Bachelor’s thesis

Method development for identification of N-linked glycans by high performance anion exchange chromatography with pulsed amperometric detection and time of flight mass spectrometry

Johanna Alm

Bachelor’s thesis carried out at Octapharma AB

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A method for separating N-linked glycans by high performance anion exchange chromatography had already been developed at the department. To develop a method for identification of the N-glycans by mass spectrometry, a desalting method on porous graphitic carbon (PGC) columns was used and optimized resulting in the eluents A (0,05% TFA in ACN:water 5:95 v/v) and B (0,05% TFA in ACN:water 50:50 v/v). Also the sample introduction on the mass spectrometer was optimized and resulted in a sensitive online liquid chromatography mass spectrometry (LC-MS) approach which gave mass spectrometric peaks with high signal to noise ratios and with high mass accuracy.

The developed procedure was then successfully used on glycans cleaved from a glycoprotein separated by high performance anion exchange chromatography with pulsed amperometric detector.

N-linked glycans, HPAEC-PAD, desalting glycans, TOF
Abstract

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The developed procedure was then successfully used on glycans cleaved from a glycoprotein separated by high performance anion exchange chromatography with pulsed amperometric detector.
### Abbreviations

<table>
<thead>
<tr>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPAEC</td>
<td>High performance anion exchange chromatography</td>
</tr>
<tr>
<td>PAD</td>
<td>Pulsed amperometric detector</td>
</tr>
<tr>
<td>PGC</td>
<td>Porous graphitic carbon</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometer or mass spectrometry</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Man-5</td>
<td>Oligomannose-5</td>
</tr>
<tr>
<td>Man-9</td>
<td>Oligomannose-9</td>
</tr>
<tr>
<td>A-1</td>
<td>Mono-sialylated galactosylated biantennary oligosaccharide</td>
</tr>
<tr>
<td>A-2</td>
<td>Di-sialylated galactosylated biantennary oligosaccharide</td>
</tr>
</tbody>
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1 Introduction

In the biopharmaceutical industry, glycan identification has become a regulatory requirement and is a part of the characterization of a glycoprotein. Glycosylation, the covalent bonding of sugars to specific amino acids in a peptide, is the most common post-translational modifications in proteins and plays an important role in many biological processes. A glycoprotein is a protein with carbohydrates covalently bound to it [1]. Carbohydrates are aldehydes or ketones with multiple hydroxyl groups. They consist of monosaccharides, containing from three to nine carbon atoms. These monosaccharides vary in size and stereochemical configuration in one or more of the carbon centers [2]. Carbohydrates contribute, for example, to a protein’s biological activity, conformation, stability and pharmacokinetic abilities [3]. To be able to correlate the functional characteristics with structural parameters, a detailed structural analysis of the glycan chains is required [4].

Proteins are glycosylated on certain amino acid side-chains. There are two main types of glycosylation, N- and O- glycosylation where in N-glycosylation the glycan is attached to a nitrogen atom in the peptide chain and in O-glycosylation the glycan is attached to a hydroxyl group. In this project, the focus was on N-glycosylation. N-linked glycans are attached to asparagine in the sequence Asn-X-Ser/Thr on the protein, where X is any amino acid except proline. N-glycans can be classified in three subgroups which all share a common core containing two N-acetylglucosamine residues and three mannoses in a branched form, on which they may have one or more antenna attached [5, 6]. The different groups are the high-mannose type containing only mannose residue attached to the core, the complex type containing various types of monosaccharides in their antennal region and the hybrid type of N-glycans which have structural features from both high-mannose structure and complex structure attached to the core structure, [5] see fig. 1.

![Figure 1. Examples of the different types of N-linked glycans. Symbols: □ Mannose □ Galactose □ N-acetylglucosamine □ N-acetylgalactosamine □ Sialic acid ▼ Fucose](image)

There are different ways to study N-linked glycosylation. One method is to release the glycans from the glycoprotein with an enzyme, N-Glycanase. This enzyme cleaves the bond between the peptide chain and the glycan and leaves most of the common N-linked glycans...
unaltered while hydrolyzing Asn to Asp [6]. The glycans released from a glycoprotein can be separated chromatographically to provide a fingerprint map, also called an oligosaccharide profile [4].

Human N-linked glycans are built mainly of the monosaccharides shown in Table I. The molecular weight and formula of the monosaccharides (Table I) and their structures (fig. 2) are shown below.

