula $C_{24}H_{39}NaO_4$. It is a cheap acid-insoluble detergent that works as a membrane-destroying surfactant. It is usually used in research that includes proteins and can be used to solubilize membrane proteins and enhance digestion [59].

Table 4.2. The structure of the detergents used in glycan release methods.

Name	Abbreviation	Chemical Structure
Sodium dodecyl sulfate	SDS	
Sodium deoxycholate	SDC	
1,4-Dithiothreitol	DTT	

Dithiothreitol (DTT) is a reducing agent that has $C_4H_{10}O_2S_2$ chemical formula[67]. It is often used alongside other detergents like SDS or SDC to denature proteins efficiently. It works by reducing disulfide chains or proteins to permit the separation of proteins [68].

In the analysis, we determined FLD chromatograms of human plasma *N*-glycans by using single detergent and detergent combinations. Figure 4.2. presents FLD

chromatograms of the human plasma *N*-glycans released from different detergent combination containing *N*-glycan release methods. The *N*-glycan profiles belonging to each method were compared by investigating *N*-glycan peaks in the Figure 4.2. The number of the detected *N*-glycans was found to be similar for each procedure. The resolution and shape of the *N*-glycan peaks were not influenced from the different detergent combination protocols. However, the early eluted *N*-glycan peaks were found to be low intensities in *N*-glycan release methods including DTT reducing agent. In order to make more reliable comparison of *N*-glycan release methods including various detergent combinations, the features of the obtained *N*-glycan peaks were extracted with a software. Then, the obtained data were compared among *N*-glycan release methods including different detergent combinations.

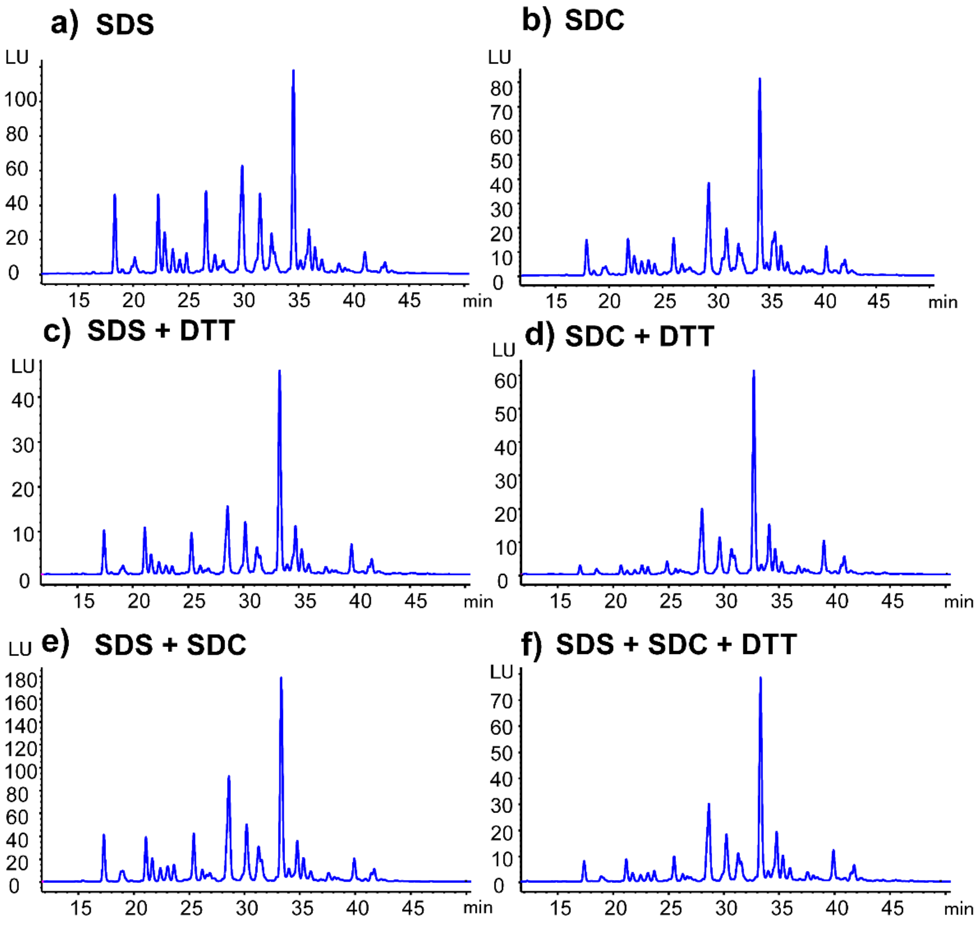

Figure 4.2. FLD chromatograms of human plasma *N*-glycans obtained from *N*-glycan release methods containing different detergent combinations (a) SDS, (b) SDC, (c) SDS + DTT, (d) SDC + DTT, (e) SDS + SDC, (f) SDS + SDC + DTT.

4.3. COMPARISON OF DETERGENT COMBINATIONS IN *N*-GLYCAN RELEASE METHODS BASED ON PEAK AREAS

The *N*-glycan release methods with different detergents were first compared by evaluating the peak areas of *N*-glycans extracted from the FLD chromatogram. The *N*-glycan areas of the detected peaks were extracted. Figure 4.3. shows the comparison of data based on peak areas of the detected *N*-glycan peaks. The total peak areas were also calculated for each detergent containing *N*-glycan release methods (Figure 4.4). The SDS + SDC detergent combination used in the *N*-glycan release methods was found to be the highest total and average peak areas. The *N*-glycan method used SDS was found to be the second-highest total and average area. Table 4.3 presents the data for *N*-glycan peak areas obtained from each method. When the data was evaluated based on total and average areas of the detected peaks, the detergent combinations were ordered from highest to lowest total and average areas as followed: SDS + SDC > SDS > SDC > SDS + DTT > SDS + SDC + DTT > SDC +DTT.

