University of South Bohemia in České Budějovice
Faculty of Science

Bachelor's Thesis

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Evaluation of different methods of glycan enrichment to their subsequent MS identification

Bachelor's Thesis

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Annotation

The main aim of this thesis was to evaluate different parameters during the sample preparation steps (such as enrichment of glycoproteins, release, purification and modification of glycans) to enable better MS identification of glycans released from different ticks related samples.

Declaration

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography.
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Also, I would like to thank Dr. Lesley Bell-Sakyi and the Tick Cell Biobank for providing of IRE/CTVM19 tick cell line.

List of abbreviations

Abbreviation	Meaning
PTMs	Post-translation modifications
CRDs	Carbohydrate-recognition domains
MS	Mass spectrometry
MALDI	Matrix-assisted laser desorption/ionization
ESI	Electrospray ionization
LC	Liquid chromatography
DHB	2,5-dihydroxybenzoic acid
THAP	2,4,6-trihydroxyacetophenone
TOF	Time-of-flight
RP	Reversed-phase
NP	Normal-phase
PNGse F	Peptide N-Glycosidase F
GlcNAc	N-acetylglucosamine
2-AB	2- aminobenzamide
2-AA	2-aminobenzoic acid
UV	Ultraviolet
DTT	Dithiothreitol
ACN	Acetonitrile
IAA	Iodoacetamide
FA	Formic acid
TFA	Trifluoroacetic acid
DMSO	Dimethylsulfoxide
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulfate
BSA	Bovine serum albumin
BCA	Bicinchoninic acid
GuHCl	Guanidine chloride
Tris-HCl	Tris hydrochloride
IRE	*Ixodes ricinus* cell line
FEP	Fluorinated Ethylene Propylene

Abstract

Glycans are biologically vital carbohydrate-based polymers that can act as immunogens for pathogen anti-transmission vaccines. Therefore, glycans structures are needed to be characterized. One of the most frequently used methods for glycans identification is a mass spectrometry that allows quick and accurate examination of glycoforms. It is known that the quality of mass spectra is crucial for successful glycans identification, and it highly depends on the method used during the sample preparation.

The aim of the following study is to evaluate different parameters during the sample preparation steps to get MS spectra of high quality for identification of glycans released from different tick related samples. In the thesis, methods for the identification of glycans enriched from a gel as well as from a solution of glycoproteins were established and applied for glycans identification from *Ixodes ricinus* tick cell line (IRE/CTVM19).

The methods established in the following project can be used in the laboratory for the study of glycan structures from various samples, including tick related ones.

Table of Contents

1. Introduction

1.1. Protein glycosylation

Glycosylation is a type of post-translation modifications (PTM). It is the binding of carbohydrate moieties to proteins. The carbohydrate can be connected to eight amino acid residues (Arey, 2012). However, the most common types of glycosylation are N-type and O-type. N-linked glycosylation is an attachment of an oligosaccharide to the amine nitrogen of asparagine (Asn) residue (Schwarz & Aebi, 2011). O-glycosylation is a connection of an oligosaccharide to the hydroxyl oxygen of serine (Ser) or threonine (Thr) residues (Steen et al., 1998).

The glycosylation is considered to be important in protein-protein interactions since it affects the three-dimensional conformation of the protein (Arey, 2012). An attachment of the carbohydrate determines the state of the protein, and thus stabilizes the protein and prolongs its half-life (Meyer & Möller, 2006). For example, N-glycosylated ribonuclease B slower degrades in the body than non-glycosylated ribonuclease A (Chatani & Hayashi, 2001). Also, it was found that glycosylation plays a physiological role in the function of gonadotropins by affecting the interaction between hormone and receptor (Nguyen et al., 2003). Therefore, glycosylation determines the biological activity of the proteins and is worth to be studied.

1.2. Importance of glycans study

Lower organisms have the recognition mechanisms for saccharides, and thus the pathogens use those abilities to interact with the host's immune system (Štěrba, 2011). Since viral pathogens use specific carbohydrate structures of host glycoproteins as attachment sites, it is possible to counteract these interactions (thus preventing viral infection) by blocking the attachment of the pathogen with a suitable sugar (Lis & Sharon, 1993). Therefore, the specificity of carbohydrate-binding proteins has to be examined.

Glycoproteins have been found to be pan-arthropod vaccine candidates (Willadsen, 1997). It has been shown that glycans are key candidates for a vaccine that can induce immunity to salivary as well as midgut glycoproteins of hematophagous arthropods due to their limited diversification and high immunogenicity (Mejia et al., 2006). It has been proven that glycosylated recombinant proteins are more immunogenic than non-glycosylated ones (de La

Fuente et al., 2006). The sugar epitopes act as immunogens for pathogen anti-transmission vaccines and increase the protective capacity of vaccines (Dinglasan et al., 2005).
Due to the fact that N- and O-linked glycans attached to arthropod glycoproteins are candidates for anti-tick vaccines, it is needed to characterize their structures (Mejia et al., 2006). For the characterization of glycan structures, several methods have been developed.

1.3. Methods for Glycan Identification

1.3.1. Glycan/lectin microarrays (lectin staining)

Lectins are proteins that are capable of binding carbohydrates. Lectins play a role in innate immunity by recognizing the pathogen's glycosylated surface via carbohydrate-recognition domains (CRDs) (Eddie Ip et al., 2009). Glycan microarray is a crucial technique for glycobiologists that allows examining the interaction between glycan-binding proteins and their ligands (Cummings & Pierce, 2014). However, the results are found to be imprecise when different array platforms are used (Oyelaran & Gildersleeve, 2009). In addition, existing arrays do not contain all carbohydrates found in nature (Wang et al., 2014). Lastly, the difficulty arises in interpretation and further usage of glycan array data (Oyelaran & Gildersleeve, 2009).

1.3.2. High-performance liquid chromatography (HPLC)

Normal-phase HPLC allows to analyze glycans' structures and to sequence N-glycans (Rudd et al., 2001). Released glycans are labelled with a fluorescent agent for subsequent detection. On the one hand, labelling of glycans with a fluorophore allows to analyze both neutral and charged glycans at the same time, and distinguish structures by their sequence and type of linkage (Campbell et al., 2008). On the other hand, fluorescent labelling of glycans provides low thresholds of their detection (compared with e.g. MS methods) (Cummings & Pierce, 2014).

1.3.3. Mass spectrometry (MS)

MS can be used for identification of both N-glycans and O-glycans in glycoproteins (North et al., 2009). Also, using MS allows to determine glycosylation sites in glycoproteins, determine the occupancy of each site as well as structure and amount of each glycan at a specific site (Harvey, 2001). There are two techniques frequently used for the analysis of glycans: matrix-

assisted laser desorption/ionization mass spectrometry (MALDI-MS), and electrospray ionization mass spectrometry (ESI-MS) combined with liquid chromatography (LC).

1.3.3.1. MALDI-MS

MALDI-MS can be used for relatively quick analysis and permits identification of both neutral and acidic glycans. The sample to be analyzed is crystallized with a UV-absorbing matrix. Usually, neutral glycans are efficiently ionized with 2,5-dihydroxybenzoic acid (2,5-DHB), or with a mixture of 2,5-DHB and 2-hydroxy-5-methoxybenzoic acid (90:10, wt %) (super-DHB) (Tsarbopoulos et al., 1994). The acidic glycans can be ionized with 6-aza-2-thiothymine, or with 2,4,6-trihydroxyacetophenone (THAP) (Hauser & Wagner, 2014). The mixture of the sample with the matrix is then irradiated with a UV light. The matrix absorbs the light and transfers the energy to the sample (Harvey, 2001). The produced ions can be analyzed by time-of-flight (TOF) analyzer. Underivatized neutral carbohydrates produce a strong $[M+Na]^+$ ion as well as weaker $[M+K]^+$ ion in the positive ion mode and have detection levels in the one picomole range (Mock et al., 1991). In comparison with neutral sugars, acidic glycans produce weaker signals, because they produce mixture of ions like $[M+Na]^+$, $[M+K]^+$, $[m\text{-}nH + (n+1)Na]^+$, $[m\text{-}nH + (n+1)K]^+$ (Harvey, 2001).

However, MALDI-MS analysis can be inaccurate in the examination of the glycoforms due to the complexity of the investigated mixtures (Harvey, 2001). Therefore, MS is often combined with LC in order to improve accuracy and obtain total glycan profiles (Harvey, 2001).

1.3.3.2. LC-MS

LC-MS technique combines properties of LC to separate a mixture and of MS to provide identification of components of the mixture with high detection sensitivity (Pitt, 2009). A sample is injected into the column and is adsorbed on a stationary phase. There are three commonly used stationary phases for LC-MS for glycans analysis: reversed-phase (RP), normal-phase (NP), and graphitized carbon. Then, the mobile phase passes through the column and separates components of the sample, based on their relative affinity to the stationary phase. Separated components can be further identified using MS.

By analyzing glycans, one may get their detailed structure. However, it is impossible to get information on the original attachment site of the glycans to a protein (Wuhrer et al., 2005). This information can be received by performing LC-MS analysis of the remaining peptides after glycan release, or by direct analysis of glycopeptides (Wuhrer et al., 2005).

Since glycans constitute only a small part of a complex glycoprotein, the enrichment of glycans prior MS analysis (MALDI-MS or LC-ESI-MS) should be performed.

1.4. Preparation of sample for subsequent MS analysis

The typical workflow for identification of glycans is depicted in Figure 1.

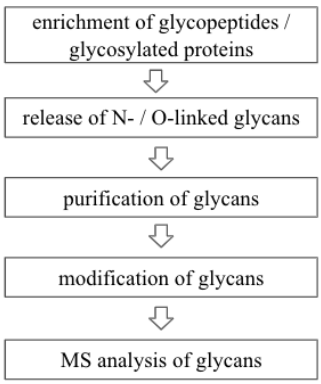


Figure 1. Typical workflow for identification of glycans

Nowadays, different approaches exist to perform each of the steps mentioned above. For example, enrichment of glycosylated proteins can be done either from a gel or from a solution of glycoproteins.

1.4.1. Enrichment of glycoproteins from a gel

Gel electrophoresis is a useful method of proteins separation based on their molecular weight. It allows to remove low molecular weight impurities such as detergents, salts, buffers, and fractionate the proteins in the complex samples (Feist & Hummon, 2015). Thus, proteins of interest are concentrated in single bands and ready for further analysis.

However, since the quality of mass spectra depends on the amount of sample used as well as on a sample purity, a poor gel resolution and an inefficient release of glycopeptides from the band can significantly complicate MS identification (Granvogl et al., 2007). The analytes losses could occur during gel destaining due to the adsorption on surfaces of pipettes, during gel drying, as well as due to incomplete extraction of glycoproteins from the gel (Granvogl et al., 2007). It has been reported that the amounts of losses vary between 15 and 50% (Stewart et al., 2001). Therefore, higher amounts of the initial glycoprotein are needed in comparison with the enrichment from a solution. In addition, in-gel digestion is a laborious and time-consuming technique.

Sample recovery for gel-based methods has been estimated to be from 70 to 80% of the efficiency of in-solution digestion (Gundry et al., 2010; Shevchenko et al., 2006). Therefore, in-solution digestion is more frequently used.

1.4.2. Enrichment of glycoproteins from a solution

In-solution enrichment is an advantageous technique over in-gel release of glycoproteins. Firstly, it is easier to handle the process in a solution. Secondly, less of glycoprotein is needed for analysis, since the losses of the sample during preparational steps are not high relatively to in-gel enrichment.

In addition to different approaches for the enrichment of glycoproteins, the second step of a typical workflow for identification of glycans (depicted in Figure 1) also varies. In order to simplify MS analysis, oligosaccharides can be removed from glycoproteins (that is deglycosylation). For removal of oligosaccharides, both chemical and enzymatic approaches exist.