Table I. The different monosaccharides in the glycans

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Sugar</th>
<th>Formula</th>
<th>Monoisotopic mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hex</td>
<td>Mannose, Galactose</td>
<td>C₆H₁₀O₅</td>
<td>162,0528</td>
</tr>
<tr>
<td>HexNAc</td>
<td>N-acetylglucosamine, N-acetylgalactosamine</td>
<td>C₈H₁₃NO₅</td>
<td>203,0794</td>
</tr>
<tr>
<td>Fuc</td>
<td>Fucose</td>
<td>C₆H₁₀O₄</td>
<td>146,0579</td>
</tr>
<tr>
<td>SA</td>
<td>Sialic acid</td>
<td>C₁₁H₁₇NO₈</td>
<td>291,0954</td>
</tr>
</tbody>
</table>

Figure 2. Structures of the monosaccharides mannose, galactose, acetylgalactosamine, acetylglucosamine, fucose and sialic acid. [Stryer et al. 2007]

1.1 Aim
The aim of this thesis was to develop a method for identification of glycans in an oligosaccharide map, i.e. to be able to identify what glycans contribute to each peak in the chromatogram from a glycoprotein in order to characterize the protein. To do so, a desalting procedure had to be optimized to get clean glycan samples and also a method for analyzing the glycans with mass spectrometry.
1.2 Techniques
To identify the glycans in the protein, they first had to be separated by anion exchange chromatography, coupled to a pulsed amperometric detector in order to get a chromatogram. The glycans were then collected in one minute fractions and the samples were neutralized with hydrochloric acid to neutralize the fractions. For further characterization of the oligosaccharides, desalting on porous graphite carbon columns was necessary. Otherwise the salts would have caused cationization, ion suppression or clogging of the inlet of the mass spectrometer and would therefore have made it impossible to get a signal. To further concentrate the samples, an on-line liquid chromatography system with a PGC-column was coupled to a time of flight mass detector with an electrospray ionization interface.

1.3 Delimitations
Only qualitative analysis was performed on four selected representative standard glycans.
2 Background

Before the practical study started a literary study was made to get information about the different methods and techniques. The background section is divided into different parts, one part about the standard glycans used in the project and several sections about the different methods used to identify the glycans.

2.1 Standard glycans

In order to optimize the analytical procedure, four standard glycans of known molecular weight and chemical properties were used. Two were high-mannose type glycans, oligomannose-5 (Man-5) and oligomannose-9 (Man-9) and two were complex type glycans, mono-sialylated galactosylated biantennary oligosaccharide (A-1) and di-sialylated galactosylated biantennary oligosaccharide (A-2). The structures of the monoisotopic standard glycans and their molecular masses are shown in fig. 3.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Mw (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man-5</td>
<td>1234,4334</td>
</tr>
<tr>
<td>Man-9</td>
<td>1882,6446</td>
</tr>
<tr>
<td>A-1</td>
<td>1931,6876</td>
</tr>
<tr>
<td>A-2</td>
<td>2222,7830</td>
</tr>
</tbody>
</table>

Figure 3. The schematic structures of the standard glycans. Structures: □ Mannose □ Galactose □ N-acetylglicosamine □ N-acetylgalactosamine □ Sialic acid □ Fucose

2.2 High performance anion exchange chromatography with pulsed amperometric detection

High performance anion exchange chromatography is a separation method used to separate ions in a complex matrix. It allows rapid and direct separation of underivatized carbohydrates [4, 8]. The method is based on the fact that carbohydrates are weak acids (pKa≈12) and form anions in an eluent with high pH (pH 13) [9].

The system consists of a gradient pump which pumps the mobile phase, a liquid, through the system. There is also an auto sampler with sample cooling, the column containing the stationary phase and the electrochemical detector that sends the gained information to a computer (fig. 4) [8].
Figure 4. The different components of a liquid chromatography system, i.e., HPAEC-PAD. Reference: [http://hiq.linde-gas.com/international/web/lg/spg/like35lgspg.nsf/repositorybyalias/image_hplc/$file/hplc.gif]

The stationary phase in an ion exchange column has charged molecules on its surface, and the ions in the mobile phase and the ionic sample molecules compete for a place on the surface. The retention is based on the attraction between negative ions in the solution and the positive ions bound to the stationary phase on an anion exchange column [10, 11] and can be regulated by varying the pH or the ion strength in the mobile phase. The separation of the compounds depends on the charge, the molecular size and the composition of the glycans [4]. The mobile phase is a gradient of sodium hydroxide and sodium acetate [12], and the column is packed with spherical porous polymer particles, so called MicroBeads. This type of column gives a very high mass transfer and high pH-stability (pH 0-14) which is necessary for separating carbohydrates due to their high pKa value [13]. The connecting tubes in the system are made of peek to withstand the high pH in the solutions.