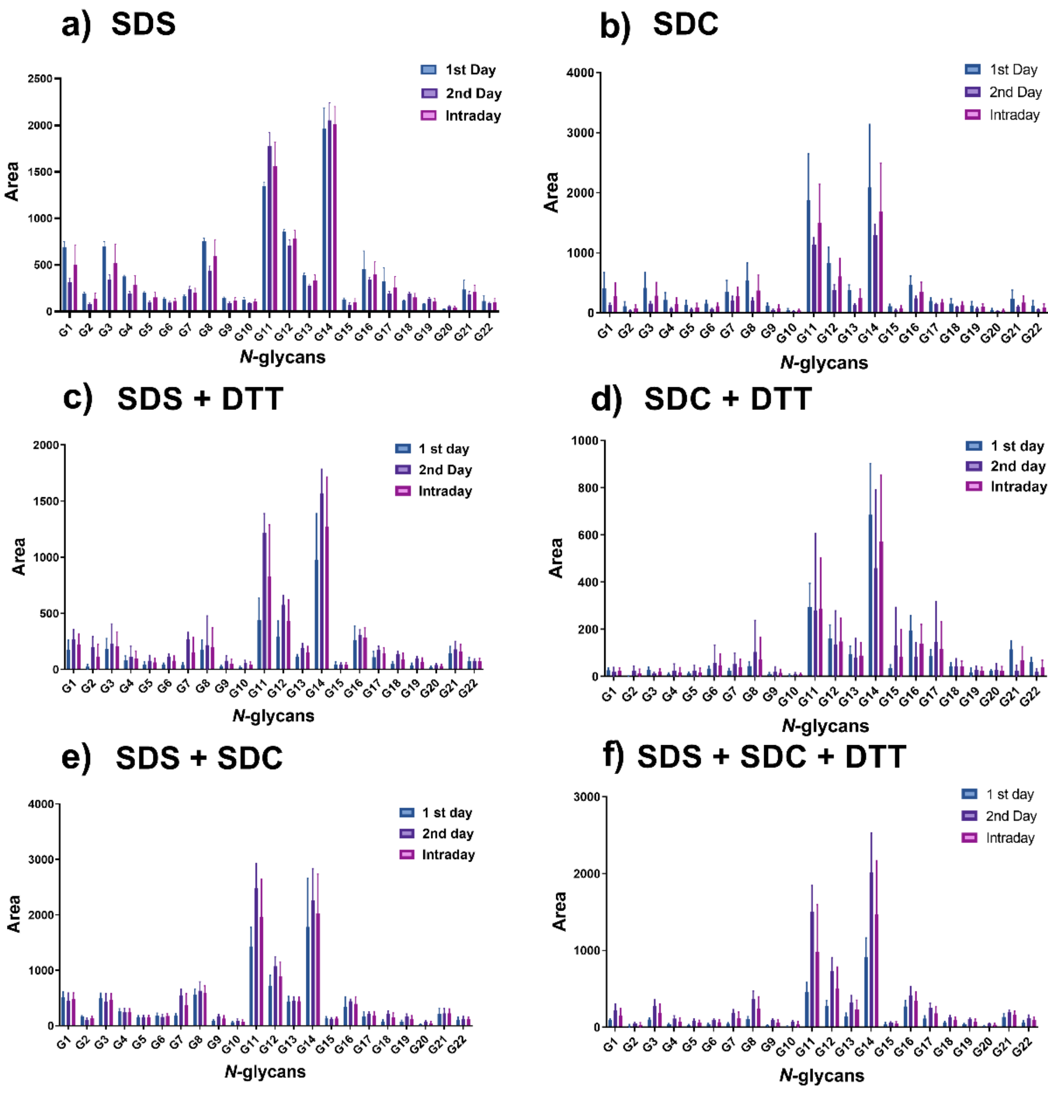

Figure 4.3. Peak areas of the *N*-glycan peaks obtained from *N*-glycan release methods containing different detergent combinations (a) SDS, (b) SDC, (c) SDS + DTT, (d) SDC + DTT, (e) SDS + SDC, (f) SDS + SDC + DTT.