1.4.2.1. Chemical release of glycans

In comparison with N-linked glycans, the release of O-linked glycans remains a problem nowadays, since there are a limited amount and specificity of O-glycanases (Simó et al., 2014). It was found that sodium hypochlorite NaClO can release both N- and O-glycans from glycoproteins. The outcome of the reaction is the formation of glycosylamines (Song et al., 2016). The reaction mechanism is shown in Figure 2. However, the oxidative release results in limited N-linked glycans yield as well as noisy background during MS detection due to impurities present (Wang et al., 2018).

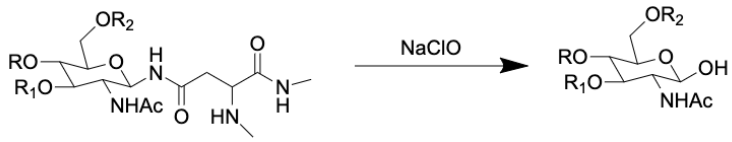


Figure 2. The reaction mechanism of the oxidative release. Adapted from "Oxidative release of natural glycans for functional glycomics", by X. Song et al., 2016, *Nature methods*, 13(6), 530.

Hydrazinolysis is another chemical approach used for the release of N-glycans (Patel et al., 1993). It is performed in anhydrous hydrazine at 90 °C for 4 hours (Patel et al., 1993). However, this approach has been found to be very toxic, and deacetylation side reactions occur (Wang et al., 2018).

Therefore, the ammonia-catalyzed release, that is one of the most used chemical methods for release of N-glycans, can be performed. N-glycans are released as a result of mild alkaline hydrolysis (Wang et al., 2018). In addition, N-glycans are protected from peeling reactions by ammonia (Wang et al., 2018). The reaction mechanism is shown in Figure 3. However, it has been reported that with decreased alkalinity and lowered reaction temperature, some O-glycans can also be released (Wang et al., 2018; Goso et al., 2017; Yang et al., 2013).

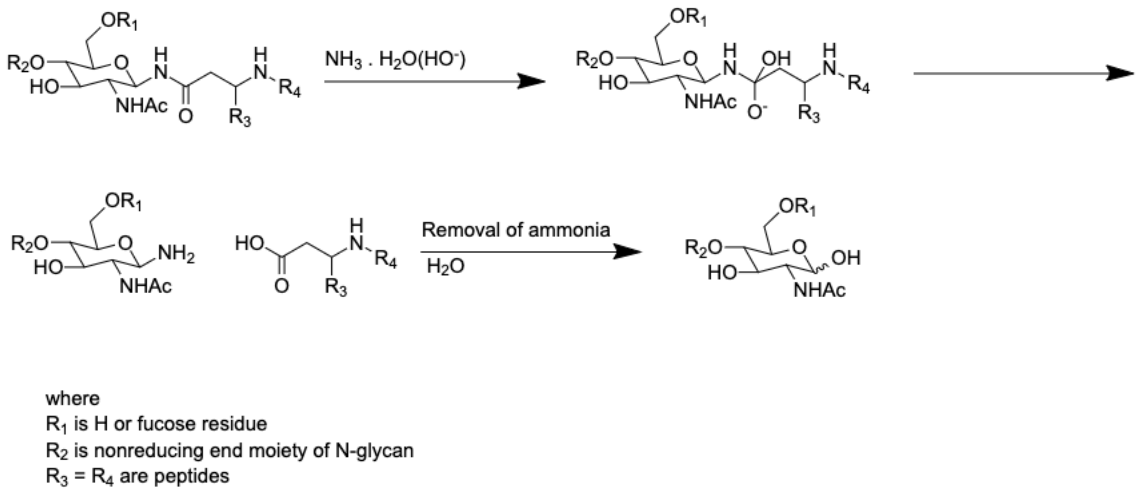


Figure 3. The reaction mechanism of the ammonia-catalyzed release. Adapted from "The ammonia-catalyzed release of glycoprotein N-glycans", by C. Wang et al., 2018, *Glycoconjugate journal*, 35(4), 414.

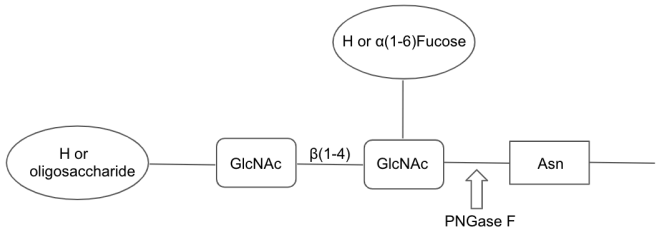
Unfortunately, chemicals methods are relatively harsh and can cause degradation of the peptide bone as well as lead to unwanted modifications (Fischler & Orlando, 2019). Therefore, enzymatic methods are employed.

1.4.2.2. Enzymatic deglycosylation

Enzymatic methods have been found to be gentler than chemical methods and can completely remove the sugars without modification of the released oligosaccharides (O'Neill, 1996).
Many enzymes for releasing N-linked oligosaccharides from glycoproteins have become available (O'Neill, 1996). Several enzymes active on N-glycans are used, such as endoglycosidases H, S and D, as well as peptide N-Glycosidase F (PNGase F) and PNGase A. Endoglycosidase H is able to remove high mannose and most hybrid N-glycans (Tarentino & Maley, 1975). Endoglycosidase F, in addition to removing high mannose and hybrids, also releases bi- and triantennary complex glycans (Elder & Alexander, 1982). The specificity of the endoglycosidase D is limited (O'Neill, 1996). It is able to cleave just some high mannose glycans (O'Neill, 1996).

The most popular enzyme is peptide N-Glycosidase F (Krenkova et al., 2013). It effectively removes N-linked oligosaccharides from glycoproteins (Tarentino et al., 1994). It cleaves between N-acetylglucosamine (GlcNAc) and asparagine residues of high mannose and complex oligosaccharides (Tarentino et al., 1994). As a result, the asparagine turns into aspartic acid via deamination process, and the oligosaccharide stays intact (Tarentino et al., 1994). The specificity of the enzyme is illustrated in Figure 4a and Figure 4b.

A.



B.

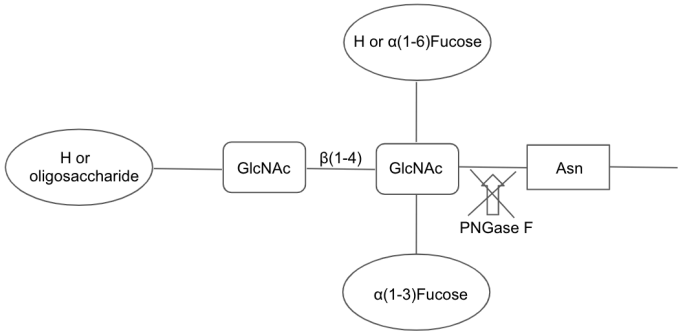


Figure 4. The specificity of PNGase F enzyme. (A) PNGase F can cleave if there is α(1-6)Fucose on the core GlcNAc; (B) PNGase F cannot cleave if there is α(1-3)Fucose on the core GlcNAc.

There are just several enzymes available for the release of O-linked oligosaccharides (O'Neill, 1996). For example, endo-α-N-acetylgalactosaminidases from *Diplococcus* and *Alcaligenes* are specific for the Gal-β-1,3-GalNAc O-linked to serine or threonine residues (O'Neill, 1996). However, these enzymes have limited substrate specificities (O'Neill, 1996). Also, it was reported that endo-α-N-acetylgalactosaminidases from *Diplococcus* has transglycosylation activities (Bardales & Bhavanandan, 1989). Therefore, glycerol has to be removed, and any buffers containing hydroxy compounds cannot be used in the presence of this enzyme (Bardales & Bhavanandan, 1989).

It has been reported by Song X. et al. (2016) that chemical release with sodium hypochlorite is as effective as enzymatic deglycosylation with PNGase F enzyme. However, it was

published later by Fischler D.A. & Orlando R. (2019) that oxidative release is less efficient than enzymatic deglycosylation with PNGase F due to glycans degradation.

Wang et al. (2018) compared chemical ammonia release to enzymatic release with PNGase F using RNase B glycoprotein. In the case of enzymatic release, five peaks were identified belonging to $[M+Na]^+$ ions of five native oligomannose type N-glycans (Wang et al., 2018). In the case of chemical release, ten peaks were identified, five of which belong to $[M+Na]^+$ ion type and others are of $[M+K]^+$ ion type (Wang et al., 2018). Those peaks were assigned to five above-mentioned oligomannose type N-glycans (Wang et al., 2018).

In addition, Wang et al. (2018) compared chemical ammonia release to enzymatic release with PNGase F and PNGase A using Ginkgo seed protein. There were three groups of detected N-glycans belonging to oligomannose type, core α-1,3-fucosylated complex type and core non-α-1,3-fucosylated complex type (Wang et al., 2018). PNGase A and ammonia-release approach could cleave all above-mentioned types (Wang et al., 2018). However, PNGase F could not cleave core α-1,3-fucosylated complex type N-glycans. Therefore, it was demonstrated that the ammonia-release method is comparable with PNGase A but is advantageous over PNGase F (Wang et al., 2018).

Finally, Wang et al. (2018) compared ammonium release to oxidative release with sodium hypochlorite using chicken ovalbumin. It was demonstrated that the ammonia-released sample shows better results since lower background noise, and more intense signals are generated (Wang et al., 2018).

1.4.3. Modification of glycans

Due to the fact that glycans are hydrophilic molecules that do not ionize in MS efficiently, the derivatization step is often needed (Banazadeh et al., 2017). The derivatization has been found to enhance ionization efficiency and abolish ionization bias between neutral and acidic glycans (Banazadeh et al., 2017). Additionally, the presence of negatively charged sialic acid residues complicates glycan analysis by MS since they decrease ionization efficiency (Nishikaze, 2019). Also, sialic acid residues are often lost during preparation stages due to their instability. Therefore, in order to promote glycan analysis by MS, chemical derivatization can be done. The derivatization can be divided into three categories: permethylation, glycan reducing end labelling, and sialic acid derivatization (Nishikaze, 2019).

1.4.3.1. Solid-phase permethylation

Permethylation is a process of addition of methyl groups to hydroxyl and N-acetyl groups as a result of the reaction of glycans with iodomethane and sodium hydroxide in dimethyl sulfoxide (Ciucanu & Kerek, 1984). It is the most common derivatization method due to its procedural simplicity and significance. It has been found to enhance measurement sensitivity, stabilize sialic acid residues, and ease tandem MS interpretation (Kang et al., 2008). In addition, it allows analysis of both acidic and neutral glycans in the positive-ion mode (Kang et al., 2008). Also, permethylation has been found to be quantitatively reproducible technique (Kang et al., 2008).

1.4.3.2. Reductive amination

The reducing end of glycans can be labelled by reductive amination (Ruhaak et al., 2010). The principle is the condensation reaction between primary amine group with the aldehyde group of a glycan, forming imine that is subsequently reduced to form a secondary amine (Ruhaak et al., 2010). One label is stoichiometrically attached to one glycan, allowing quantitation by analyzing fluorescence or UV-absorbance intensity (Ruhaak et al., 2010). There are different labels used for reductive amination, such as 2-aminobenzamide (2-AB) and 2-aminobenzoic acid (2-AA) (Anumula, 2006).

1.4.3.3. Esterification

Esterification is a standard derivatization method. It has been found by Powell A. K. and Harvey D. J. (1996) that methyl iodide can be used for methyl esterification of carboxyl groups on sialic acids. Therefore, the sialic acid residue becomes stable against laser-induced decomposition during analysis with MALDI-MS (Powell & Harvey, 1996).

