4 Chapter Four: Glycosylation analysis - method evaluation

4.1 Establishment and optimization

The following analytical scheme was used to meet the requirements mentioned in the thesis objective (Figure 21). The described methods were already known and have been described in the literature [101, 140].

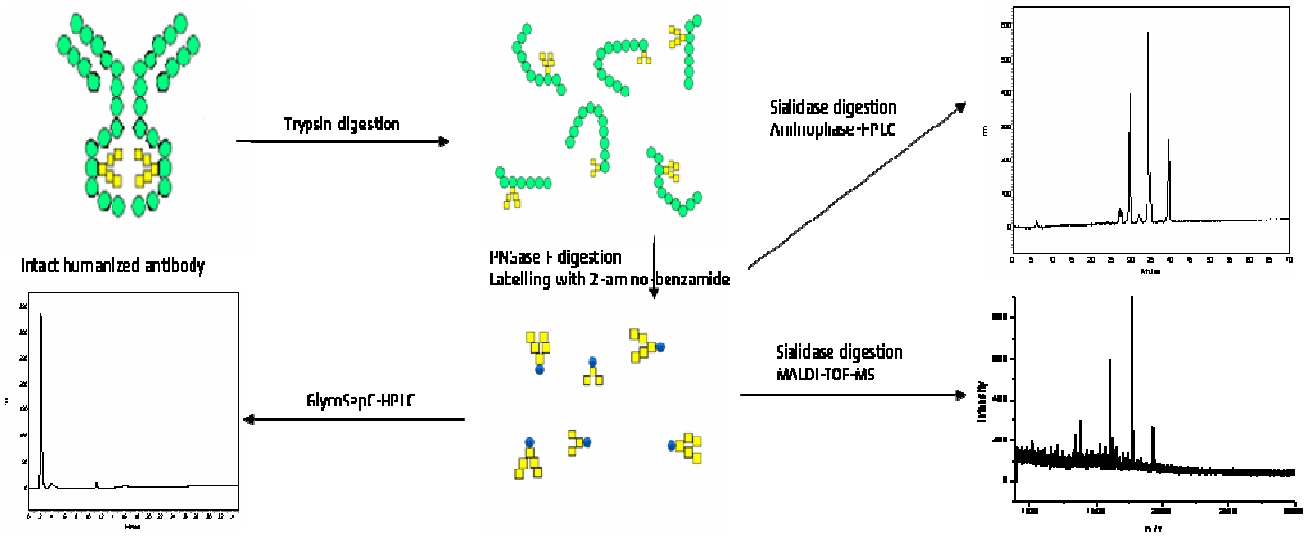


Figure 21: Established analytical scheme for glycosylation analysis[9]

As mentioned in the introduction, positive attributes of the above methods in comparison to HPAEC-PAD are its sensitivity (fluorimetric detection), good signal separation properties (resolution), the comparable response factors of the analytes, the robustness of the methods and the low costs of the conventional HPLC-system (Shimadzu) compared to the relative high costs of a specialized Bio-LC-system (Dionex).

The major disadvantage of these methods is the pre-column derivatization step (2-AB-labelling) which increases the total time of the analysis. Besides

[9] *GlycoSepC-HPLC = AEX, Aminophase-HPLC = HILIC*

additional validation effort is necessary to validate the labelling step.

The analytical techniques were established with qualified equipment (see Appendix) and standardized by the description in standard operation procedures (SOPs).

4.1.1 Short description of the analytical scheme

At first, the purified protein is desalted and after that digested with trypsin to obtain peptide fragments with bound glycans. Deglycosylation of the peptides is performed by PNGase F digestion. After that, the cation exchange purification follows. The isolated glycans are labelled with 2-aminobenzamide which makes fluorimetric detection possible. The labelled glycans are purified by paper chromatography to separate them from label excess. One part of the glycans is digested with sialidase to get uncharged oligosaccharides. The sialidase-digested glycans are purified by mixed bed chromatography consisting of cation exchange and anion exchange resins. Then, two different HPLC methods follow. The desialylated glycans are separated on an aminophase column (HILIC). The sialylated glycans are separated on a DEAE-column (AEX). To identify unknown glycan structures MALDI-TOF-MS is used. For standardization, working protocols were created for the sample preparation procedure (see Appendix).