The pulsed amperometric detector is a sensitive and selective detection method for molecules lacking a chromophore or other easily detectable groups [1]. The detection is based on the measurement of the change in current, which is proportional to the oxidation rate of the analyte. A repeating triple potential waveform is applied in a flow-through cell. First the analyte is oxidized on the surface of a gold electrode and a potential, E1, is measured. The product of this oxidation yields a contamination on the electrode and the potential is raised to clean the surface (E2). In the last step the potential is lowered and the gold electrode is deoxidized (E3). Detection is given by repeating this sequence of potentials, E1, E2 and E3 [3, 13].

2.3 Desalting
Desalting can be performed on a porous graphitic carbon drip column. PGC is a highly hydrophobic material. It allows salts to pass through while the glycans are adsorbed onto the matrix. The sample is loaded onto the column in an aqueous buffer. The contaminants, the salts, which have a low binding affinity for the carbon matrix, are eluted using a solvent with a low percentage of an organic modifier. Finally, the glycans are eluted using a solvent with a higher percentage of an organic modifier [14].
2.4 Liquid chromatography
Liquid chromatography is a separation method used to separate compounds in a complex matrix. Like the HPAEC system, the LC system consists of a mobile phase and a stationary phase. After injecting the sample the analytes flow into the column with the mobile phase. The separation occurs due to the fact that the compounds in the sample have different binding affinity to the stationary phase. Compounds with higher binding affinity to the stationary phase interact more strongly with it than the compounds with low binding affinity for the stationary phase. Therefore, the compounds in the sample with the lower binding affinity for the stationary phase will elute first and the ones with higher binding affinity elute later [11].

2.5 Mass spectrometry
Mass spectrometry is a method widely used to identify unknown analytes in a sample. The analyte is first ionized in the ion source. Then the ions are separated in the mass analyzer and finally, the detector measures the ions according to their mass-to-charge ratio, m/z (fig 5) and sends the information to a computer which converts the signals to a mass spectrum [15]. If an ion has the charge of +1 then the m/z is equal to the mass of the ion ([M+H^+] for a protonated ion) but if the charge instead is +2 then the m/z is half of the mass ([M+2H^+] / 2), the general formula for multiple charges is described as ([M+nH^+] / n), where n is the charge of the ion [11].

![Diagram of mass spectrometer](https://www.smbo.fr/spectrometrieuk.php)

**Figure 5. Simplified schematic picture of a mass spectrometer. The analytes are ionized in the ion source, separated in the mass analyzer and measured in the detector. Reference: [http://www.smbo.fr/spectrometrieuk.php](http://www.smbo.fr/spectrometrieuk.php)**

2.5.1 Electrospray ionisation
Electrospray ionization is a soft ionization technique capable of ionizing large molecules, such as proteins, with little or no fragmentation from an aqueous solution. The liquid is sprayed into a chamber through a capillary or a needle. A potential difference is applied between the capillary and the opposing chamber wall, creating an electrical field at the capillary exit. The electrical field disperses the droplets into a fine spray of charged droplets that migrates towards the mass spectrometer inlet. Heat or drying gas (N_2) evaporates the solvent from the droplets, making the droplets shrink. The decreasing diameter of the droplets creates an increase in the charge density on the droplets surface. The repulsive forces will eventually get too close together making the droplets burst into smaller and smaller ones until the analyte is desolvated and transferred into the mass spectrometer (fig. 6) [11, 15, 16].
2.5.2 Time-of-flight mass spectrometry

A time of flight mass spectrometer is a high resolution mass spectrometer that can measure ions in both negative and positive mode with a high mass accuracy down to a few ppm. In a time of flight mass spectrometer the m/z is determined by measuring the time it takes for the ions to move through a field free region between the source and the detector. The ions are accelerated into the tube by applying a high voltage pulse. They are then separated in the tube due to their different velocities, resulting in different flight times. In the flight tube, heavier ions travel slower than lighter ones and therefore the lighter ions will reach the detector first.

Ions with the same m/z can get different kinetic energy when accelerated into the flight tube. To correct the energy dispersion a reflector is placed at the end of the flight tube. Ions with more kinetic energy will penetrate the reflector more deeply and spend more time in the reflector and thus reach the detector at the same time as slower ions with the same m/z (fig 7). [15, 17].

Figure 6. The principle of electrospray ionisation. Reference: [Manz et al. 2003]

Figure 7. A schematic picture of a time of flight mass spectrometer. Reference: [http://media.wiley.com/CurrentProtocols/NC/nc1001/nc1001-fig-0003-1-full.gif]
3 Experimental procedures

The experiments in this project were divided into five different parts; (1) preparation of the N-linked glycans, (2) separation and fractionation of the glycans, (3) optimization of the desalting procedure, (4) optimization of the sample introduction to the mass spectrometer and finally applying the method on the fractions (5).