1.4.4. Purification of glycans

The third step of a typical workflow for identification of glycans (shown in Figure 1) is purification of glycans. Purification is used to remove detergents that may affect the quality of MS spectra. There are different methods available for this step that are worth trying in the laboratory.

1.4.4.1. Glycan purification with a non-porous graphitized carbon column

Activated charcoal solid-phase extraction columns can be used for glycans cleanup (Davies et al., 1993; Fan et al., 1994; Koizumi, 1996). Due to the fact that oligosaccharides bind to the carbon beads, unbound moiety such as salts, detergents, proteins and chemicals used for glycans release, can be washed away (Packer et al., 1998). The separation is based on the size, charge, and linkage of the sugars (Lee, 1996). The desalting with carbon can also be used to fractionate neutral oligosaccharides from acidic ones (Packer et al., 1998).

1.4.4.2. Glycan purification with reversed-phase C18 StageTips

The purification of glycans can be performed using a microgradient device connected to a capillary packed with core-shell C18-based particles (Rehulka et al., 2018). The microgradient technique allows preconcentrating of permethylated glycans as well as removing of polar contaminants (Rehulka et al., 2018). C18 sorbent is a hydrophobic silica-based bonded phase (McDonald & Bouvier, 1995). It allows binding, concentration, desalting and elution of peptides as well as permethylated glycans.

It can be concluded that nowadays different workflows exist for the preparation of glycans to their subsequent MS analysis. Therefore, the main goal of the project was to evaluate different parameters during the sample preparation steps (such as enrichment of glycoproteins, the release of glycans, purification and modification of glycans) to enable their identification using MALDI-MS.

2. Materials and Methods

The chemicals used in the study can be seen in Table 1.

Table 1. The description of chemicals used

Chemical	Description
ELECTROPHORESIS	
Transferrin	Sigma-Aldrich (Steinheim, Germany)
SDS loading buffer (5x)	Roth (Karlsruhe, Germany)
Dithiothreitol (DTT)	Sigma-Aldrich (Steinheim, Germany)
Prestained protein marker VI (10-245)	Appli-Chem (Darmstadt, Germany)
Acetic acid	Sigma-Aldrich (Steinheim, Germany)
Coomassie Brilliant Blue G-250	Sigma-Aldrich (Steinheim, Germany)
REDUCTION AND ALKYLATION	
Ammonium hydrogen carbonate	Lach-Ner (Neratovice, Czech Republic)
Iodoacetamide (IAA)	GE Healthcare (UK)
DEGLYCOSYLATION	
PNGase F (Glycerol-free, recombinant)	New England Biolabs (USA)
GlycoBuffer 2 (10x)	New England Biolabs (USA)
NP-40 (10%)	New England Biolabs (USA)
Glycoprotein denaturing buffer (10x)	New England Biolabs (USA)
TRYPSINISATION	
Trypsin (modified)	Promega (USA)
Formic acid (FA)	Merck (Darmstadt, Germany)
PURIFICATION	
Trifluoroacetic acid (TFA)	Sigma-Aldrich (Steinheim, Germany)
PERMETHYLATION	
Sodium hydroxide	Sigma-Aldrich (Steinheim, Germany)
Dimethylsulfoxide (DMSO)	Sigma-Aldrich (Steinheim, Germany)
Methyl iodide	Sigma-Aldrich (Steinheim, Germany)
Sodium chloride	Merck (Darmstadt, Germany)
MATRIX PREPARATION	
Sodium acetate	Sigma-Aldrich (Steinheim, Germany)
2,5-Dihydroxybenzoic acid (DHB)	Sigma-Aldrich (Steinheim, Germany)
super-DHB (sDHB)	Sigma-Aldrich (Steinheim, Germany)
OXIDATIVE RELEASE	
Sodium borate	Sigma-Aldrich (Steinheim, Germany)
Sodium hypochlorite	Sigma-Aldrich (Steinheim, Germany)
AMMONIA-CATALYZED RELEASE	
Ammonium hydroxide	Sigma-Aldrich (Steinheim, Germany)
CELL LYSATES	
Phosphate buffered saline (PBS)	Sigma-Aldrich (Steinheim, Germany)
Sodium dodecyl sulfate (SDS)	Carl Roth (Karlsruhe, Germany)
Sodium phosphate	Sigma-Aldrich (Steinheim, Germany)
DETERMINATION OF PROTEIN CONCENTRATION	
Bovine serum albumin (BSA)	Thermo Fisher Scientific (Waltham, MA, USA)

Table 1 (continued)

DISSOLUTION	
Guanidine chloride (GuHCl)	Sigma-Aldrich (Steinheim, Germany)
Sodium hydrogen phosphate	Sigma-Aldrich (Steinheim, Germany)
Sodium dihydrogen phosphate	Sigma-Aldrich (Steinheim, Germany)
Urea	Sigma-Aldrich (Steinheim, Germany)
Tris hydrochloride (Tris-HCl)	
MALDI-MS	
Peptide Calibration Standard II	Bruker (Bremen, Germany)

Organic solvents were from various suppliers.

The samples were applied onto MSP AnchorChip ™ 384 target plate (Bruker Daltonics). MS measurements were performed on an Autoflex Speed MALDI mass spectrometer (Bruker Daltonics). As standards, peptide calibration standard II by Bruker was used.

The processing of the acquired spectra was done using flexAnalysis v.3.4 (Bruker Daltonics). In addition, mMass software was used to visualize spectra and compare them. Lastly, GlycoWorkbench software was used for drawing of glycan structures.

Gel images were captured using a gel documentation system G:Box Chemi XX6 by Syngene (Cambridge, UK).

2.1. In-gel approach

2.1.1. Preparation of a gel

The SDS-PAGE was done using 1 µg of the model glycoprotein – transferrin (sialylated glycoprotein). The electrophoresis was performed according to the method described by Laemmli U. K. (1970). For the application onto the gel, 1 µl of protein was mixed with 11 µl of water, and 3 µl of SDS loading buffer (5x) containing 0.5 M dithiothreitol (DTT). The samples were then incubated at 100 ˚C for 15 minutes, cooled to room temperature, centrifuged, and transferred to the gel. Color prestained protein marker with a range from 10 kDa to 245 kDa was used. The gels were run in a Mini-PRO-TEAN II unit (Bio-Rad, USA). The voltage was set to 125 V, and the electrophoresis was performed till the bromophenol blue dye migrates to the bottom of the gel. Then the gel was washed with water, and the fixation solution of 40% methanol/ 10% acetic acid (vol/vol) was added. The gel was incubated in the fixative solution for 20 minutes. Afterwards, the gel was washed with water three times for 5 minutes each. The gel was stained with Coomassie Brilliant Blue G-250 dye overnight (Candiano et al., 2004). Then, the gel was destained by washing with water.

2.1.2. Reduction and alkylation of glycoproteins

Bands were cut with a scalpel under dust-free environment (Laminar Flow Box) and then destained with a solution 100 mM ammonium hydrogen carbonate in 50% acetonitrile (1:1, vol/vol), following by washing with water 3 times for 10 minutes. Then, the pieces were dehydrated with acetonitrile 3 times for 5 minutes and dried at room temperature. The reduction of disulfide bridges of protein was done using 10 mM DTT in 100 mM ammonium hydrogen carbonate followed by incubation at 56 ˚C for 30 minutes. The alkylation was done using 10 mM iodoacetamide in 100 mM ammonium hydrogen carbonate. The pieces were incubated at room temperature for 30 minutes in the dark. Lastly, the gel pieces were dehydrated 3 times with acetonitrile for 5 minutes and dried at room temperature.

2.1.3. Approaches for release of glycans in-gel

Gel pieces were subjected in the first case to the digestion with trypsin following by deglycosylation with PNGase F enzyme, and in the second case, proteins were directly deglycosylated with PNGase F.

2.1.3.1. Deglycosylation using PNGase F

The deglycosylation with PNGase F procedure was performed similarly for both in-gel and in-solution digestion. For in-gel approach, gel bands were treated with the enzyme, and in the case of in-solution approach, a solution of glycoproteins was directly treated with PNGase F. The glycoproteins (in gel or solution) were treated with 3 µl of PNGase F enzyme, 2 µl of GlycoBuffer 2 (10x), 2 µl NP-40 (10%), 15 µl of MS water. The deglycosylation in-solution was terminated by acidifying using 1% TFA obtaining a solution with 0.1% TFA concentration after incubation at 37 ˚C for 20 h. The gel pieces were covered with 20 µl of 50 mM ammonium hydrogen carbonate buffer and incubated at 37 ˚C for 20 hours. Then, extraction of glycans from the gel pieces was done twice with 100 µl of MS water and twice with 150 µl of 50% acetonitrile solution, with sonication for 30 minutes each. All extracts, as well as incubation buffer, were combined and dried in a SpeedVac at 30 ˚C.

2.1.3.2. Trypsin digestion

Dehydrated gel pieces were treated with 5 µl of a solution of trypsin with concentration 12.5 ng μl^{-1} dissolved in 50 mM ammonium hydrogen carbonate solution, and then put in a

fridge for 1 hour. Then, 25 µl of 25 mM ammonium hydrogen carbonate solution was added, and the samples were incubated at 37 ˚C overnight. Peptides were extracted from the gel by incubation in 100 µl of 5% formic acid in acetonitrile (2:1, vol/vol) at 37 ˚C for 15 minutes. The supernatant was collected, and analytes were dried in a SpeedVac at 30 ˚C. The deglycosylation of glycans was performed according to the above-mentioned procedure in section 2.1.3.1. The obtained extracts with the incubation buffer were removed, combined and dried in a SpeedVac at 30 ˚C.

2.1.4. N-glycan purification with a non-porous graphitized carbon column

Glycans were subjected to the purification using a non-porous graphitized carbon spin columns. The samples were prepared for loading onto a column by redissolving in 250 µl of 5% ACN/ 0.1% TFA (vol/vol) followed by vortexing and 1-minute centrifugation at 2500×g. The columns were conditioned by firstly washing with 400 µl of 85% ACN/ 0.1% TFA (vol/vol) three times and centrifugation for 2 minutes at 1800×g. Then, the columns were washed three times with 400 µl of 5% ACN/ 0.1% TFA (vol/vol) and centrifuged for 2 minutes at 1800×g. The samples were loaded onto columns and centrifuged for 2 minutes at 1800×g. The reloading of samples was done three times following by centrifugation for 2 minutes at 1800×g. The salts were removed by washing a column twice with 200 µl of 5% ACN/ 0.1% TFA (vol/vol). The N-glycans were recovered by passing through the column 200 µl of 30% ACN/ 0.1% TFA (vol/vol) twice and centrifugation for 2 minutes at 3000×g each. The obtained glycans were dried in a SpeedVac at 30 ˚C.

2.1.5. Permethylation

About one-fourth of the empty spin column by Harvard apparatus was filled in with 100% ACN, and the rest was packed with sodium hydroxide beads to approximately 1 cm below the top. The columns were centrifuged for 1 minute at 1600×g to remove ACN. The columns were washed with 100 µl of DMSO three times and centrifuged for 1 minute at 1600×g each. The N-glycans were dissolved in 5 µl of MS grade water. Then, 65 µl of DMSO and 35 µl of methyl iodide were added, subsequently. The analytes were applied onto the packed column and were left to lie horizontally on a tissue for 20 minutes. The samples were centrifuged for 1.5 minutes at 1600×g. Then, 35 µl of methyl iodide were added to the samples, and the glycans were reapplied onto the column. Afterwards, the column was centrifuged for 1.5 minutes at 3000×g and washed with 200 µl ACN to ensure complete collection of the sample. The mixture was subjected to liquid-liquid extraction. Permethylated

glycans were extracted from the solution with 400 µl chloroform and 1 ml 0.5 M sodium chloride solution. The aqueous phase was discarded while the chloroform layer was washed twice with 1 ml of MS water. Following the extraction, permethylated N-glycans in chloroform were dried in a SpeedVac at 30 ˚C.