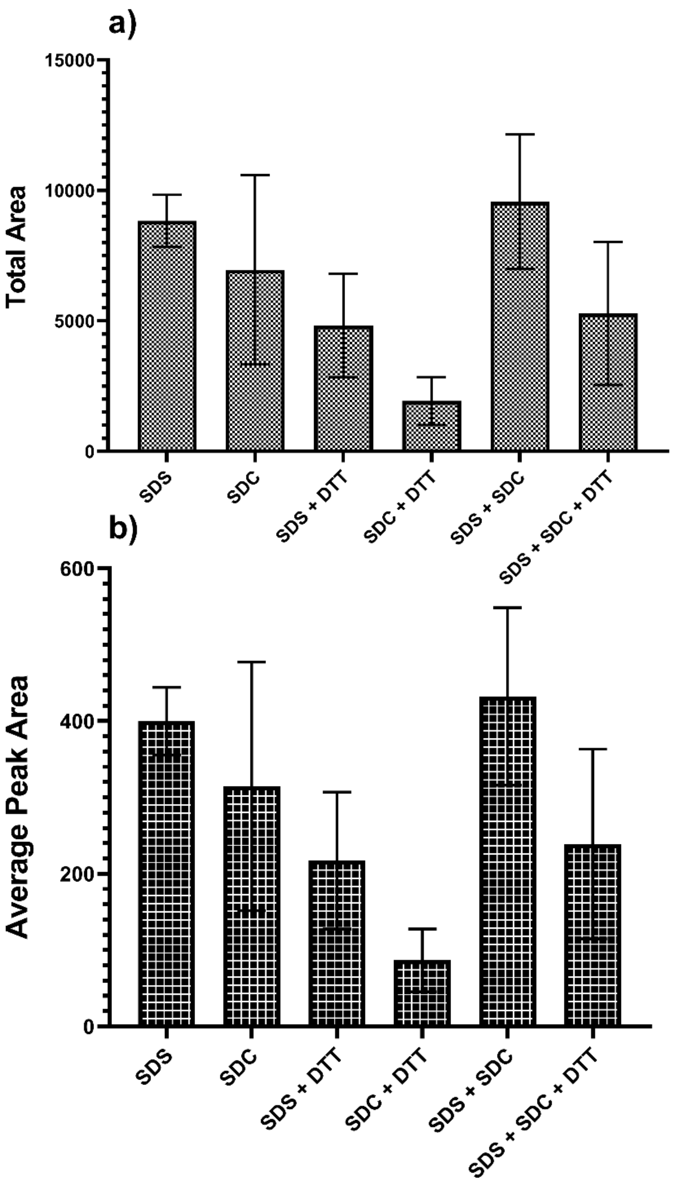

Figure 4.4. Total and average peak areas of the *N*-glycan peaks obtained from *N*-glycan release methods including different detergent combinations.

Table 4.3. Peak intensities of the *N*-glycan peaks obtained from *N*-glycan release methods including different detergent combinations.

Glycan Peak	SDS		SDC		SDS + DTT		SDC + DTT		SDS + SDC		SDS + SDC + DTT	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
G1	504.85	212.20	271.85	224.50	221.80	91.53	22.36	14.21	481.84	110.66	155.73	88.52
G2	137.24	62.04	75.37	61.50	110.33	113.07	12.48	18.01	133.79	36.75	33.27	26.38
G3	523.16	200.22	285.32	218.54	204.90	128.51	19.51	12.12	467.96	107.00	188.07	111.88
G4	283.74	102.59	140.93	111.39	95.53	67.14	18.04	19.18	248.65	56.16	76.71	46.76
G5	152.85	57.35	93.95	64.27	60.77	37.42	17.90	16.93	147.57	34.01	57.79	34.43
G6	113.86	26.35	109.38	61.56	76.19	42.61	45.57	49.68	175.75	39.78	67.49	29.61
G7	203.97	45.91	274.98	154.77	152.18	131.69	37.99	34.14	370.62	209.68	116.95	83.20
G8	597.21	175.55	370.56	263.65	196.01	174.76	73.52	91.48	597.46	124.30	240.17	156.79
G9	119.01	30.41	81.35	49.34	51.14	39.90	14.83	15.03	129.44	49.13	57.35	39.46
G10	107.95	25.13	38.82	18.18	40.29	25.73	8.67	4.67	79.34	28.44	41.45	33.71
G11	1561.01	258.01	1502.19	641.47	828.08	458.37	286.51	216.48	1959.35	679.63	981.15	616.90
G12	782.00	93.36	605.62	301.23	432.50	187.88	148.52	98.43	897.28	252.15	504.93	279.53
G13	330.61	64.77	254.01	146.75	153.47	49.80	87.95	55.44	443.23	72.79	235.22	118.22
G14	2010.39	189.24	1692.01	799.94	1273.15	439.57	571.95	280.00	2024.43	712.47	1466.12	704.02
G15	99.37	38.58	79.98	38.60	41.28	18.53	82.86	115.40	129.82	22.71	52.43	18.92
G16	397.02	141.47	353.07	155.22	284.04	87.20	139.21	81.11	394.20	126.68	343.14	117.53
G17	258.38	118.66	168.41	46.91	141.94	51.56	116.51	114.30	188.57	65.53	182.94	87.90
G18	152.75	42.00	125.60	60.14	90.09	53.28	42.77	22.22	146.02	86.24	91.81	43.57
G19	111.15	31.33	99.57	48.69	65.88	36.06	23.52	16.37	116.73	63.50	71.09	38.65
G20	42.92	15.55	38.84	17.84	29.64	13.80	25.24	16.30	46.80	27.14	34.11	18.05
G21	209.11	72.97	170.91	113.99	160.90	61.87	68.80	57.38	220.54	82.35	165.52	46.14
G22	99.52	39.97	87.88	62.72	74.60	23.46	40.18	28.19	113.41	42.06	93.43	41.36

4.4. COMPARISON OF DETERGENT COMBINATIONS IN *N*-GLYCAN RELEASE METHODS BASED ON PEAK INTENSITIES

The *N*-glycan release methods with different detergents were also compared by evaluating the peak intensities of *N*-glycans extracted from the FLD chromatograms. Figure 4.5 shows the comparison of the data based on peak intensities of the detected *N*-glycan peaks. As expected, the SDS + SDC detergent combination was the highest total and average peak intensities in the FLD chromatogram when the obtained intensity values were compared in the *N*-glycan release methods (Figure 4.6 and Table 4.4). The SDS detergent containing the *N*-glycan release method was found to be the second-highest total and average intensity values. The data was ordered based on total and average intensities as followed: SDS + SDC > SDS > SDC > SDS + DTT > SDS + SDC + DTT > SDC + DTT. These results were consistent with the data obtained from area-based experiments.