3.1 Chemicals and materials

The chemicals used were Oligomannose 5, Man-5 (Prozyme, product No GKM-002500), Oligomannose 9, Man 9 (Prozyme, product No GKM-002900), mono-sialylated galactosylated biantennary oligosaccharide, A-1 (Prozyme, product No GKC-124300), di-sialylated galactosylated biantennary oligosaccharide, A-2 (Prozyme, product No GKC-224300), water of Milli-Q quality. The materials used were GlycoClean™ H cartridges (Prozyme, product No GKI-4025).

3.2 Instrumentation

The ion exchange chromatography was performed on an ICS-3000 High Performance Ion Chromatography system (Dionex, Sunnyvale, CA) and the LC-MS system consisted of a 1200 series LC system (Agilent Technologies, Santa Clara, CA) where the mass spectrometer was a micrOTOF (Bruker Daltonics, Billerica, MA)

3.3 Separation with HPAEC-PAD

The following solutions and sample preparation were made for the separation of the oligosaccharides.

3.3.1 Mobile phases

Two mobile phases were prepared; 125 mM NaOH in water and a mixture of 250 mM sodium acetate and 125 mM NaOH in water.

Mobile phase A, 125 mM NaOH in water
2000 ml MilliQ water was added to a 2000 ml eluent plastic bottle and then degased by helium for about 10 minutes. 13 ml 50% NaOH solution were added and then the solution was degased for additionally 5 minutes.

Mobile phase B, 250 mM sodium acetate, 125 mM NaOH in water
20,5 g sodium acetate were dissolved in 1000 ml MilliQ water in a 2000 ml eluent plastic bottle and the air was removed by sparging helium for about 10 minutes. Then 6,5 ml 50% NaOH were added to the solution and bubbled with helium for additionally 5 minutes.

Both mobile phases were connected to corresponding inlets with a helium over-pressure.

3.3.2 Glycan standard sample

10 µl of each of the standard glycan stock solutions (Man-5, Man-9, A-1 and A-2) were added to a tube with 50 µl water to get the total concentration of 5 µg/ml of each of the glycans. The sample was then transferred to a Dionex vial.
3.3.3 Separation
The separation of the glycans was performed with an ion chromatography system with an auto sampler and a CarboPac PA200 column of dimension 250 mm x 3 mm ID and particle size 5,5 µm. The PAD detector was an ICS-3000 CD electrochemical detector with a 3mm gold membrane.

The flow rate through the column was held at 0,5 ml/min and the temperature over the column was 30°C. A gradient of 125 mM NaOH in water and 250 mM sodium acetate and 125 mM NaOH in water was used for elution of the glycans (gradient not shown in public report).

The detector worked in a pulsed mode with an oxidation-reduction cycle where each cycle had a duration time of one second as shown in fig. 8.

![Figure 8 The oxidation-reduction cycle. The analyte is oxidized, E1. The potential is raised, cleaning the surface of the electrode, E2. The potential is lowered, deoxidizing the electrode, E3.](image)

The Dionex vial was inserted in the auto injector and 50 µl of the standard glycan mix was injected onto the column. After separation by HPAEC-PAD, 0,5 ml glycan fractions were collected (1 minute fractions) and then neutralized with 503 µl 0,1 M HCl to avoid unwanted degradation of the glycans due to the high pH. The fractions were then frozen in -70°C for storage.

3.4 Optimization of the desalting procedure
The desalting procedure was performed on GlycoClean H cartridges packed with porous graphitic carbon. Optimization was made to find the most efficient washing procedure and which eluent to use when desalting the fractions from the HPAED-PAD separation of the glycans. Without the desalting, the contaminants in the sample could clog the mass spectrometer inlet or suppress the signal.

To optimize the desalting method different tests were made using four different standard glycans.
### 3.4.1 Standard glycans

Four representative standard glycans were used, A-1, A-2, Man-5 and Man-9, for optimization of the desalting method. In each experiment, 10 µl of the selected standard glycan with a concentration of 50 µg/ml were diluted in 90 µl eluate buffer (20 % B) (see section 3.2.1) to simulate the elution condition from the HPAEC-PAD separation, and then the sample was neutralized with 105 µl 0,1M HCl. All four glycans were not used in each test, representative glycans were chosen to reduce the amount of cartridges used.

### 3.4.2 Optimization of eluents and wash

Various tests with different acids and different amount of acid in the eluents were performed to choose which eluent to use when desalting the glycans. Tests were also done to optimize the washing procedure. The solvents were prepared as described in Table II.