2.1.6. Preparation of the DHB/sDHB matrices for MS analysis

The DHB/sDHB matrices with a concentration of 10 mg ml^{-1} were obtained by dissolving the corresponding matrix in methanol: 1 µM sodium acetate (1:1, vol/vol) solution.

2.1.7. Deposition of native glycans on the MS target plate for further analysis with MALDI MS

One microliter of a solution containing purified glycans was deposited onto MSP AnchorChip ™ 384 target plate by pipetting and was instantly covered with 1 µl of the respective matrix. The mixture was allowed to dry prior MS measurements.

2.1.8. Deposition of permethylated glycans onto the MS target plate using microgradient device.

Permethylated glycans were dissolved in 10 µl of 0.1% (vol/vol) TFA solution for subsequent separation on the target plate using a microgradient technique. The setup consisted of a gas-tight microsyringe connected to a small packed capillary. This capillary was prepared from FEP tubing that was packed with core-shell 3.0 µm C18-based core-shell particles (Rehulka et al., 2018). The capillary was firstly washed with 7 µl 80% ACN/ 0.1% TFA and equilibrated with 10 µl of 0.1% TFA (vol/vol) solution. Afterwards, the sample was loaded onto the capillary by placing 5 µl of 30% ACN/ 0.1% TFA (vol/vol) and 2 µl of a sample into the microsyringe. The microsyringe was inserted into the capillary, and the solution was pushed through. Elution of glycans was done using ACN gradient with increasing concentration. The volumes and percentages of ACN solution used can be seen in Table 2. The eluted glycans were spotted on the MALDI target plate in 0.5 µl steps and were instantly covered with 0.5 µl of sDHB matrix.

Table 2. The concentration and volume of ACN solutions used for microgradient separation

w (ACN) / %	***V* (ACN) / µl**
24	1
35	1
42	2
50	2
56	2
68	2
80	2

2.2. In-solution approach

2.2.1. Approaches for release of glycans in-solution

2.2.1.1. Oxidative release of glycans

The procedure was performed according to (Song et al., 2016). For the oxidative release, transferrin glycoprotein (25 µl, 10 mg ml^{-1}) was mixed with 25 µl saturated sodium borate solution, following by addition of 50 µl of NaClO (1%). The mixture was shaken for 1 minute, and 5 µl of formic acid were added. The solution was cooled on ice for 2 minutes and then centrifuged at 10000×g for 2 minutes. To the supernatant, 1% TFA was added, resulting in a concentration of 0.1% TFA in a solution. The resulting mixture was purified using a non-porous graphitized carbon column.

2.2.1.2. Ammonia-catalyzed release of glycans

The procedure was performed according to (Wang et al., 2018). For the ammonium release, transferrin glycoprotein (25 µl, 10 mg ml^{-1}) was mixed with 200 µl of 25% aqueous ammonia solution (weight/weight). The resulting solution was incubated in a sealed eppendorf tube at 60 ˚C for 16 hours. After incubation, the solution was dried in a SpeedVac at 30 ˚C and then redissolved in 400 µl of 0.1% TFA. The solution was purified using a non-porous graphitized carbon column.

2.3. Enrichment of glycans from biological samples

2.3.1. Preparation of cell lysates and precipitation of proteins

The cell lines suspensions were prepared as described in (Loginov et al., 2019). The *Ixodes ricinus* cell line (IRE/CTVM19) suspensions were centrifuged for 3 minutes at 300×g, and the pellets were washed three times with 2 ml of phosphate buffered saline, pH 7.4 (PBS). The lysis was performed with 500 µl of a lysis buffer (4% SDS in 50 mM sodium phosphate buffer, pH 8.0). The suspension was then vortexed for 20 minutes, incubated at 95 ˚C for 5 minutes, subjected to ultrasonication for 10 minutes, and again vortexed for 10 minutes. The protein concentration was measured using a BCA Protein Assay Kit by Thermo Fischer Scientific (USA). To 350 µl (around 300 µg of a total protein) of a lysate 1400 µl of methanol, 350 µl of chloroform, and 1050 µl of water were added. The mixture was vortexed thoroughly after each addition. Subsequently, the mixture was centrifuged for 1 minute at 12000×g, and two layers (aqueous and chloroform) were formed, within which a protein film was formed. The aqueous layer was removed, and 1400 µl of methanol was added, following by vortexing and centrifugation for 5 minutes at 12000×g. The methanol was removed, and the precipitated proteins were air-dried.

Afterwards, the proteins were redissolved in two solutions containing chaotropic salts: in one case with 8 M urea in sodium phosphate buffer (pH 7.5) and in the other case with 6 M guanidine hydrochloride (GuHCl) in Tris-HCl buffer (pH 8.0) followed by vortexing, and centrifugation for 3 minutes at 10000×g. The resulting solutions were subjected to desalting on Zeba Spin Desalting Columns according to the instruction provided by Thermo Fischer Scientific. The samples were then deglycosylated with PNGase F enzyme. The glycans were purified with activated charcoal and permethylated.

2.4. Data processing

mMass software was used for visualization and analysis of glycans spectra. Firstly, the spectrum of interest was uploaded into the system. Then, the parameters for the analysis were set: signal-to-noise ratio was set to 5.0 and relative intensity threshold was 1.0%. Baseline correction option was chosen, that allows to visually adjust the spectrum baseline. Also, Savitsky-Golay filter was used to remove signal distortion. Inessential data were removed with cropping option. Deconvolution was done, with help of which the multiply-charged species were recalculated into a singly-charged form. Lastly, peak picking option was chosen, that allows automatic choosing of peaks according to signal-to-noise ratio and intensity threshold.

Obtained peaks were then checked manually to control their shape. The identification of glycans was done using obtained MS spectra, and it was based on the existing data (MS fingerprinting). For native glycans released from transferrin, a region of spectra above 1000 m/z was analyzed. In case of permethylated glycans released from transferrin, the region above 1500 m/z was examined, since permethylation increases molar mass of glycans. For glycans released from tick cell lines, the region from 1000 m/z was studied.

3. Results and Discussion

In the following section, different methods for the identification of glycans enriched from a gel as well as from a solution of glycoproteins will be compared by means of an examination of obtained MS spectra and identification of the released glycans.

3.1. Choosing a matrix for glycans analysis

The first goal of the project was to choose a suitable matrix for MALDI MS analysis of glycans. Thus, two matrices were examined, namely DHB and sDHB. For this purpose, the glycans released form a gel, digested with trypsin, deglycosylated with PNGase F enzyme, and purified with a non-porous graphitized carbon column were used. Obtained spectra were compared (see Figure 5). There were 38 peaks detected using sDHB matrix, while just 10 peaks were found with DHB matrix. Four glycans were detected using sDHB matrix, with the intensities below 20%. Whereas by using DHB matrix, it was possible to identify one glycan with the intensity of *ca*. 40%. The noise of both spectra was comparable.

One may conclude, that sDHB matrix is preferential to use, since more glycans were detected using this matrix. Therefore, sDHB was used for all further experiments.

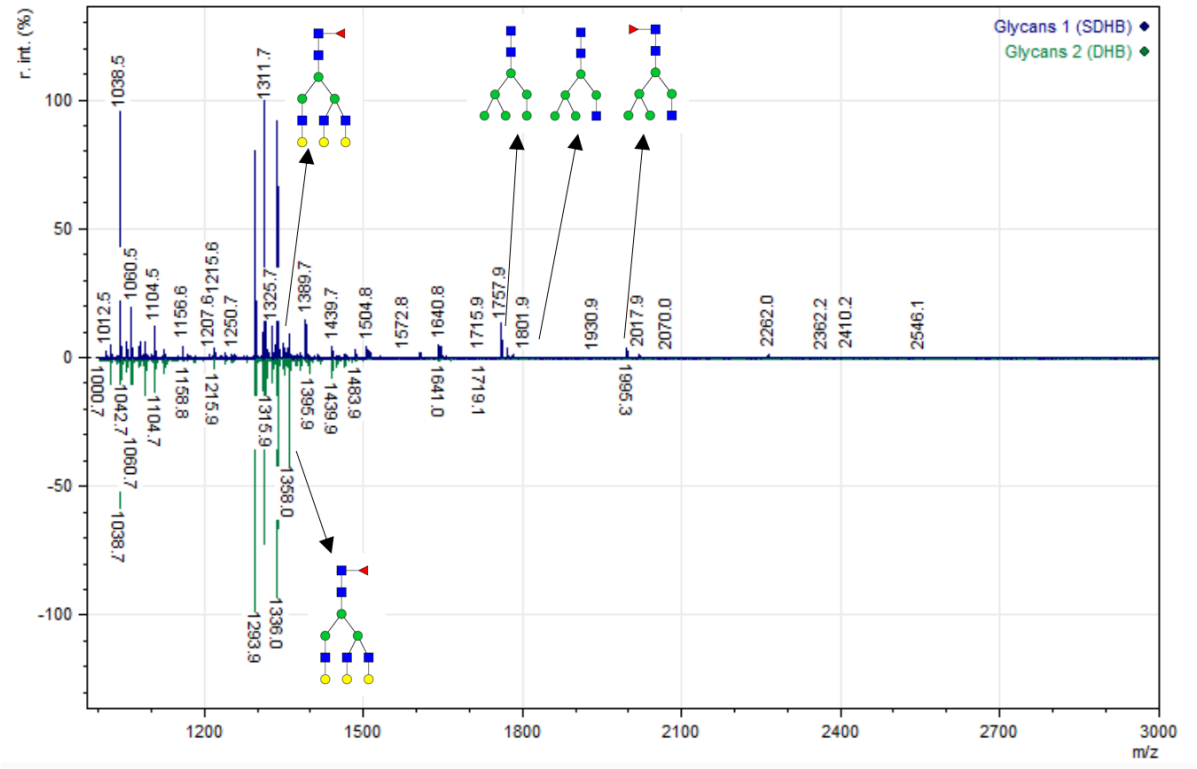


Figure 5. Mass-spectrometric profiles of permethylated glycans with the corresponding matrix. Symbol key: blue square: N-acetylglucosamine; green circle: mannose; yellow circle: galactose; red triangle: fucose; white rhombus: N-acetylneuraminic acid.

3.2. The influence of the permethylation

The next aim was to examine the effect of the permethylation on the quality of spectra and identification of glycans. For this purpose, the spectrum of permethylated glycans was compared to that of native glycans obtained after digestion with trypsin, deglycosylation with PNGase F enzyme, and active charcoal purification (see Figure 6). It can be seen that the noise of those two spectra is comparable. In case of permethylated glycans, there were 34 peaks detected, whereas in case of native glycans, there were 15 peaks detected. There was one native glycan identified with relative intensity below 7%, while it was possible to detect seven permethylated glycans with the intensity below 14%.

One may conclude that the spectrum of glycans without permethylation is significantly worse. Thus, permethylation is an essential step that should not be skipped during the sample preparation. That result is in accordance with previously published one by Kang P. et al. (2008). Therefore, the permethylation was performed for all further experiments.