All studied glycan release methods including different detergent combinations resulted moderately reproducible total and average intensities. The result clearly showed that SDS + SDC detergent combination was found to provide the highest total areas and intensities. The total areas and intensities of SDS, SDC, and SDS + SDC detergent combination-containing methods were decreased when these detergent combinations included DTT reducing agent. This result is probably due to the activity loss of the PNGase F enzyme in DTT reducing agent containing detergents.

The average areas and intensities obtained from each method were also evaluated to test the efficiency of *N*-glycan analysis. The results obtained from the total area and intensity values of the *N*-glycans were matched with the results from average areas and intensities (Figure 4.4 and 4.6). Deglycosylation using the method with SDC + DTT resulted in lower total and average areas and intensities. The *N*-glycan release method with SDS detergent provided more reproducibility based on peak areas and intensities.

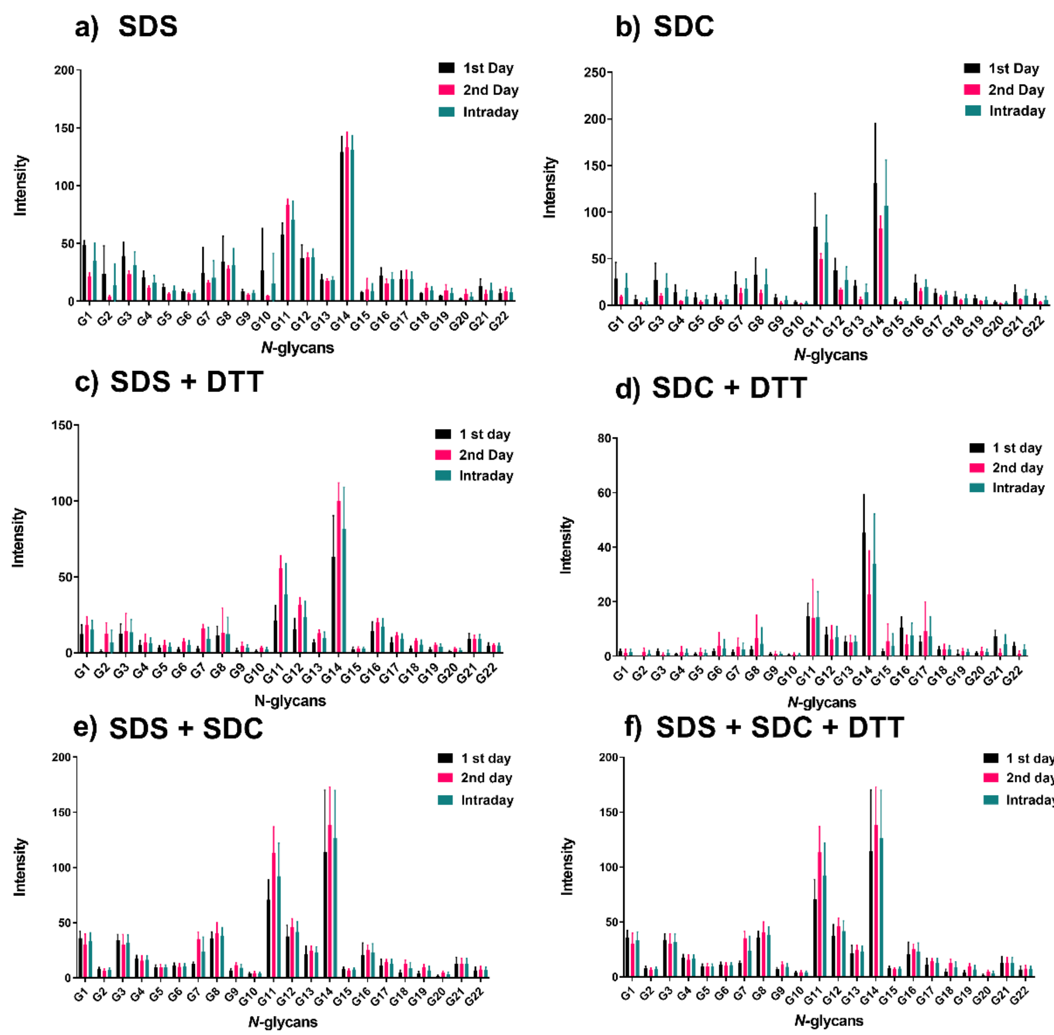

Figure 4.5. Peak intensities of the *N*-glycan peaks obtained from *N*-glycan release methods containing different detergent combinations (a) SDS, (b) SDC, (c) SDS + DTT, (d) SDC + DTT, (e) SDS + SDC, (f) SDS + SDC + DTT.