**Table II.** The three pairs of eluents prepared for the desalting procedure, one with FA and two with different percentage of TFA.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Water (%)</th>
<th>Acetonitrile (%)</th>
<th>TFA (%)</th>
<th>FA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td>95</td>
<td>5</td>
<td>-</td>
<td>0,1</td>
</tr>
<tr>
<td>B₁</td>
<td>50</td>
<td>50</td>
<td>-</td>
<td>0,1</td>
</tr>
<tr>
<td>A₂</td>
<td>95</td>
<td>5</td>
<td>0,05</td>
<td>-</td>
</tr>
<tr>
<td>B₂</td>
<td>50</td>
<td>50</td>
<td>0,05</td>
<td>-</td>
</tr>
<tr>
<td>A₃</td>
<td>95</td>
<td>5</td>
<td>0,1</td>
<td>-</td>
</tr>
<tr>
<td>B₃</td>
<td>50</td>
<td>50</td>
<td>0,1</td>
<td>-</td>
</tr>
</tbody>
</table>

**Test 1**

In the first test the solvents with FA (A₁ and B₁) were used. The cartridge was first washed with 6 ml water to make sure it was free from contaminants. Then it was primed with 6 ml solvent B₁ followed by 9 ml solvent A₁ to get the right condition in the column before the sample was added. Then the cartridge was washed with 4 ml solvent A₁ to elute the salts and other contaminations and finally the glycans were eluted with 4*0,5 ml solvent B₁ and collected in tubes. The tubes were evaporated using a centrifugal evaporator (SpeedVac) and then resuspended in 60 µl solvent A₁. A-2 and Man-9 were desalted using this procedure, on separate columns.
Figure 9. The different steps of the desalting procedure. First the cartridge is washed and prepared for sample addition. The sample is added and the cartridge is washed to remove salts. Finally the glycans are eluted and collected in tubes.

Test 2
In the second test the formic acid was exchanged for trifluoroacetic acid (TFA) in two different proportions (Table II).

Three cartridges were used in the second test. On the first cartridge, Man-9 was desalted and on the second cartridge A-2 was desalted. Both samples were desalted using the same procedure as in the first test but with the solvents with the low percentage of TFA (A$_2$ and B$_2$).

On the third cartridge another sample of A-2 was used and this time the solvents with the higher percentage of TFA were used. This time the second wash procedure was altered. The cartridge was first washed with 6 ml water, primed with 6 ml solvent B$_3$ followed by 9 ml A$_3$ (the same procedure as previously). After addition of the sample the cartridge was washed with 3 ml water followed by 3 ml solvent A$_3$ (instead of only 4 ml solvent A) before elution with solvent B$_3$, evaporation and resuspension in solvent A$_3$.

Test 3
This time, instead of 4 ml, the cartridge was washed with 6 ml solvent A$_2$ after the sample, Man-9, had been added. Then the glycan was eluted, evaporated and resuspended.

Test 4
In test four, the procedure was similar to the previous one with the high percentage of TFA (0,1 %) although now, the solvents with the lower percentage of TFA (0,05%) were used. The cartridge was first washed with 6 ml water and then primed with 6 ml solvent B$_2$ followed by 9 ml solvent A$_2$. Then the sample, A-2, was added. The cartridge was washed with 3 ml water followed by 3 ml solvent A$_2$. The samples were then eluted with 4*0,5 ml solvent B$_2$ and collected in tubes. The tubes were then evaporated and resuspended in solvent A$_2$.

Test 5
Four fractions collected from HPAEC-PAD separation were desalted using the cartridges. Two of them were desalted using the washing procedure of only 6 ml of solvent A$_2$ and the
other two were washed with 3ml water followed by 3 ml solvent A₂ before eluted with solvent B₂. All fractions were then evaporated using the SpeedVac system.

3.4.3 Repeatability
The Glycoclean cartridges are sold as disposable columns that should only be used once. Because of economical as well as environmental aspects, these tests were made to see if the cartridges could be reused without degradation of the column or any trace of previously used standards.

Two of the cartridges used in the optimization of the desalting procedure were reused a total of five times with both the high-mannose- and the complex type glycans.

All desalted samples, both from the optimization of the desalting procedure and the repeatability tests, were eluted in four tubes. The first two fractions (tubes) and the last two of each sample were pooled together after being resuspended in 60 µl solvent A (total volume of 120 µl). Then they were analyzed by direct infusion electrospray ionization TOF MS (see paragraph 3.4.1).

3.5 Optimization of the sample introduction to the mass spectrometer
To find the best method to analyze the glycans in the fractions from the HPAEC-PAD and to find which glycans the protein contained the sample introduction to the MS had to be optimized. Therefore different approaches were made to optimize the analysis. Direct infusion with two different syringes and also an on-line capillary-LC system coupled to the mass spectrometer were investigated.