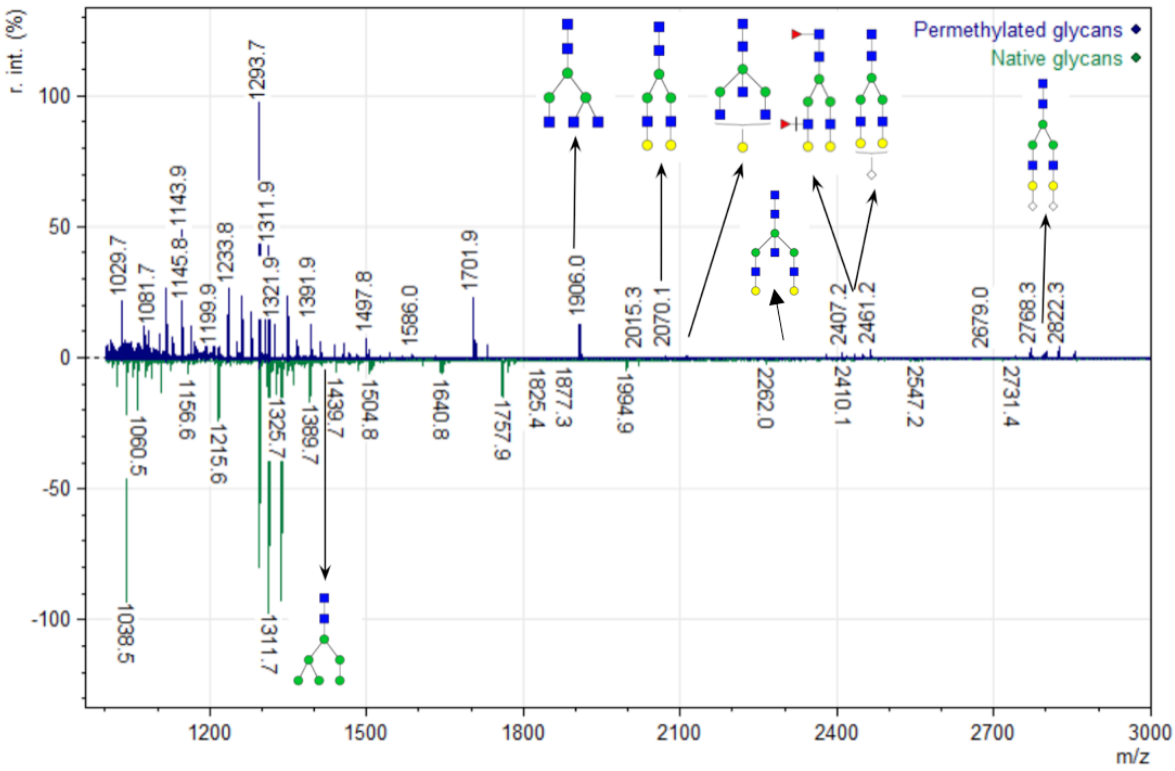


Figure 6. Mass-spectrometric profiles of permethylated and native glycans. Symbol key: blue square: N-acetylglucosamine; green circle: mannose; yellow circle: galactose; red triangle: fucose; white rhombus: N-acetylneuraminic acid.

3.3. In-gel digestion approaches

The next goal was to choose the most efficient approach for identification of glycans in-gel. The essential steps of the in-gel enrichment used in the present study are shown in Figure 7. There are two major variants: in the first case, trypsin digestion coupled with deglycosylation with PNGase F enzyme was performed; and in the second case, glycoproteins were directly treated with the PNGase F enzyme. Therefore, the influence of the digestion with trypsin step on the glycans identification was examined. Also, the effect of an extra purification step with StageTip C18 on the glycans identification was determined.

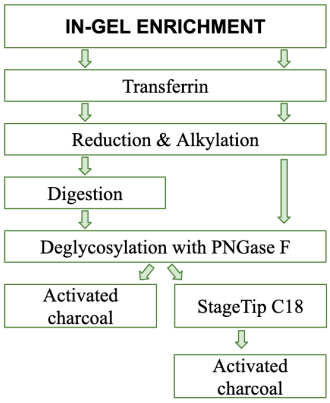


Figure 7. The scheme of in-gel enrichment of glycans

3.3.1. The influence of the extra trypsin digestion step

The influence of the digestion with trypsin step on the glycans identification was examined. The spectrum of glycans obtained after digestion, deglycosylation with PNGase F and purification with a non-porous graphitized carbon (depicted as Glycans 1) was compared to that obtained after deglycosylation with PNGase F and purification with a non-porous graphitized carbon (depicted as Glycans 2) (see Figure 8). It can be seen that the noise of the spectrum obtained without the additional digestion step is considerably higher. Also, there were 32 peaks detected in that case, and it was possible to detect five glycans with relative intensities below 3%. Whereas seven glycans were identified using the sample subjected to both digestion with trypsin and deglycosylation with PNGase F.

Therefore, it was proven that the additional digestion step is advantageous for glycans identification. This result is in accordance with previously published one by Y. Kita et al. (2007) who reported that tryptic digestion improved deglycosylation efficiency.

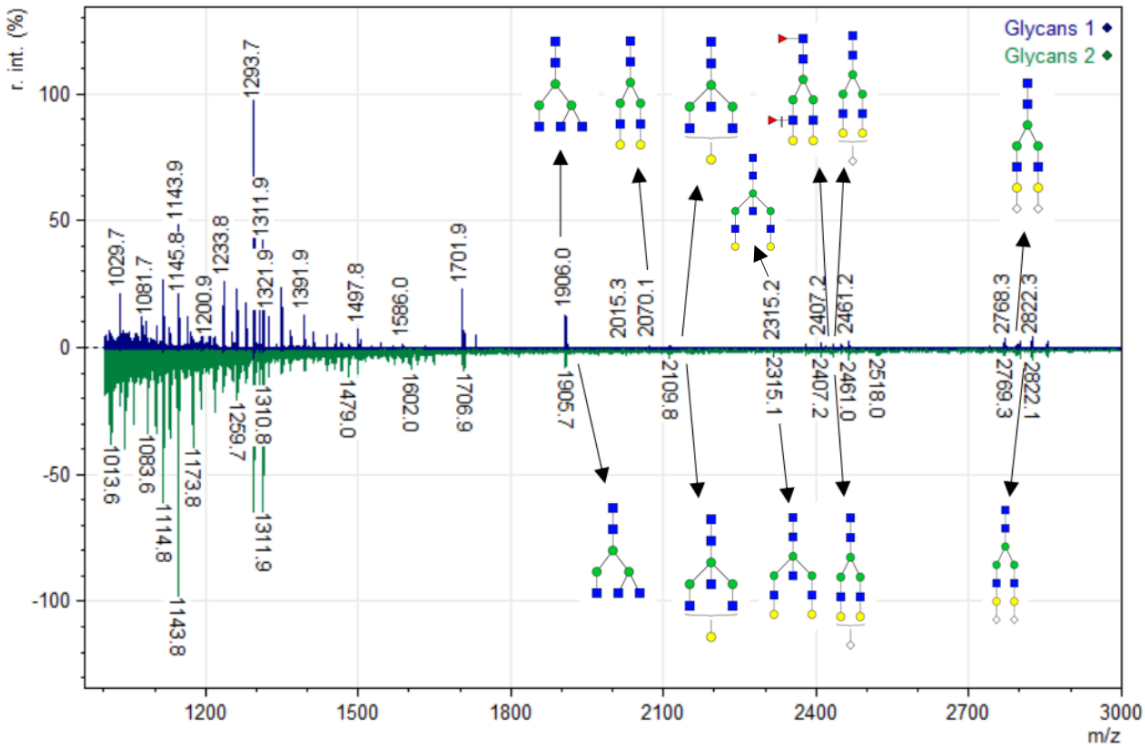


Figure 8. Influence of digestion step onto mass-spectrometric profiles of glycans. Symbol key: blue square: N-acetylglucosamine; green circle: mannose; yellow circle: galactose; red triangle: fucose; white rhombus: N-acetylneuraminic acid.

3.3.2. The influence of the extra purification step

Firstly, the influence of an additional purification step with StageTip C18 was tested. The glycans, obtained after digestion with trypsin and deglycosylation with PNGase F, were used for the comparison. The spectrum of glycans subjected to purification with a non-porous graphitized carbon was compared to that obtained after cleaning-up with a StageTips C18 followed by non-porous graphitized carbon, depicted as Glycans 1 and Glycans 2 in Figure 9, respectively. It can be seen that the noise of the spectrum obtained with activated charcoal purification only is higher. Nevertheless, it was possible to detect seven glycans following that approach. In case of two purification steps, there were nine peaks detected, and two glycans with the relative intensities below 1% were identified.

It can be concluded that two purification steps worsen the spectrum. That can be caused by loss of the sample during the preparation steps.

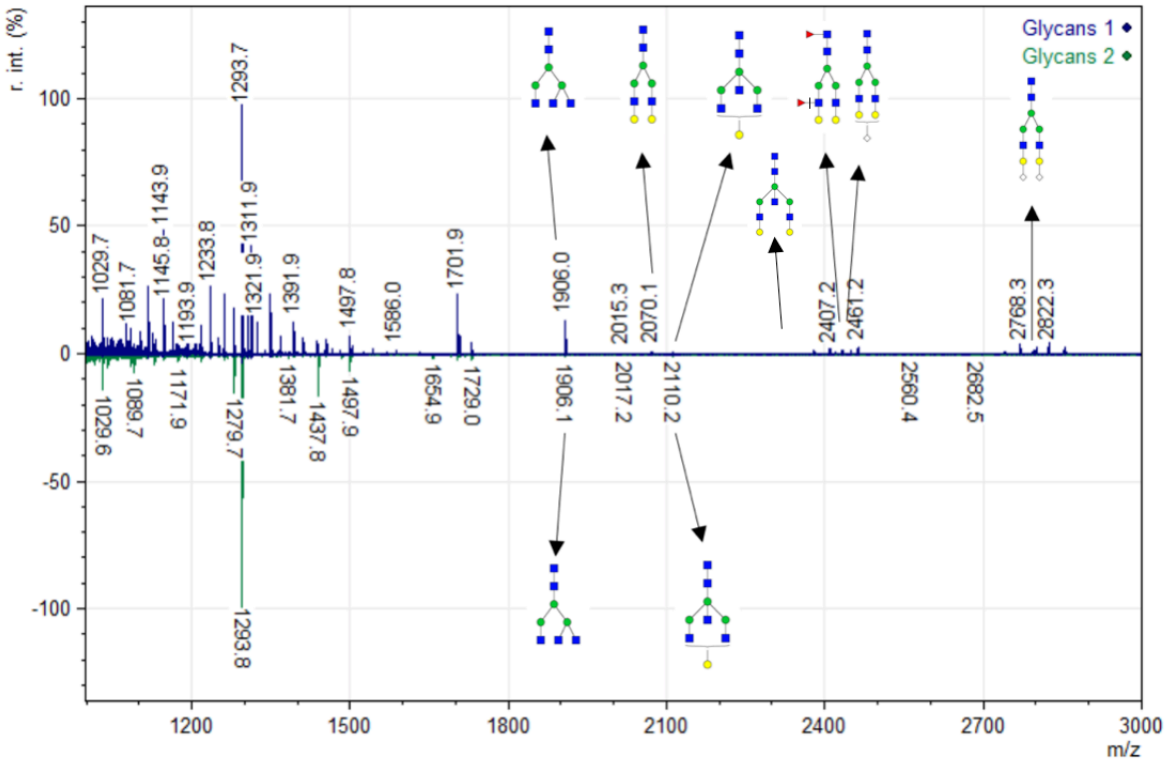


Figure 9. Mass-spectrometric profiles of glycans obtained using different purification steps. Symbol key: blue square: N-acetylglucosamine; green circle: mannose; yellow circle: galactose; red triangle: fucose; white rhombus: N-acetylneuraminic acid.

3.3.3. Choosing the most effective in-gel approach

Comparing the number of glycans identified and their relative intensity as well as a number of detected peaks, one may conclude that approach involving digestion with trypsin, deglycosylation with PNGase F and subsequent purification with a non-porous graphitized carbon column is a privileged approach for glycans identification in-gel.