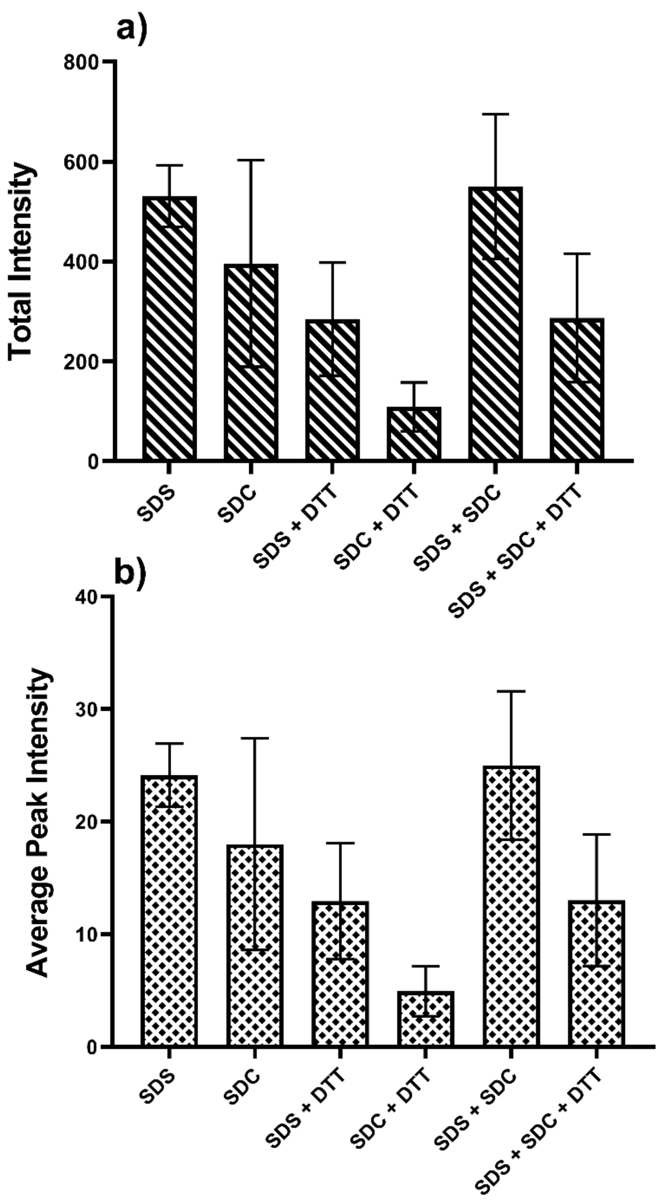

Figure 4.6. Total and average peak intensities of the N-glycan peaks obtained from N-glycan release methods including different detergent combination.

Table 4.3. Peak intensities of the *N*-glycan peaks obtained from *N*-glycan release methods including different detergent combinations.

	SDS		SDC		SDS + DTT		SDC + DTT		SDS + SDC		SDS + SDC + DTT	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
G1	35.06	15.12	18.71	15.46	15.17	6.21	1.51	1.06	33.28	7.64	10.11	4.85
G2	13.93	18.61	4.37	3.29	6.99	7.76	0.85	1.23	7.32	1.70	1.85	1.55
G3	31.25	11.58	18.94	14.59	13.46	8.43	1.29	0.88	31.97	7.08	12.03	6.55
G4	16.30	6.06	8.99	7.40	6.09	3.89	1.20	1.30	16.50	3.67	4.83	2.81
G5	9.53	3.55	6.14	4.10	4.07	2.45	1.13	1.03	9.59	2.10	3.57	1.98
G6	7.35	1.83	6.73	3.48	4.93	2.99	2.86	3.33	10.70	2.23	4.13	1.79
G7	20.30	14.72	18.09	10.07	9.23	7.56	2.52	2.28	24.01	13.07	7.60	5.21
G8	31.10	14.37	22.82	16.04	12.21	11.13	4.58	5.84	37.93	7.70	14.95	9.25
G9	7.33	1.79	5.58	3.73	3.12	2.18	0.87	0.60	9.21	3.02	3.76	2.39
G10	15.68	25.84	2.64	1.16	2.32	1.29	0.61	0.35	4.21	1.08	1.78	1.10
G11	70.57	15.72	67.17	29.43	38.55	20.37	14.36	9.32	92.27	29.71	42.38	22.85
G12	37.78	7.47	27.28	13.90	23.39	10.37	7.05	3.78	41.68	9.26	23.00	11.47
G13	18.18	3.05	14.02	8.54	9.88	3.68	5.23	2.02	23.10	5.26	12.12	5.42
G14	131.20	12.04	106.83	49.05	81.51	27.48	33.99	18.18	126.43	43.44	86.26	33.33
G15	8.97	6.14	5.00	2.05	2.61	1.03	3.69	4.50	7.51	1.45	3.40	1.37
G16	18.97	5.89	19.80	7.23	17.12	5.34	7.54	4.58	23.01	7.81	19.33	6.81
G17	19.15	6.47	11.27	3.53	9.16	3.25	7.36	7.13	12.68	4.33	10.44	4.04
G18	9.31	3.35	7.64	3.86	5.35	3.03	2.52	1.32	8.91	5.02	5.51	2.49
G19	7.21	3.91	5.76	2.69	3.77	2.00	1.41	1.02	6.80	3.61	4.00	2.10
G20	4.19	3.26	2.78	1.23	1.87	1.11	1.58	0.99	3.26	2.03	2.32	1.19
G21	9.72	5.84	10.08	6.53	9.18	2.70	4.26	3.60	12.86	4.87	8.54	1.83
G22	7.77	3.14	5.53	3.82	4.75	1.44	2.43	1.79	7.17	2.71	4.88	1.42

4.5. COMPARISON OF DETERGENTS AND DETERGENT COMBINATIONS IN *N*-GLYCAN RELEASE METHODS BASED ON RELATIVE ABUNDANCES

The *N*-glycan release methods with different detergent combinations were compared by evaluating the relative abundances of *N*-glycans. The percentage of the relative abundances from peak areas and peak intensities of the *N*-glycan peaks were curated by the total area normalization approach. Figure 4.7A and 4.7B shows the comparison of data based on relative abundances of the detected *N*-glycan peaks. The % relative abundances obtained from peak areas and peak intensities of the detected peaks are shown in Table 4.5 and Table 4.6, respectively. The data showed that there were different profiles for human plasma *N*-glycans regarding *N*-glycan release methods, including different detergent combinations. The *N*-glycans abundantly found in human plasma differed among protocols. For example, the *N*-glycan G14 was found to be higher in DTT-containing protocols (Table 4.5 and Table 4.6). In addition, the relative abundances of tri-antennary sialylated species were determined to be high in DTT reducing agent-containing methods.