4.1.2 Material and methods

- For sample preparation:

Desalting columns:

Amersham Biosciences, PD-10 columns (Sephadex G-25 M), Product-No. 17-0851-01

Trypsin:

Roche, Product-No. 109819

PNGase F:

Roche, Product-No. 1365193

Incubation buffer: 50 mM N-Methyl-2,2'-iminodiethanol, pH 8.0, adjusted with concentrated TFA

N-Methyl-2,2'-iminodiethanol, VWR Merck, Product-No. 8.05851.0100

Concentrated Trifluoracetic Acid, VWR Merck, Product-No. 1.08262.0025

Ion Exchange Resins:

Cation exchange resin: BioRad AG 50W X12 Resin, 100-200 mesh, hydrogen form, Product-No. 142-1641

Anion exchange resin: BioRad AG 4 X4, 100-200 mesh, free base, Product-No. 140-4341

Disposable Polystyrene Columns:

PIERCE, Product-No. 29920

Lyophilisator:

ISMATEC, Ser. No. 215106

Chromatography Paper 3 mm Chr:

Whatman, Product-No. 3030917, 100 Sheets, 46*57 cm

Eluent for paper chromatography:

Mixture of 4 parts butanol, 1 part ethanol and 1 part purified water

Butanol and Ethanol were from VWR Merck and water was produced by a Millipore Milli-Q and Direct-Q system.

3 ml syringes for elution of the oligosaccharides from the chromatography paper:

BD, 3 ml Syringe Luer-Lok, Latex Free Syringe, Product-No. 309585

Membrane filter for the separation of cellulose particles after the paper chromatography:

Millipore, Millex-HV Syringe Driven Filter Unit, pore size 0.45 µm, Product-No. SLHV R04 NL

Sialidase:

Roche, Product-No. 269611

Sialidase buffer: 500 mM sodium acetate solution, pH 5.0

Mixture of acetic acid and purified water and adjusted with NaOH-solution

Acetic acid (Product-No. 1.00066.0250) and sodium hydroxide solution (Product-No. 1.05428.0250) were from VWR Merck and water was produced by a Millipore Milli-Q and Direct-Q system.

2-Aminobenzamide:

Sigma, Product-No. A8,980-4

Dimethylsulfoxide:

VWR Merck, Product-No. 41647

Sodium cyanoborhydride:

Fluka, Product-No. 71435

Glas chamber for paper chromatography:

Dimensions: 2.5 cm x 8 cm x 7 cm

UV-lamp:

BENDA, NU-8 k1, 254 nm and 366 nm

Centrifuge tubes:

Biochrom, 15 ml, Product-No. P91015

Screwing vials:

Fisher, 0.5 ml, Product-No. 9479900

PCR-Tubes:

Cotech, 0.2 ml, Product-No. AXY-PCR-02Y

Drying oven:

Integra Biosciences, Cellsafe

Thermo controller:

MJ Research, Programmable Thermal Controller, PTC-100™

- For HPLC-methods:

HPLC-device:

QC-HPLC

Acetic acid:

VWR-Merck, Product-No. 1.00066.0250

Sodium hydroxide solution:

VWR-Merck, Product-No. 1.05428.0250

Acetonitrile:

Acetonitrile HPLC Grade S, Rathburn, Product-No. RH1016

4.1.3 Pre-calculations for the enzymatic digestions

For the tryptic digestion, a trypsin solution with a concentration of 1 mg/ml was produced. The addition of trypsin solution to the protein sample was performed in two steps. In the first step, trypsin solution in a ratio of 1:50 (m/v) based on the total amount of protein in µg was added. That meant that for 1600 µg protein, 32 µl of trypsin solution were necessary. In the second step, trypsin solution in a ratio of 1:25 (m/v) was added to the protein and the sample was filled up to a volume of 950 µl with incubation buffer. After performing the tryptic digestion and after inactivation of the trypsin, PNGase F (1 mU/µl) was added in two fractions à 25 µl to the sample solution. So the digestion was standardized with 50 mU PNGase F/1000 µl sample volume (Table 2).