3.4. In-solution digestion approaches

The next goal was to adapt the method for the identification of glycans in-solution. The essential steps of the experiment are shown in Figure 10. However, the amount of PNGase F enzyme needed for successful N-deglycosylation had to be determined first in order to proceed.

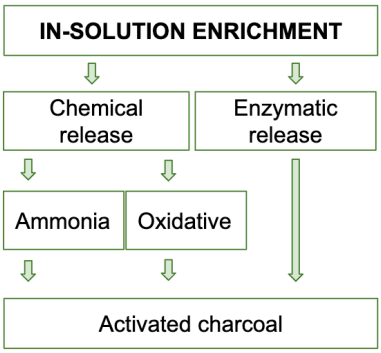


Figure 10. The essential steps of in-solution enrichment of glycans

3.4.1. Determination of amount of PNGase F enzyme needed for successful deglycosylation

Since an amount of glycoproteins in samples might vary a lot, it is necessary to add deglycosylation enzyme in sufficient amount to ensure a complete glycan release. Therefore, four different amounts of the transferrin, namely 50, 100, 250 and 500 micrograms, were treated with the same amount of the PNGase F (1500 Units). The obtained picture of the gel can be seen in Figure 11. Deglycosylation of transferrin by PNGase F enzyme caused a band shift from *ca*. 80 kDa to *ca*. 70 kDa. The band at *ca*. 30 kDa corresponds to PNGase F enzyme. Therefore, it was determined that 1500 Units of PNGase F enzyme was sufficient for complete deglycosylation of transferrin in a range from 50 µg to 500 µg.

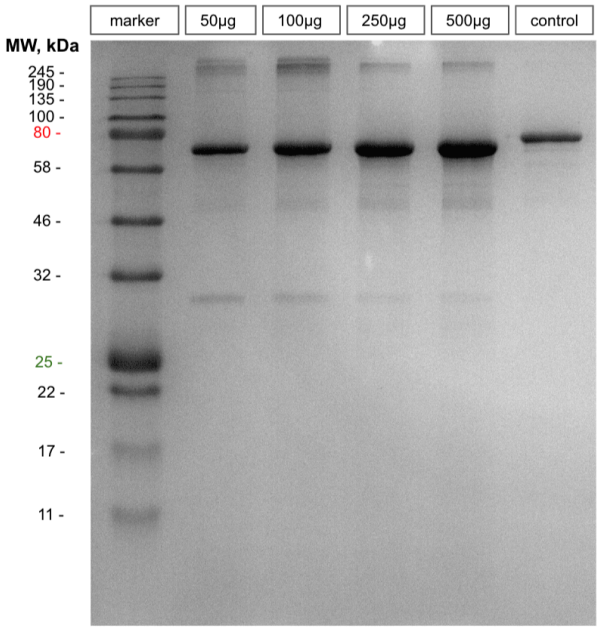


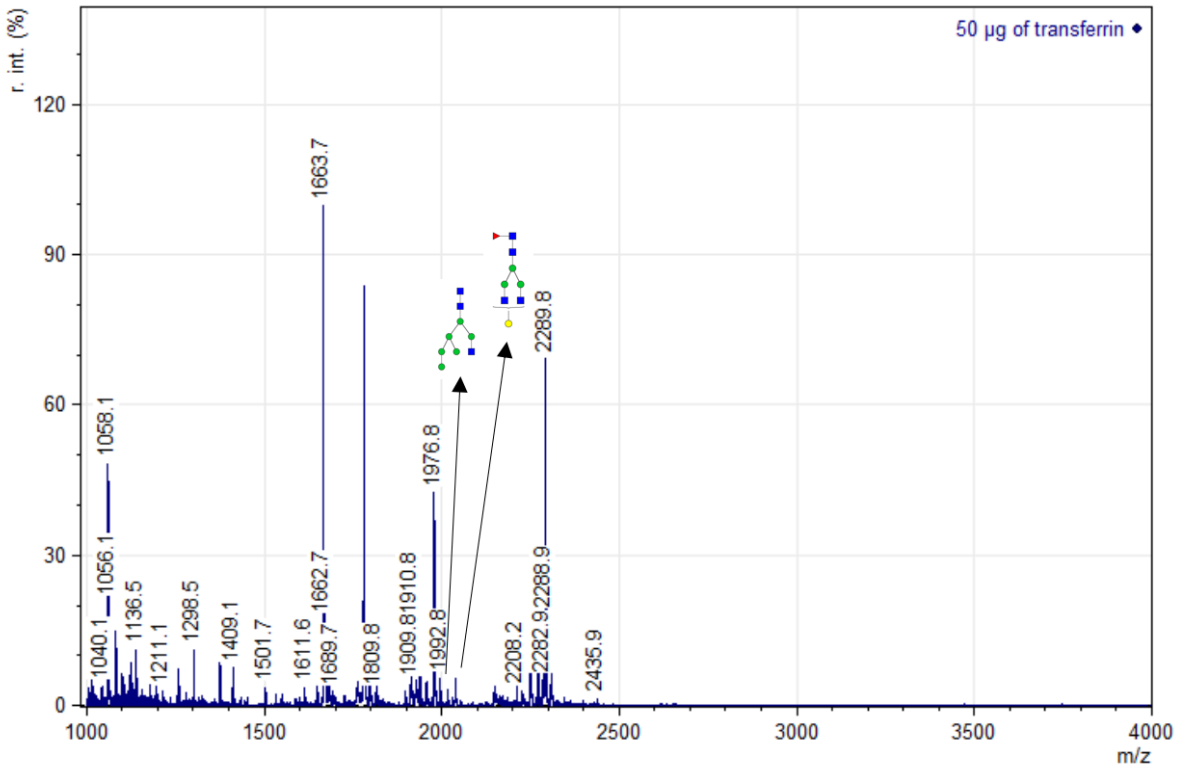
Figure 11. Comparison of separation of transferrin before and after deglycosylation

The resulting MS spectra can be seen in Figures 12 (a-d). It can be seen that the noise of spectra obtained is comparable. In case of 50 µg of transferrin used, there were 56 peaks

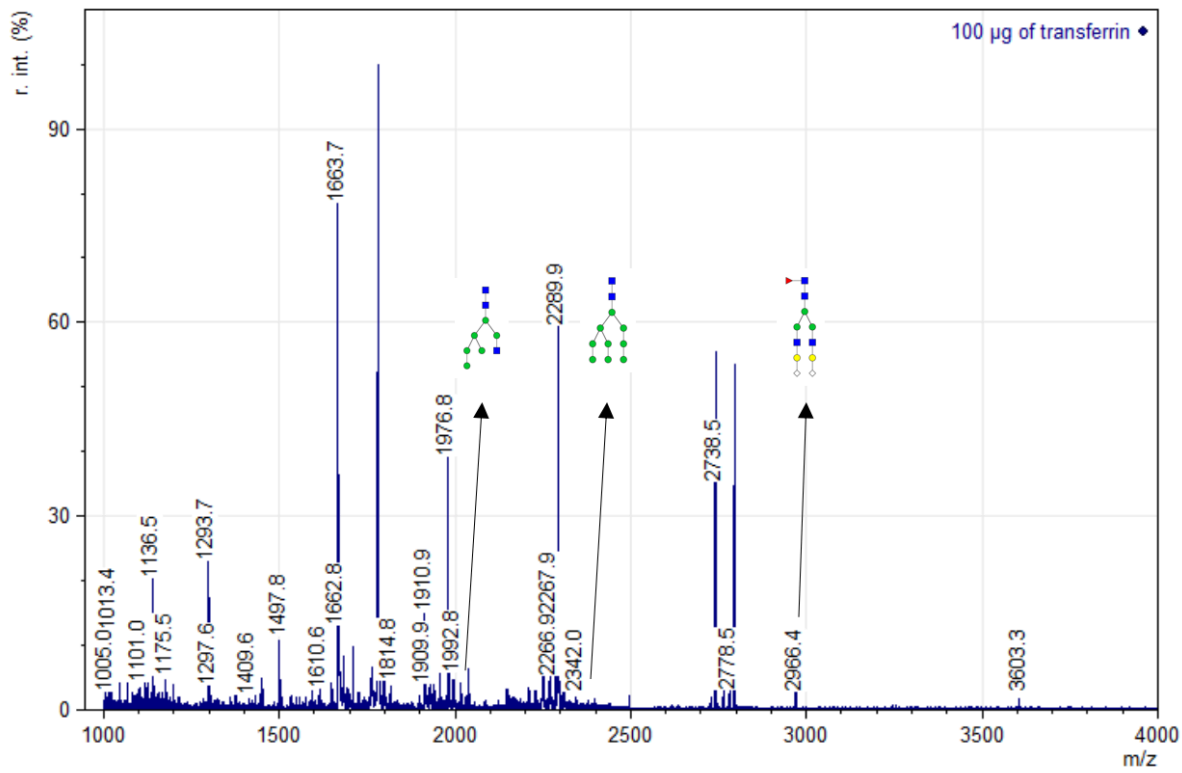
detected, and two glycans with relative intensities below 6% were identified. For 100 µg of transferrin, there were 40 peaks detected, and it was possible to identify three glycans with relative intensities below 3%. In case of 250 µg of transferrin used, there were 82 peaks detected, and fourteen glycans were identified. Thirteen peaks assigned to the glycans had the relative intensity below 16%, and one had the intensity 69%. That is the highest amount out of three other spectra. Therefore, that was the most informative spectrum. Also, the shape of peaks was better than in other cases, meaning the peaks were not broad, that allows more precise identification. However, in case of 500 µg of transferrin used, there were 28 peaks detected, and one glycan was identified with the relative intensity below 2%. The spectrum of such a low quality can be explained by some mechanical mistakes during sample preparation. The experiment was not repeated, since it was proven that 250 micrograms of transferrin were already sufficient for qualitative analysis.

Since it was found that using 250 micrograms of transferrin provided the most useful spectrum, this spectrum was compared further with a chemical method to find the most efficient approach of glycans identification in-solution.

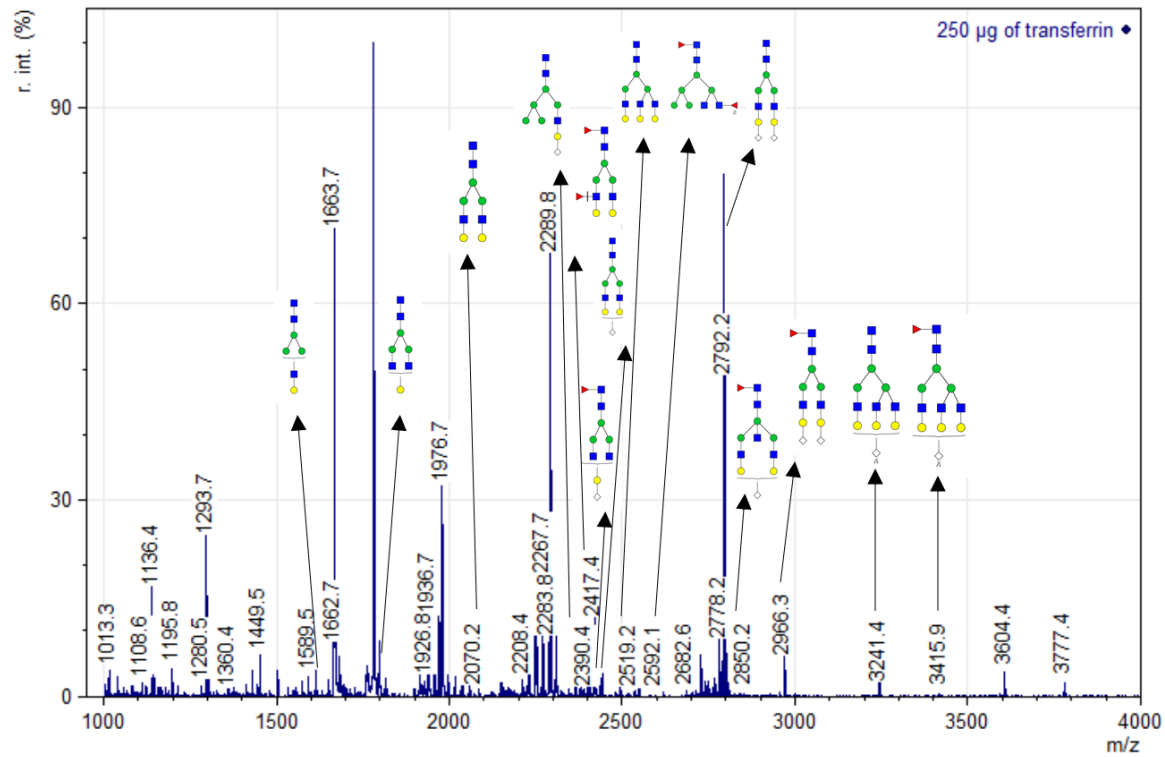
A.