The area-based CV% (coefficient of variation) of the peak abundances was also investigated for *N*-glycan release methods with different detergent combinations (Figure 4.8). It was found that SDS + DTT and SDC + DTT containing *N*-glycan release methods had higher area CV% (average area CV% was 32% and 62% for SDS + DTT and SDC + DTT). However, the area-based CV% values of plasma *N*-glycan abundances for SDS, SDC, and SDS + SDC + DTT detergent containing *N*-glycan release method were detected to be relatively low. The average CV% of these *N*-glycan release methods was 18%, 26%, and 20%, respectively.

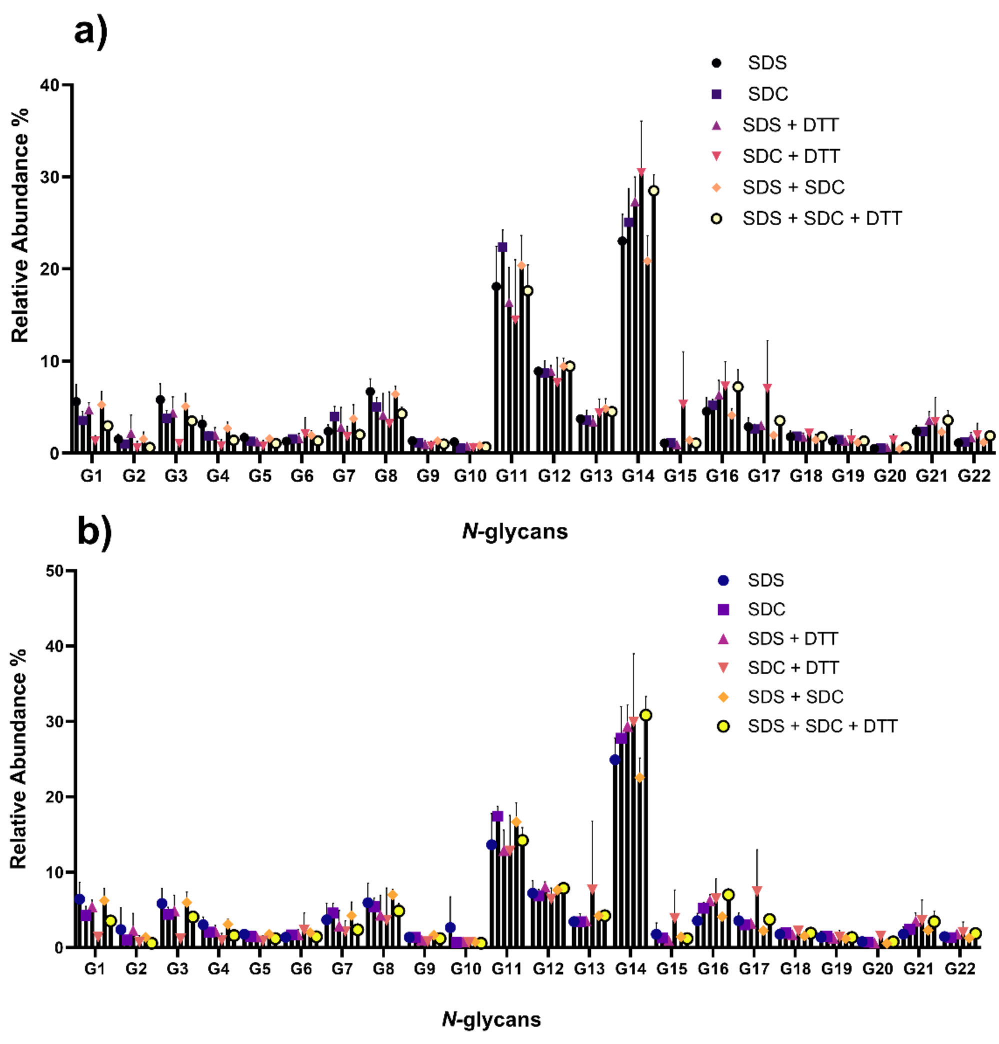

Figure 4.7. Relative abundances of the detected *N*-glycans calculated using (a) peak areas and (b) peak intensities.

Table 4.4. Relative abundances of *N*-glycan peaks based on total area.