Table 2: Pre-calculations for the enzymatic digestions of three different concentrated glycoprotein samples

Sample number	Protein (mg)	Volume incubation buffer	1. Trypsin addition	2. Trypsin addition	Sum trypsin	1. PNG-aseF	2. PNG-ase F	Sum PNGase F
1	1.6	854 µl	32 µl	64 µl	96 µl	25 µl	25 µl	50 mU
2	2.1	824 µl	42 µl	84 µl	126 µl	25 µl	25 µl	50 mU
3	1.8	842 µl	36 µl	72 µl	108 µl	25 µl	25 µl	50 mU

4.1.4 Basic working procedures

Transfer of samples: Centrifuge tubes -> centrifuge tubes, 0.2 ml PCR-vials or 0.5 ml screwing vials

When samples were transferred between the above described vessels, centrifuge tubes had to be rinsed four times. Rinsing was defined as the addition of 200 µl water, vortexing, centrifugation and the pipetting in the new vessel. Pipette tips were rinsed 10 times after each pipetting.

Concentration of the samples:

Concentration of a sample was defined as the procedure of dissolving and lyophilizing a sample up to a special predefined volume. For example, if a sample was concentrated from 100 µl to 10 µl, the 100 µl sample was lyophilized to dryness. After that, it was dissolved in 50 µl of water, was vortexed, centrifuged and lyophilized again. The same procedure was repeated with 20 µl and 10 µl. This operation was necessary to guarantee a quantitative accumulation of the sample in a predefined volume.

4.1.5 Desalting of protein samples

Desalting of proteins took place with Sephadex PD-10 columns (Figure 22). At first, the bottom cap had to be cut off, the top cap had to be removed and the excess liquid had to be poured off (1). After that, the PD-10 column had to be equilibrated with 25 ml of purified water (2+3). The sample had to be solved in a volume of 2.5 ml and this volume had to be pipetted on the column (4). The flow-through was discarded in 15 ml centrifuge tubes. At last, the sample was eluted from the column with 3.5 ml of purified water and the flow-through was collected.

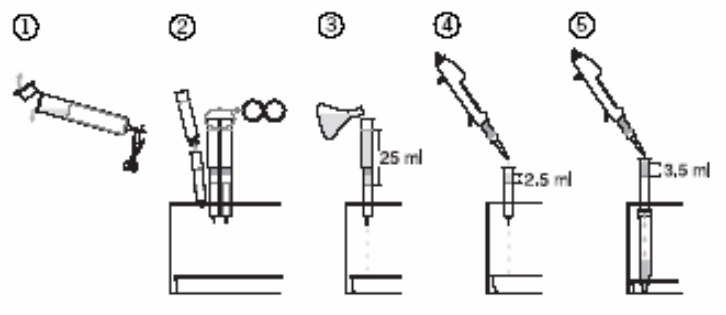


Figure 22: Desalting with PD-10 columns

4.1.6 Digestion with trypsine

To assure a complete cleavage of N-glycans by PNGase F, it had to be guaranteed that the enzyme reached all glycosylation sites. To avoid steric hindrances due to the orientation of polypeptides in the aquatic phase, a digestion with trypsin was performed before the PNGase F-digestion. Trypsin cuts the polypeptide chain after the amino acids arginine and lysine. After that, the resulting glycopeptides were ready for complete deglycosylation. An advantage of this method is that one does not need any detergents for deglycosylation [141].

The lyophilized desalted protein sample was dissolved in the pre-calculated amount of incubation buffer. As described above, the first addition was in ratio 1:50 (m/V), the second in ratio 1:25 (m/V). After each trypsin addition,

the sample was incubated for 11 hours at 37°C in a drying oven. The trypsin solution was only durable for one hour. After the incubation, the solution was boiled at 100°C in a water bath for 10 minutes to inactivate the trypsin. A cooling down of the solution in crushed ice for 30 minutes followed. This step was necessary because the trypsin would also digest the PNGase F in the next digestion step.

4.1.7 Digestion of glycopeptides with PNGase F

The peptide-N4-(N-acetyl-β-glucosaminyl)-asparagine amidase F cleaves selectively N-glycans from the asparagine rest of the peptide [97].

At first, the acquired PNGase F had to be desalted. The eluate was lyophilized and after that solved in a volume of 250 µl incubation buffer. So the PNGase F-solution was standardized with 1 U/µl. For one sample, 50 PNGase F units were used for a complete digestion. Therefore 250 µl PNGase F-solution could be used for five samples. As described above, the addition of PNGase F was performed in two steps à 25 µl. After each addition, incubation at 37°C in a drying oven for 11 hours followed.