B.


100 µg of transferrin
r. int. (%)
m/z
1005.0
1013.4
1101.0
1136.5
1175.5
1293.7
1297.6
1409.6
1497.8
1610.6
1662.8
1663.7
1814.8
1909.9
1910.9
1976.8
1992.8
2266.9
2267.9
2289.9
2342.0
2738.5
2778.5
2966.4
3603.3

C.


250 µg of transferrin
r. int. (%)
m/z
1013.3
1108.6
1136.4
1195.8
1280.5
1293.7
1360.4
1449.5
1589.5
1662.7
1663.7
1926.8
1936.7
1976.7
2070.2
2208.4
2283.8
2267.7
2289.8
2390.4
2417.4
2519.2
2592.1
2682.6
2778.2
2792.2
2850.2
2966.3
3241.4
3415.9
3604.4
3777.4

D.

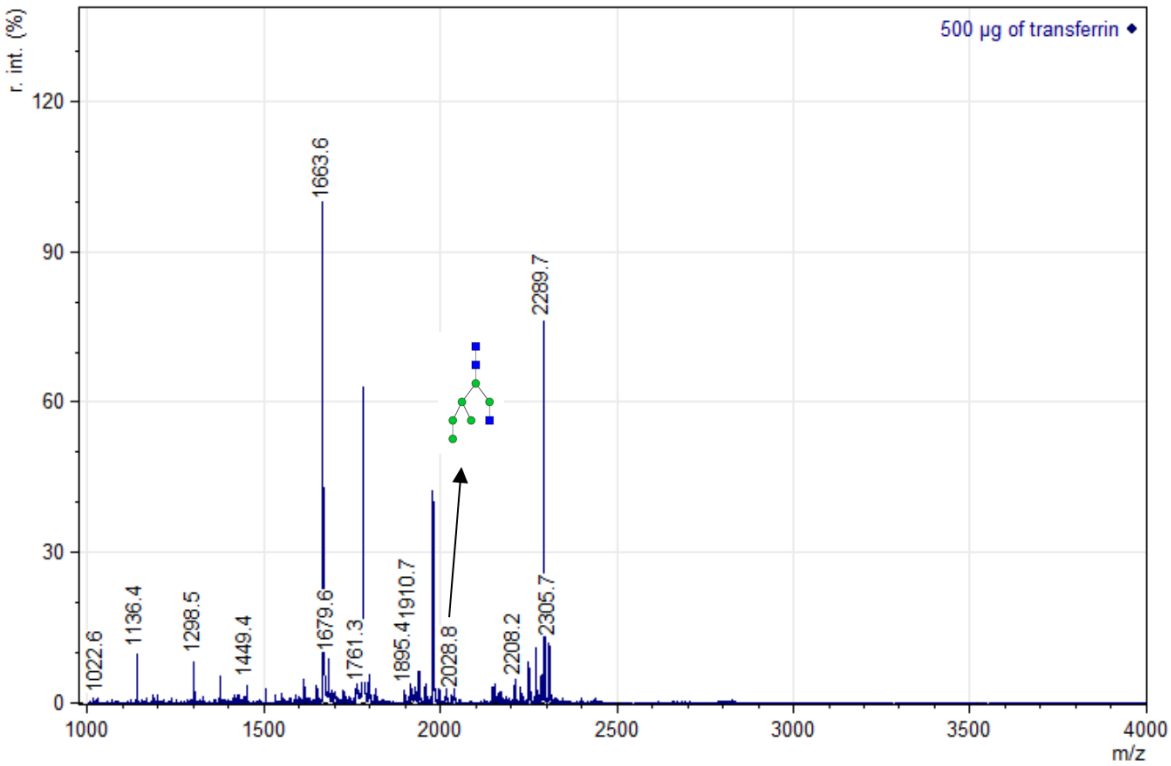


Figure 12. The spectra obtained using different amount of glycoprotein (A) 50 µg of transferrin; (B) 100 µg of transferrin; (C) 250 µg of transferrin; (D) 500 µg of transferrin. Symbol key: blue square: N-acetylglucosamine; green circle: mannose; yellow circle: galactose; red triangle: fucose; white rhombus: N-acetylneuraminic acid.

3.4.2. Comparison of two chemical methods for in-solution digestion

The best chemical method was found by comparing the spectra of glycans released by ammonia-catalyzed and oxidative approaches (see Figure 13). There were 61 peaks detected using ammonia release, and six glycans were identified. Five of those glycans had relative intensity below 2%, and one has the intensity of *ca*. 25%. In case of oxidative release, there were 35 peaks detected, and one glycan was identified with the relative intensity *ca*. 3%.

All in all, it can be concluded that ammonia-catalyzed release is more effective, since more glycans were identified in comparison with the oxidative release. That result is in agreement with previously published results by Wang et al. (2018).

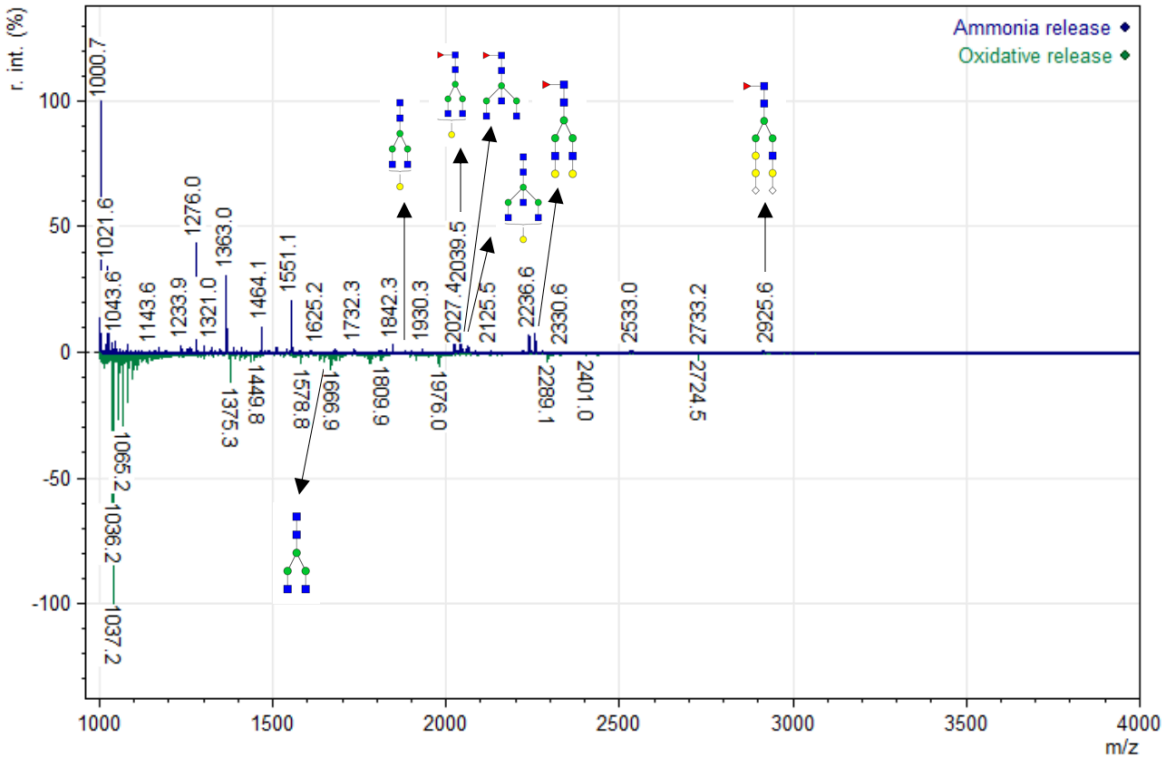


Figure 13. The comparison of two chemical methods. Symbol key: blue square: N-acetylglucosamine; green circle: mannose; yellow circle: galactose; red triangle: fucose; white rhombus: N-acetylneuraminic acid.

3.4.3. Choosing the most effective in-solution approach

The enzymatic method of glycans release was compared with a chemical ammonia-catalyzed release. Usage of the enzymatic release allowed to identify more glycans. Thus, one may conclude that the enzymatic release with PNGase F enzyme is advantageous over the ammonia-catalyzed release in-solution.

Therefore, it was shown that, in our case, the most effective in-solution methodic of glycans identification from model protein includes the following steps: deglycosylation with PNGase F enzyme and purification of the released glycans with a non-porous graphitized carbon column.

3.5. Enrichment of glycans from biological samples

The aim of the second part of the project was to determine the suitable method for glycans identification from *Ixodes ricinus* tick cell line (IRE/CTVM19) lysates. The best approach was found by evaluating the results obtained after ammonium release, oxidative release and deglycosylation of the cell lysates.

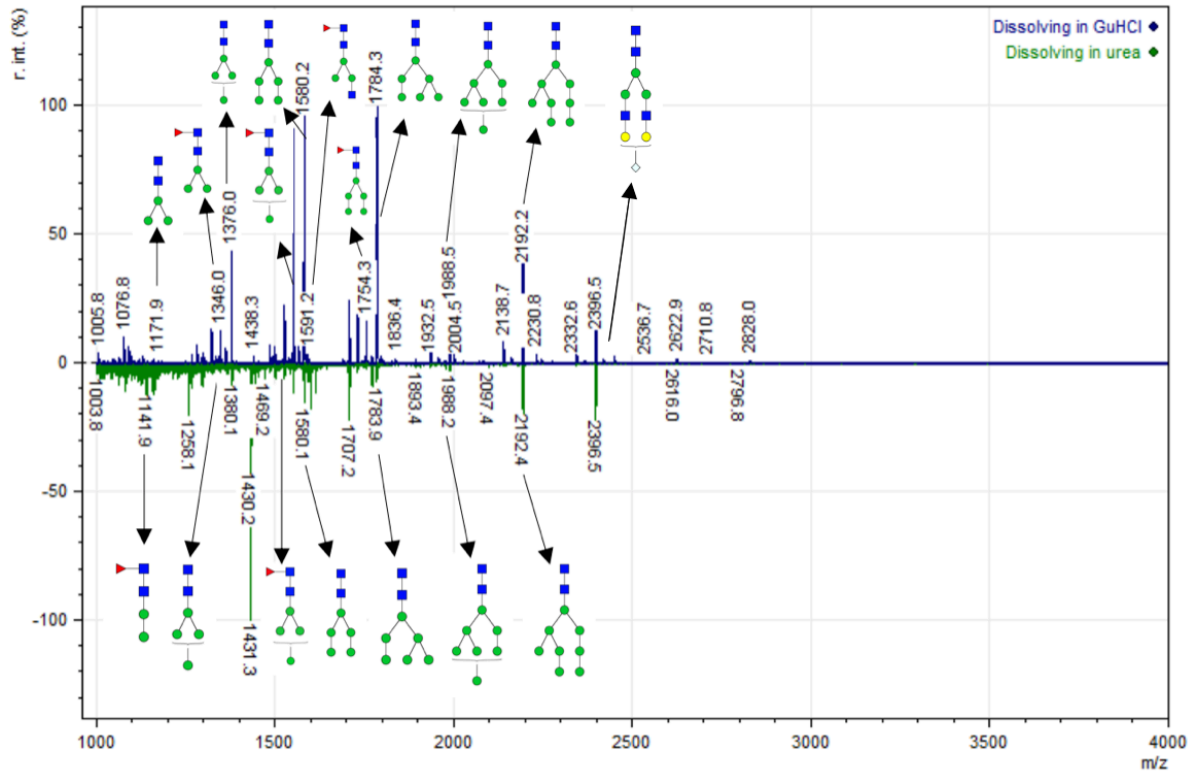
3.5.1. Choosing the most efficient chaotropic agent for dissolving of precipitated glycoproteins and subsequent glycans identification