3.5.1 Direct infusion
First the analysis was made using direct infusion due to the fact that it is a simpler method with no additional chromatographic step. In the first tests, 100 µl of the desalted and resuspended standard glycan sample, from the optimization procedure, was loaded in a 500 µl syringe and analyzed by the time of flight mass spectrometer. The time of flight mass spectrometer operated over the chosen mass range of 500-3000 m/z in a positive ion mode and the flow rate into the MS was held at 180 µl/h.

Later, a different approach was tested where a smaller syringe was used. The advantage of this strategy was that the samples could be further concentrated prior to the MS analysis. The samples were evaporated again and this time resuspended in a total volume of 25 µl and loaded in a 25 µl syringe. Then the sample was analyzed in the TOF with the same flow rate and mass range as previously.

3.5.2 On-line coupling of a PGC-column
To concentrate the samples further, an on-line LC system with a PGC-column was coupled to the mass spectrometer. The column was a Hypercarb capillary column of the dimension 100mm*0.5 mm ID and 3µm particle size. The mobile phases used were the same as the eluents used in the desalting procedure, A₂ and B₂ (the solvents with the low percentage of TFA), and the flow rate was held at 0,02 ml/min. The different steps of the desalting procedure were recalculated (see Calculations) and transferred to the on-line system, resulting in a gradient (fig. 10). The wash procedure with water was omitted.
Two samples of A-2 with different concentrations, 1 µg/ml and 0,25 µg/ml, and one sample of Man-9 with the concentration of 0,50 µg/ml were injected onto the column.

3.6 Analysis of glycans from the HPAEC-PAD

Four fractions from the standard glycan mix sample, corresponding to the peaks from the HPAEC-PAD chromatogram, were selected and desalted using the optimized desalting procedure that was developed during the project. The cartridge was washed with 6 ml water and then primed with 6ml solvent A2 followed by 9 ml solvent B2 before the sample was added. Then the cartridge was washed with 3 ml water followed by 3 ml solvent B2. The sample was then eluted with 2*0,5 ml solvent A2 and collected in tubes. The tubes were evaporated using a centrifugal evaporator (SpeedVac) to complete dryness.

After evaporation with the SpeedVac system the samples were dissolved in a total volume of 20 µl. Six micro liters of each sample were injected to the on-line LC-TOF MS system.
4 Calculations

In order to transfer the desalting method from the cartridges to the on-line column calculations were made on the volume of the column and what it would translate to on the on-line column.

Cartridge diameter: approximately 10*10mm

Column diameter: 100*0,5mm

\[ v = \pi r^2 h \]

Volume cartridge = \( \pi (0,5 \text{cm})^2 \times 1 \text{cm} = 0,785 \text{cm}^3 = 0,785 \text{ml} \)

Volume column = \( \pi (0,025 \text{cm})^2 \times 10 \text{cm} = 0,0197 \text{cm}^3 = 19,7 \mu\text{l} \)

Number of column volumes = cartridge volume * amount of ml used in each step in the desalting procedure

Ex; 6ml solvent B: \( \frac{6 \text{ml}}{0,785 \text{ml}} = 7,639 \rightarrow 7,639 \times 19,7 \mu\text{l} = 150,0 \mu\text{l} \), which means that 6ml is equal to 7,6 column volumes on the cartridge and 150,0\( \mu\text{l} \) in the on-line column.

The flow rate was held at 20\( \mu\text{l/min} \) \( \rightarrow \frac{150,0 \mu\text{l}}{20 \mu\text{l/min}} = 7,5 \text{ min} \) \( \rightarrow \) 6ml solvent B on the cartridge is approximately equal to 7,5 minutes flow in the on-line system.

<table>
<thead>
<tr>
<th>Prime cartridge</th>
<th>Column volume (no.)</th>
<th>Equal volume (( \mu\text{l} ))</th>
<th>Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>6ml solvent B</td>
<td>7,6</td>
<td>150,0</td>
<td>7,5</td>
</tr>
<tr>
<td>9ml solvent A</td>
<td>11,5</td>
<td>225,0</td>
<td>11,3</td>
</tr>
<tr>
<td>Apply sample</td>
<td>Sample elution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4*0,5ml solvent B</td>
<td>0,6</td>
<td>12,5*4</td>
<td>2,5</td>
</tr>
</tbody>
</table>

To determine the mass accuracy on the time of flight mass spectrometer calculations in ppm was made.

\[
\text{Mass accuracy in ppm} = \frac{\text{theoretical mass} - \text{experimental mass}}{\text{theoretical mass}} \times 10^6
\]

Example: \( \text{Mass accuracy} = \frac{1234,4334 - 1234,4149}{1234,4334} \times 10^6 = 15 \text{ ppm} \)
5 Results and discussion

5.1 Separation with HPAEC-PAD
From the separation of the standard glycan mix sample, with HPAEC-PAD, a chromatogram with four prominent high intensity peaks was obtained (fig. 11) where the glycans were known.