4.1.8 Cation exchange purification

At first, the digested sample was vortexed and centrifuged. After that, 100 µl of 10% (V/V) acetic acid were added to the sample. The pH of the solution changed from 8 to 3 - 4. Now the 2 ml disposable polystyrene columns were filled with a corresponding frit. This frit was rinsed with 1 ml of purified water. Now 0.5 ml of cation exchange material was pipetted onto the frit. The cation exchange resin was flushed with 5 x 0.5 ml of purified water and with 5 x 0.5 ml of 1% (V/V) acetic acid. After that, the disposable column was put into a 15 ml centrifuge tube and the sample was transferred onto the resin. The whole flow-through was collected. The column was flushed with 4 x 0.5 ml of purified water. Then, the filtrate was lyophilized to dryness in the centrifuge tubes. The lyophilisate was solved in 100 µl of purified water, transferred into 0.2 ml PCR-tubes and concentrated to a volume of 10 µl.

4.1.9 Labelling of the oligosaccharides with 2-aminobenzamide

For labelling of the sialylated and desialylated oligosaccharides with the fluorophor 2-aminobenzamide, the Signal™ 2-AB-Labelling-Kits from Ludger Ltd or Prozyme® were used at first. Thereby, the N-glycans were marked. The reaction based on the characteristic of aromatic amines to create Schiff's bases with aldehyde functions, in this case with the aldehyde functions of the hexoses, under secession of water. To achieve a quantitative marking as well as to make the product more stable against hydrolysis, sodium cyano-borohydride was added to the reaction mixture, which worked as a strong reducing agent and transferred the Schiff's base to a secondary amine (Figure 23). The product was extricated from the chemical equation and the reaction could go on. A reduction from aldehyde to alcohole was not observed. The original labelling conditions were developed by Bigge et al in 1995 [142].

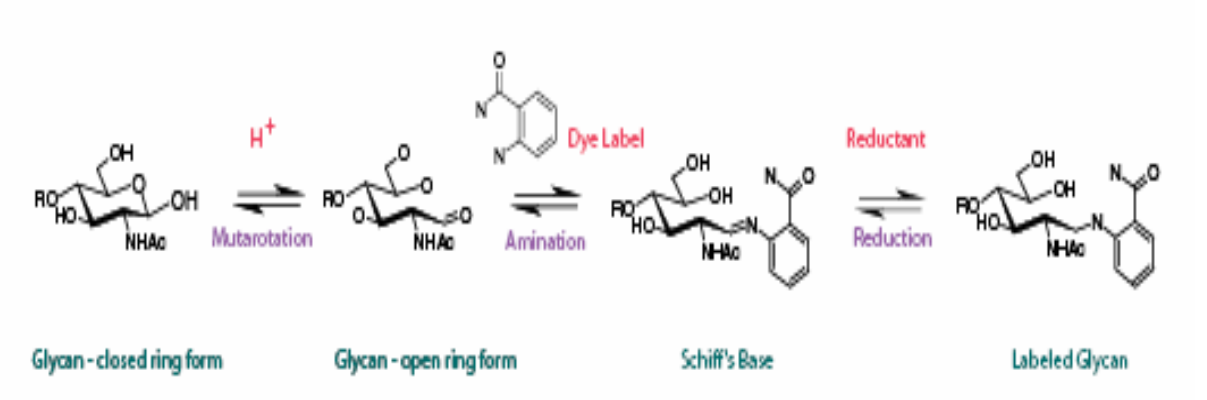


Figure 23: Reaction of a glycan with 2-aminobenzamide

Later, labelling of oligosaccharides was performed with the raw chemicals and not with the labelling kit to reduce costs. As Bigge et al already described in 1995, the labelling temperature was a compromise between the reaction efficiency in a limited time and the contrary reaction, namely the loss of sialic acids at too high temperature [142] (Figure 24).