The first and crucial step for the identification of glycans from the cell lysates is a dissolving of proteins in a suitable reagent. There are two frequently used chaotropic agents for dissolving of glycoproteins, namely guanidine hydrochloride (GuHCl) and urea. They have been used for protein extraction from various tissues (Jiang et al., 2007; Hsueh et al., 2016; Ngoka, 2008; Su & Dias, 2017). Addition of these denaturants results in losing of ordered protein structure. The resulting mixture of glycoproteins in a solvent was desalted and deglycosylated with PNGase F enzyme. Four hundred microliters of the corresponding denaturant were added to 340 µg of the precipitated proteins. After dissolving in the chaotropic agents, the total protein content in urea was 100 µg, whereas in GuHCl it was 185 µg. After desalting, the content of proteins in a sample dissolved in urea was 80 µg (resulting in *ca*. 24% recovery), while of that dissolved in GuHCl was 150 µg (resulting in *ca*. 44% recovery). That result is in accord with the publication of Pace C. N. (1986), who reported that GuHCl is 1.5 to 2.5 times more effective as a protein denaturant than urea. In addition, West S. M. et al. (1997) compared urea and GuHCl effect on protein refolding and reported that GuHCl is a stronger denaturant than urea.

The influence of two chaotropic agents (guanidine hydrochloride and urea) on the detection of glycans from biological samples was examined (see Figure 14). In the case of dissolving in guanidine chloride, there were 61 peaks detected, and 11 glycans were identified. Three of these glycans had the relative intensities below 4%, five had the intensity below 45%, and three had the intensity higher than 90%. In addition, the noise of spectra was comparatively low. In case of dissolving in urea, there were 28 peaks detected, and seven glycans were detected with the relative intensities below 26%.

Therefore, it can be concluded that the approach of dissolving in guanidine chloride is preferential to use since it allows to identify more glycans and give the signals with higher intensity.

A.



B.

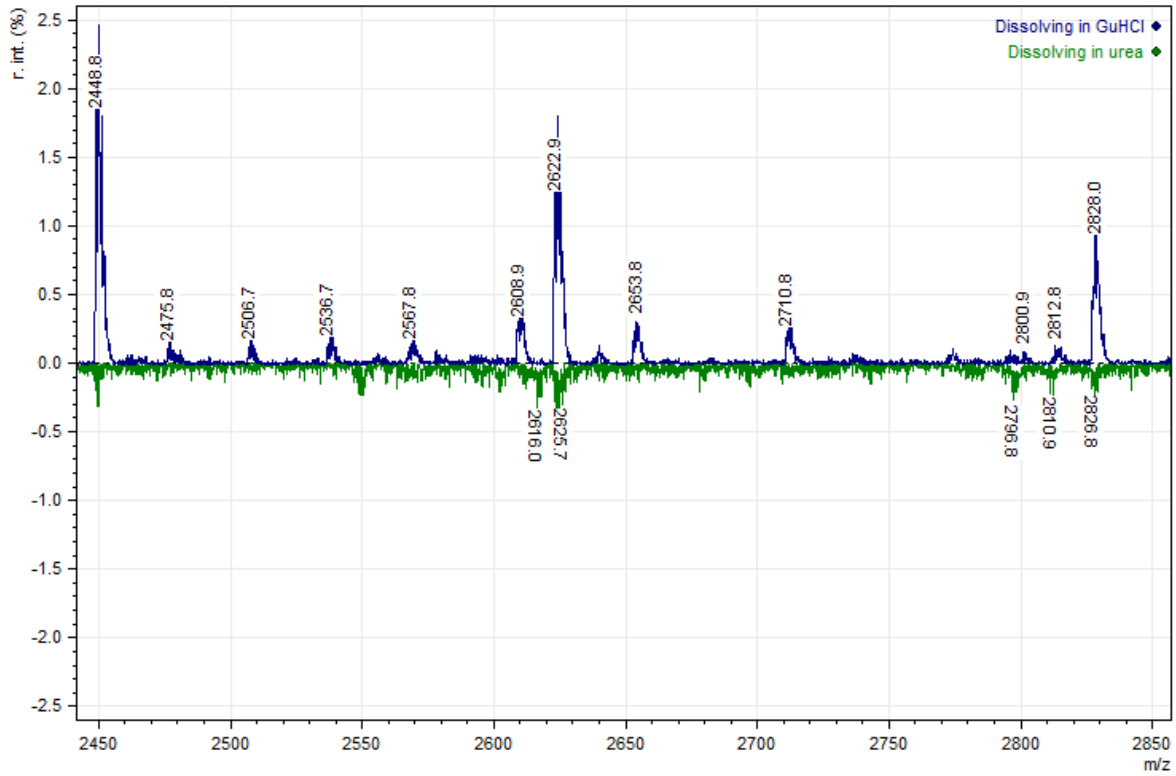


Figure 14. The comparison of glycans spectra dissolved in different chaotropic agents (A) the region from 1000 to 4000 m/z; (B) the region from 2450 to 2850 m/z.

3.5.2. Choosing the most efficient chemical method

It was reported by Wang et al. (2018) that ammonia-catalyzed release is mostly used for the enrichment of N-linked glycans. However, in tick cell line samples both N- and O-glycosylation may be present. Therefore, it was necessary to test the effectivity of ammonia-catalyzed as well as oxidative release. For this purpose, the spectra of glycans released from cell lysates by these two chemical methods were compared (see Figure 15). In case of ammonia-catalyzed release, there were 38 peaks detected, from which six glycans were identified with relative intensities below 16%. In comparison, in case of oxidative release, there were 11 peaks detected, and two glycans were identified with relative intensities lower than 15%.

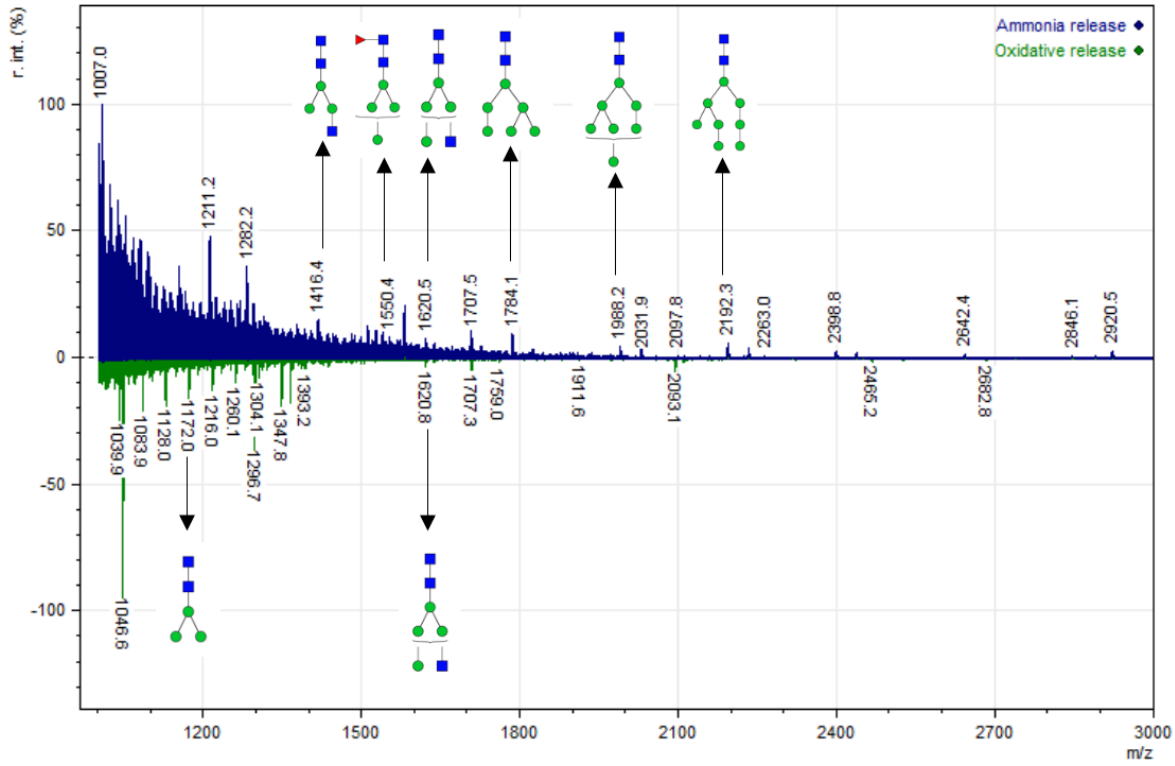


Figure 15. The comparison of two chemical methods. Symbol key: blue square: N-acetylglucosamine; green circle: mannose; yellow circle: galactose; red triangle: fucose; white rhombus: N-acetylneuraminic acid.

3.5.3. Choosing the most effective approach for glycans detection from cell lysates

By using the results of the previous experiments, the most efficient method for the identification of glycans from the tick cell lysates was found by comparing enzymatic release and chemical ammonia-catalyzed release. It can be concluded that ammonia-catalyzed release

approach gives considerably higher noise in comparison with the enzymatic method. Usage of the enzymatic release allowed to identify more glycans.

Therefore, one may conclude that dissolving of protein pellet from tick cell lysates in guanidine hydrochloride with subsequent deglycosylation with PNGase F enzyme is the preferential method for detection of glycans, since it allows to identify more glycans from tick cell lines IRE/CTVM 19.

It should be mentioned that, at the present moment, there is no complete map of possible glycans occurred in tick related samples. Thus, using more sensitive mass spectrometers (Orbitrap, FT-ICR) coupled with LC will allow to identify new structures of glycans released by different methods, including oxidative release.

4. Conclusion

The goal of studying different preparatory approaches was to establish the reproducible methods that could be used for identification of glycans released from real tick cell line samples using MALDI MS.

Firstly, the suitable matrix for the identification of glycans was found by examination of DHB and sDHB matrices. It was found that spectra obtained with sDHB matrix are of higher quality due to the number of peaks, their intensity, and the number of identified glycans.

Secondly, the method for identification of glycans in-gel from transferrin was established. This method consists of the following steps: reduction and alkylation, digestion with trypsin, deglycosylation with PNGase F and subsequent purification of glycans with a non-porous graphitized carbon column.

Thirdly, the method for glycans identification from biologically relevant samples (*Ixodes ricinus* (IRE/CTVM19) tick cell lysates) was established. The following method comprises of dissolution of glycoproteins in guanidine hydrochloride and subsequent deglycosylation with PNGase F enzyme.

The established methods can be used for identification of glycans, the subsequent study of their structures, and glycosylation in ticks related samples.

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