	SDS		SDC		SDS + DTT		SDC + DTT		SDS + SDC		SDS + SDC + DTT	
	Mean	sd	Mean	sd	Mean	sd	Mean	Sd	Mean	sd	Mean	sd
G1	5.59	1.87	3.55	0.99	4.72	0.77	1.32	0.60	5.26	1.46	2.97	0.35
G2	1.52	0.56	0.99	0.26	2.17	1.96	0.60	0.68	1.54	0.76	0.62	0.53
G3	5.82	1.71	3.82	0.80	4.40	1.72	1.08	0.40	5.10	1.38	3.49	0.33
G4	3.16	0.89	1.86	0.48	1.99	0.79	0.79	0.70	2.70	0.68	1.41	0.15
G5	1.70	0.50	1.29	0.26	1.26	0.36	0.78	0.62	1.59	0.34	1.06	0.10
G6	1.28	0.17	1.55	0.30	1.56	0.57	2.12	1.75	1.92	0.53	1.34	0.16
G7	2.37	0.71	3.99	1.09	2.81	2.17	1.81	1.10	3.75	1.51	2.01	0.58
G8	6.69	1.38	5.03	1.01	4.11	2.37	3.19	3.47	6.41	0.86	4.29	0.71
G9	1.34	0.24	1.14	0.32	0.97	0.39	0.77	0.52	1.34	0.27	1.00	0.28
G10	1.22	0.15	0.57	0.09	0.79	0.25	0.59	0.45	0.82	0.13	0.68	0.29
G11	18.09	4.38	22.36	1.87	16.37	3.81	14.46	6.54	20.39	3.26	17.62	2.81
G12	8.89	0.44	8.73	1.30	8.91	0.61	7.64	2.73	9.44	0.90	9.43	0.57
G13	3.73	0.36	3.58	1.09	3.39	0.67	4.35	1.53	4.84	1.08	4.52	0.18
G14	23.04	2.89	25.07	3.68	27.33	2.67	30.45	5.62	20.88	2.74	28.50	1.72
G15	1.10	0.34	1.16	0.16	0.93	0.39	5.28	5.75	1.42	0.34	1.10	0.39
G16	4.53	1.48	5.21	0.66	6.34	1.58	7.27	2.67	4.12	0.73	7.21	1.85
G17	2.87	0.99	2.62	0.51	3.03	0.30	7.01	5.22	1.95	0.29	3.54	0.21
G18	1.78	0.63	1.85	0.22	1.75	0.45	2.17	0.26	1.44	0.59	1.78	0.20
G19	1.30	0.46	1.47	0.18	1.31	0.24	1.44	1.12	1.16	0.39	1.33	0.13
G20	0.50	0.22	0.58	0.07	0.62	0.06	1.44	0.59	0.46	0.19	0.64	0.10
G21	2.36	0.65	2.37	0.26	3.55	0.96	3.44	2.63	2.29	0.48	3.57	1.05

Table 4.5. Relative abundances of *N*-glycan peaks based on total intensity.

	SDS		SDC		SDS + DTT		SDC + DTT		SDS + SDC		SDS + SDC + DTT	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
G1	6.44	2.24	4.27	1.22	5.40	0.92	1.39	0.58	6.25	1.64	3.55	0.45
G2	2.40	2.91	1.02	0.25	2.27	2.29	0.73	0.87	1.38	0.40	0.58	0.39
G3	5.87	1.97	4.43	0.92	4.88	2.08	1.16	0.46	5.98	1.40	4.08	0.52
G4	3.05	1.02	2.05	0.59	2.15	0.80	0.96	0.93	3.09	0.73	1.62	0.25
G5	1.78	0.58	1.49	0.29	1.41	0.41	0.88	0.71	1.78	0.34	1.20	0.15
G6	1.37	0.20	1.69	0.26	1.67	0.71	2.34	2.26	2.00	0.45	1.46	0.11
G7	3.70	2.19	4.60	1.28	2.88	2.04	2.10	1.45	4.26	1.80	2.38	0.79
G8	5.95	2.62	5.43	1.14	4.31	2.66	3.59	4.31	7.01	0.75	4.87	0.99
G9	1.38	0.28	1.34	0.44	1.02	0.33	0.77	0.31	1.67	0.29	1.21	0.30
G10	2.66	4.08	0.68	0.06	0.77	0.18	0.66	0.39	0.77	0.09	0.58	0.12
G11	13.65	4.12	17.41	1.36	12.89	2.70	12.82	4.72	16.69	2.50	14.21	1.70
G12	7.23	1.63	6.87	0.86	8.04	0.70	6.44	1.48	7.66	0.50	7.87	0.43
G13	3.46	0.61	3.42	1.10	3.55	0.44	7.69	9.11	4.25	0.36	4.25	0.19
G14	24.91	2.91	27.76	4.22	29.32	2.87	29.92	9.06	22.56	2.59	30.84	2.47
G15	1.79	1.50	1.30	0.16	0.97	0.30	3.89	3.78	1.42	0.35	1.21	0.18
G16	3.57	0.98	5.25	0.78	6.24	0.84	6.49	2.67	4.12	0.58	7.01	0.81
G17	3.57	1.02	3.03	0.52	3.27	0.23	7.44	5.55	2.27	0.32	3.74	0.29
G18	1.78	0.69	1.95	0.18	1.76	0.43	2.20	0.45	1.54	0.63	1.91	0.14
G19	1.37	0.77	1.49	0.17	1.26	0.24	1.40	0.92	1.18	0.42	1.35	0.15
G20	0.80	0.64	0.73	0.10	0.61	0.15	1.51	0.61	0.56	0.27	0.79	0.12
G21	1.80	0.93	2.45	0.23	3.51	1.10	3.60	2.70	2.30	0.43	3.45	1.38
G22	1.46	0.55	1.33	0.17	1.80	0.53	2.02	1.37	1.27	0.20	1.87	0.55