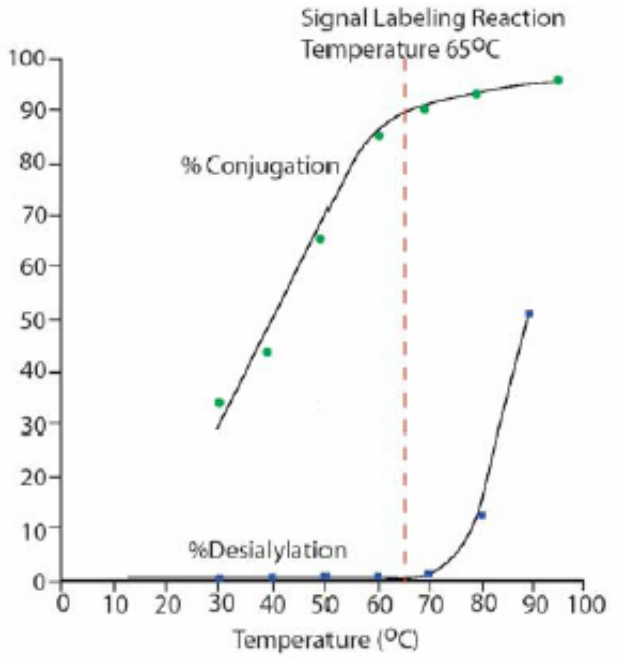


Figure 24: Labelling conditions found out by Bigge et al in 1995

In 2000 Watanabe et al found out that the labelling conditions could be optimized for quantitative analysis in a way that the recovery of sialylated structures could be increased at an equivalent level of reaction efficiency. Therefore the labelling temperature had to be reduced to 37°C and the reaction time was increased to 16h [143]. I could confirm these results by comparing different samples, each labelled at 65°C for 3 hours and at 37°C for 16 hours. All samples labelled at 37°C for 16 hours showed higher GlycoSepC-A2-, -A3- and -A4-fractions than the corresponding samples labelled at 65°C for 3 hours (Figure 25 and Figure 26). Comparing both diagrams, there was a shift from the lower sialylated structures (A0, A1) to the higher sialylated ones (A2, A3, A4) in relative percentages.