![Figure 11. The chromatogram obtained from the separation of the standard glycan mix (Man-5, Man-9, A-1 and A-2 in order).](image)

From the standard glycan mix 20 fractions with a volume of 0,5 ml were collected in tubes for further analysis on the mass spectrometer.

5.2 Optimization of the desalting procedure
As described in the experimental part, different tests were performed to optimize the desalting procedure for glycans eluted from the HPAEC-PAD separation. After desalting, all standard glycans were analyzed by direct infusion electrospray mass spectrometry.

5.2.1 Optimization of eluents and wash
In the experiments with TFA in the eluent (test 2-4, paragraph 3.4.2) all the standard glycans could be detected by mass spectrometry as high intensity peaks. Thus, the desalting was efficient both with 0,1 % and 0,05 % TFA. Although the peak was higher when using 0,1 % TFA it was decided to use the eluent with 0,05 % TFA because this condition is gentler towards the mass spectrometer. The quality of the mass spectra were good enough, i.e. high intensity peaks were detected when using 0,05 % TFA in the eluents.

The tests to optimize the washing procedure showed that using 6 ml of solvent A₂ (see Table II) gave a higher peak than when using only 4 ml and that using water followed by solvent A₂ gave the highest intensity peaks. Although using water followed by solvent A₂ gave the
highest intensity peak the first choice was to use only solvent A₂ because it would be more convenient to use only two mobile phases when transferring the method onto an on-line system. However, this did not work when desalting the fractions from the HPAEC-PAD separation of the glycans. In the fractions desalted using only 6 ml of solvent A₂ in the washing procedure of the glycans, salt remained in the tubes after evaporating the samples and those therefore had to be desalted again before they could be analyzed.

When using FA in the eluents, when analyzing A-2 (containing two sialic acids) by mass spectrometry no peak could be detected. The reason for this is probably because the pH in the eluents with FA were higher (measured to 2,90 and 2,63) than the pKₐ value for sialic acid which is 2,6. For Man-9, only sodium-adducts could be detected in this experiment. The two glycans used in this test were representative for the four standard glycans and therefore the other two were not chosen during this test.

The result from optimizing the desalting method was the following desalting procedure: first wash the cartridge with 6ml water and then prime it with 6ml solvent B (0,05% TFA in ACN:water 1:1 v/v) followed by 9ml solvent A (0,05% TFA in ACN:water 5:95 v/v) before the sample is added. Then wash the cartridge with 3ml water followed by 3ml solvent A and elute the sample with 2*0,5ml solvent B and collect in tubes, as shown in fig. 12.

![Diagram of desalting procedure](image)

**Figure 12.** The different steps of the optimized desalting procedure.

In the cartridge instructions from the manufacturer, it is proposed that the glycan samples are eluted in 4*0,5ml fractions from the Glycoclean cartridges. When doing so, it was discovered that all glycans eluted in the first two fractions from the desalting procedure and nothing in the last two fractions. Therefore the fraction glycans were eluted with only 2*0,5ml solvent B₂.

### 5.2.2 Repeatability

In the tests with reused cartridges, no trace of any previously desalted glycan could be detected, only the ones desalted with each cartridge in each test. No significant difference in the intensity of the peaks could be detected when comparing the glycans desalted on an unused cartridge with a reused one. The experiments show that the cartridges can be reused at least five times without any interference or degradation of the cartridge.
5.3 Optimization of the sample introduction to the mass spectrometer
As described in the experimental part, different tests were performed to optimize the sample preparation for the analysis on the mass spectrometer.

5.3.1 Direct infusion
Analysis from direct infusion was successful for the standard glycans (from paragraph 3.3.1). Peaks with very good signal to noise ratios could be detected.

The final method was aimed for a classified protein. HPAEC-PAD was performed on glycans from this protein and it was demonstrated that the chromatographic peaks, and thus the concentration of the glycans, originating from this protein were much lower (data not shown) than in the standard glycan analysis. Therefore the method was tested on two of those fractions. Using the larger syringe (500 µl), one glycan could be detected in the first fraction with low signal to noise ratio (fig. 13) and nothing in the second fraction.

![Figure 13. The mass spectrometric peak from the analysis with the 500µl syringe. One glycan is detectable, Man-5.](image)

In the analysis with the smaller syringe (25 µl), the signal to noise ratio did improve somewhat but the analytes were still difficult to detect. From the fraction where nothing had been detected with the larger syringe, one glycan was now detectable, although the signal to noise ratio was very low (fig 14). Direct infusion was not a very sensitive method to use and the method needed to be improved.