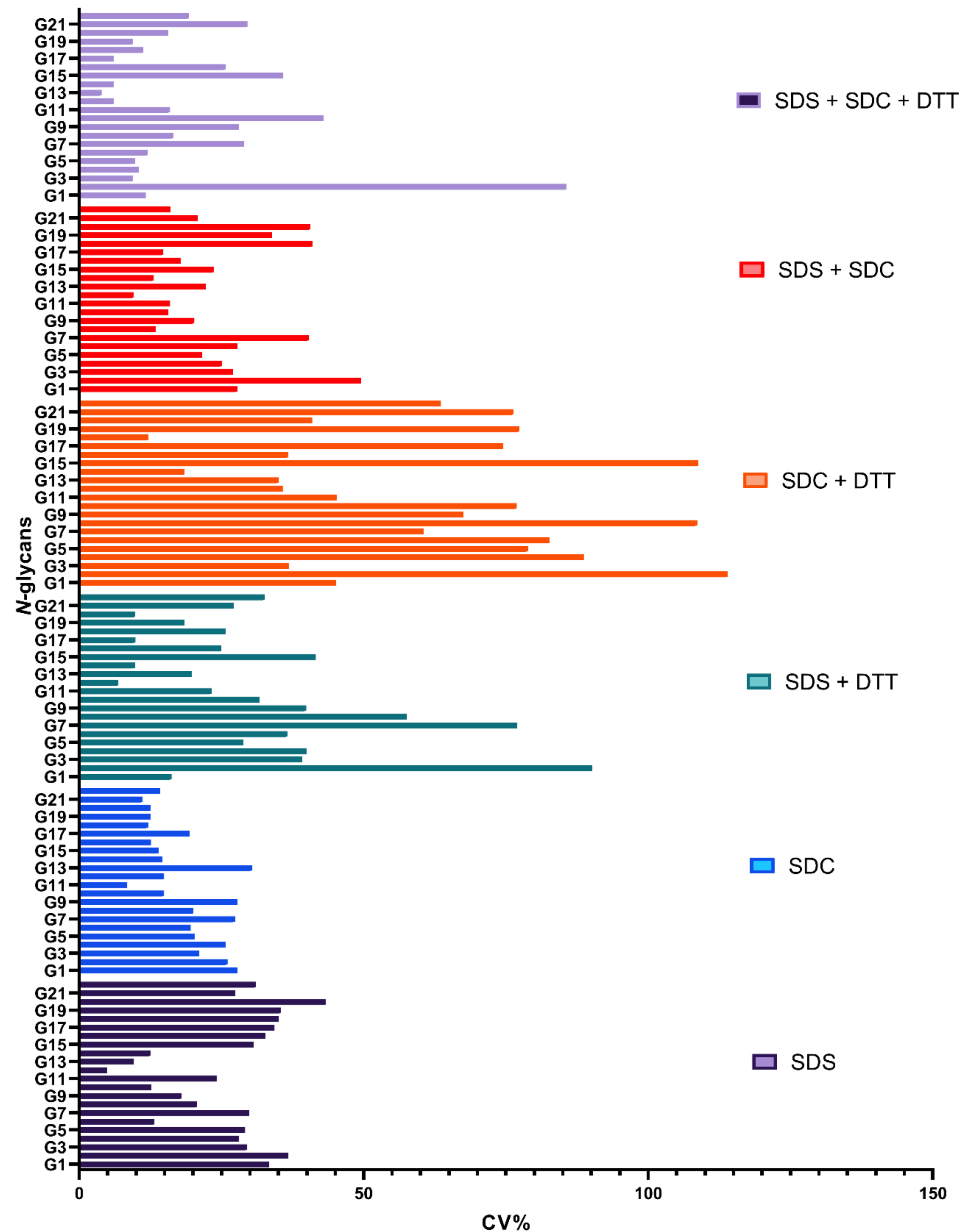

Figure 4.8 . Comparison of six different detergent combinations used in *N*-glycan release methods. Reproducibility of human plasma *N*-glycome quantification represented by area coefficient of variation (CV%) values for 22 *N*-glycan peaks.

The main limitation of this study was to compare three different detergents and their combinations, whereas many others are available in *N*-glycan release methods. Many manufacturers producing the PNGase F enzyme offer SDS-based *N*-glycan release methods. These methods were applied with DTT reducing agents.

Therefore, the most widely used chemicals for that purpose were evaluated in the study. On the other hand, SDC has been recently evaluated in releasing *N*-glycans from glycoproteins extracted from biological samples [69]. They have determined that SDC assisted approach was found to be more efficient compared with filter-aided sample preparation.

In our study, the *N*-glycan profiles of human plasma were found to change in different detergent combinations containing *N*-glycan release methods. It could be concluded that the denaturation agents influence the efficiency of *N*-glycan release. In addition, the release of *N*-glycan types was found to differ based on *N*-glycan release methods with different detergent combinations. A recent study was evaluated the PNGase F enzymes produced by three manufacturers regarding the *N*-glycan profiles. They have found that deglycosylation with PNGase F enzymes manufactured by different companies resulted in different IgG and plasma *N*-glycosylation HILIC-FLD profiles [70]. These results indicated that the applied *N*-glycan release method, including denaturation agents and PNGase F enzymes, provided different *N*-glycosylation HILIC-FLD profile.

PART 5

CONCLUSION

In conclusion, the SDS + SDC detergent combinations provided more yield for releasing *N*-glycans from human plasma by the analysis of the released and procainamide labeled *N*-glycans with HPLC-HILIC-FLD. The SDS detergent-containing protocol was more reproducible regarding the total or average areas and intensities. It was determined that DTT reducing agents decreased the total areas and intensities of the detected *N*-glycans when it was used with SDS and SDC. Finally, the *N*-glycan release methods with different detergent combinations resulted in varied *N*-glycosylation profiles. These results showed that the denaturing agents used in the *N*-glycan release methods are critical for efficient *N*-glycan analysis.

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RESUME

Rokia SAKHTA completed its first training in Syria, elementary and high school education in Saudi Arabia. She started an undergraduate program at Zarqa University Department of medical analysis in 2013 in Jordan. Then in 2018, she started M Sc. Education at Karabük University in Turkey.