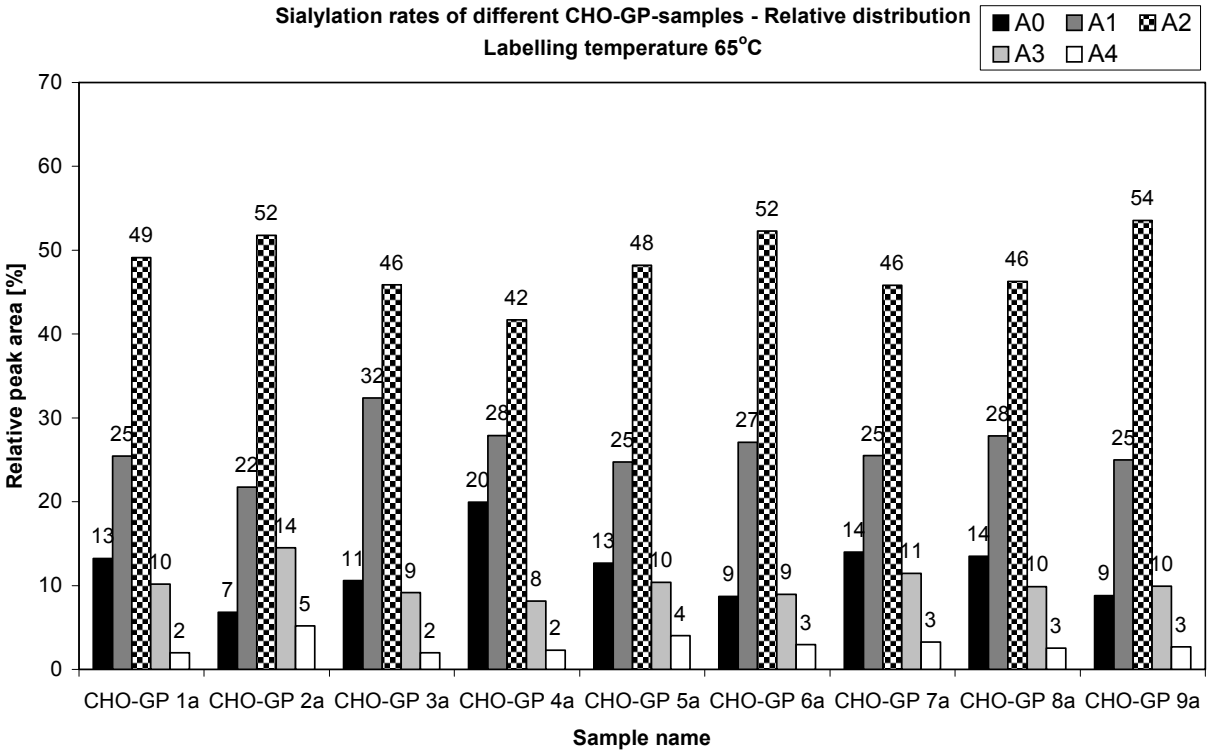


Figure 25: Labelling at 65°C

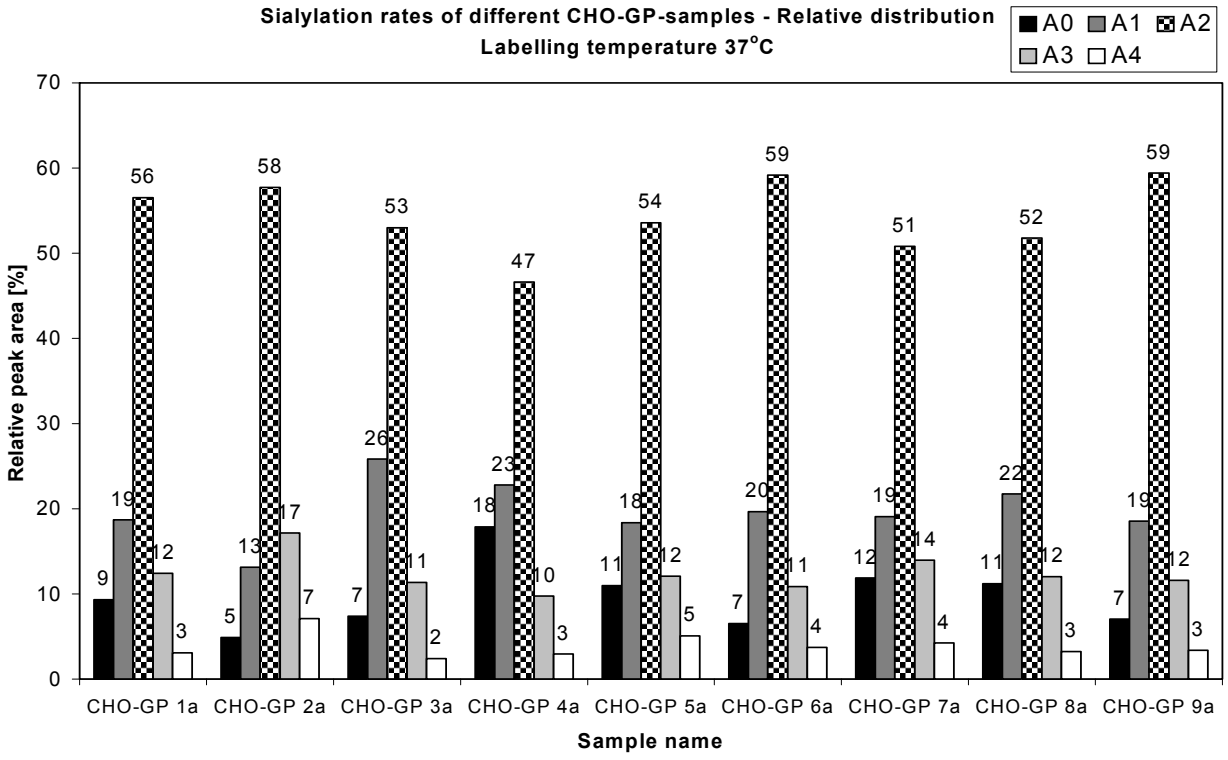


Figure 26: Labelling at 37°C

Procedure:

The lyophilized, concentrated oligosaccharide samples were mixed with a labelling solution, consisting of

- 30 mg 2-aminobenzamide
- 2100 µl dimethylsulfoxide
- 900 µl acetic acid
- 36 mg sodium cyanoborohydride

The factor 6 higher inserted amounts compared with the SIGNAL™ 2-AB labelling kit resulted from the fact that the best weighing machine at ProBioGen had a minimum initial weight of 30 mg.

Manufacturing of the labelling mixture:

- Addition of 900 µl acetic acid to 2100 µl DMSO
- Dissolving of 30 mg 2-aminobenzamide in 600 µl DMSO-acetic acid-mixture
- Complete dissolution of 36 mg sodium cyanoborohydride in the 2-AB-mixture
- Usage of the labelling mixture within 60 minutes

To each sample 10 µl of labelling mixture were added and this solution was centrifuged. An incubation for 16 hours at 37°C in a thermo controller followed.