![Figure 14. The mass spectrometric peak from the analysis with the 25µl syringe. One glycan is detectable, Man-6.](image)
5.3.2 On-line coupling of a PGC-column

Since direct infusion was not sensitive enough for analyzing the glycans, another approach was applied to concentrate the analytes further. An on-line PGC-column was coupled to the mass spectrometer.

The procedure from the PGC drip columns was transferred onto the on-line system (see section 4). The first test on the on-line system, with the standard samples, was successful. After about 13 minutes, a peak appeared in the chromatogram for the Man-9 standard glycan sample and the other glycan standards, A-2, eluted after about 15 minutes (fig 15). These two standard glycans were chosen to represent the neutral glycans (Man-9) and the charged glycans (A-2) to get an indication of when the glycans would elute when analyzing the glycans from the HPAEC-PAD fractions.

![Figure 15. Base peak chromatogram for standard glycan Man-9 (0.5µg/ml as peak 1) and A-2 (1µg/ml as peak 2 and 0.25µg/ml as peak 3).](image)

The peaks in the mass spectra of each of the standard glycans gave peaks with high signal to noise ratios (fig 16-18).

![Figure 16. On-line analysis of the standard glycan A-2 with the concentration 1µg/ml. A peak with high signal to noise ratio can be detected.](image)
Figure 17. On-line analysis of the standard glycan A-2 with the concentration 0,25µg/ml from the optimization of the MS-method. Also here, a peak with high signal to noise ratio can be detected.

Figure 18. On-line analysis of the standard glycan Man-9 with the concentration 0,5µg/ml. Also here, a peak with high signal to noise ratio can be detected.

When analyzing two fractions from the classified protein both samples analyzed gave peaks in the LC-chromatogram about 14 minutes after the samples were injected. The peaks in the mass spectra were easily detected and the signal to noise ratio was high as shown in fig. 19 where the glycan Man-6 is detected (compare to fig.15). This proved to be a sensitive and good method to use when analyzing the glycans.

Figure 19. Mass spectrometric peak of Man-6 from the analysis with the on-line PGC-column.
5.4 Analysis of standard glycans from the HPAEC-PAD separation

The optimized procedure was finally applied to the standard glycan mix separated with HPAEC-PAD (fig. 11). The four peaks found corresponded to the four standard glycans used and they were all high intensity peaks.

The first peak in the HPAEC-PAD chromatogram corresponds to the standard glycan Man-5 with a mass accuracy of 15ppm.

Figure 20. Mass spectrometric peak, corresponding to Man-5.

The second peak corresponds to the glycan Man-9 with a mass accuracy of 18ppm.

Figure 21. Mass spectrometric peak, corresponding to Man-9.

The third peak from the HPAEC-PAD chromatogram corresponded to A-1 with a mass accuracy of 23ppm.

Figure 22. Mass spectrometric peak, corresponding to the standard glycan A-1.
The fourth and last peak from the chromatogram corresponded to the glycan A-2 with a mass accuracy of 20ppm.

![Figure 23. Mass spectrometric peak, corresponding to the standard glycan A-2, with two additional peaks.](image)

From this, it is shown that high mannose type glycans elute in the beginning of the HPAEC-PAD chromatogram and that the fewer mannose residues attached, the earlier the glycan will elute due to the seize of the glycans. It is also shown that glycans with sialic acid attached elutes later in the chromatogram and the more sialic acid attached (more charged glycan), the later the glycan will elute due to higher attraction to the stationary phase in the ion chromatograph.

In the last glycan fraction (fig. 23), two more mass spectrometric peaks are found (circled in fig. 23), one with the mass addition of 22 which corresponds to the attachment of sodium. The other peak, which has a mass addition of 53 is still unknown, no clarification has been made of what could have caused this.
6 Conclusions

The conclusion drawn from the project is that a method for identification of the glycans was successfully developed. The glycans were successfully identified by a combination of chromatographic separation by HPAEC-PAD and identification with LC/MS.

When optimizing the sample introduction to the mass spectrometer, the conclusion was that an on-line PGC column coupled to the mass spectrometer was the method to use when analyzing the glycans. The four standard glycans were successfully identified and gave mass spectrometric peaks with high signal to noise ratio with a mass accuracy of about 20 ppm.

The method was also successful when analyzing the glycans from a classified glycoprotein (confidential data not shown in public report) due to the ability to ionize the glycans with unique molecular masses and because of the high mass accuracy of the mass spectrometer.
Reference


[12] Instruction from Octapharma, HPAEC-PAD