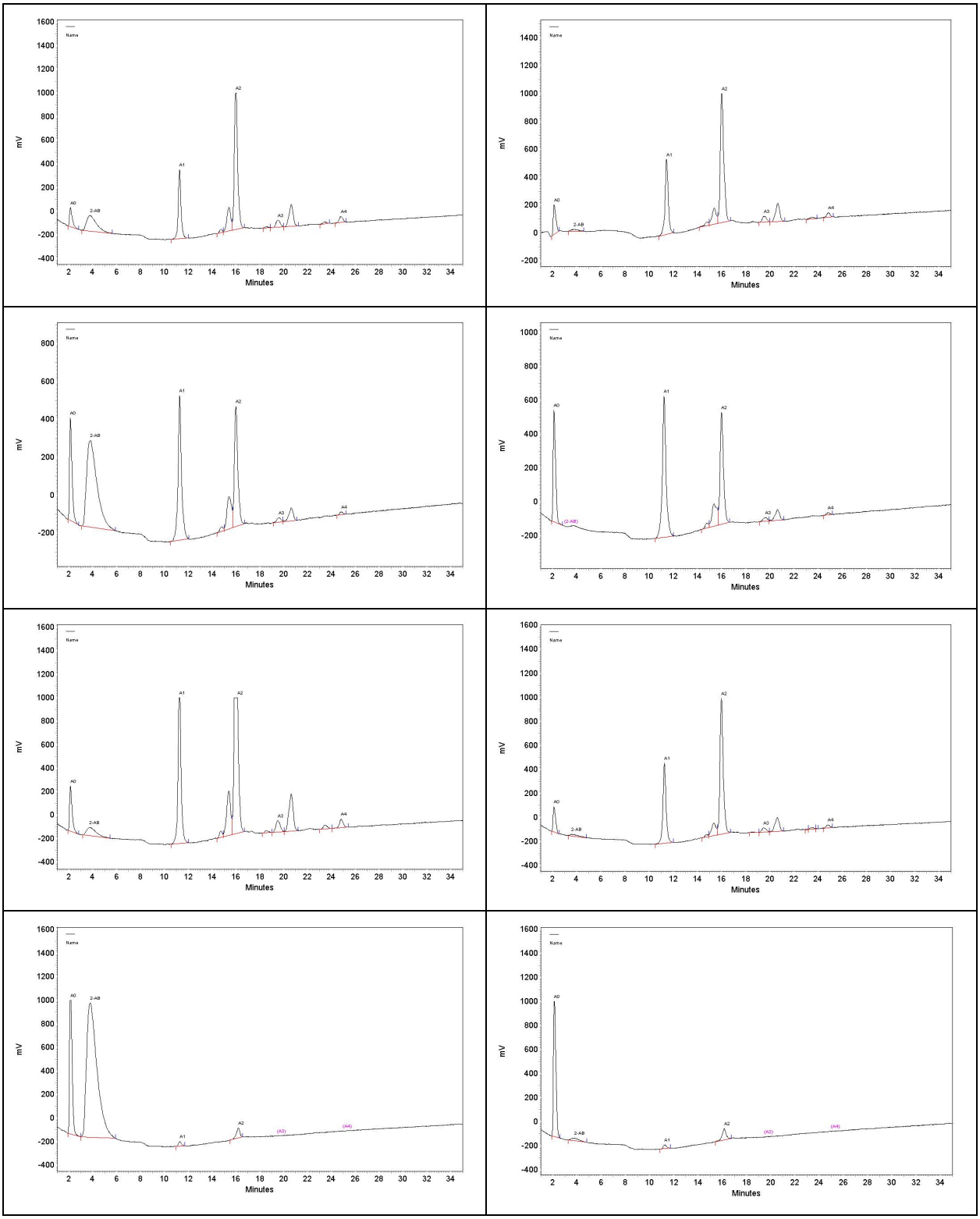
4.1.10 Purification of labelled oligosaccharides

After the incubation, the samples were centrifuged again. Separation of reagent excess was performed by paper chromatography. Each sample was halved in two equal parts à 5 µl and pipetted on paper stripes (2.5 cm x 8 cm). One part was termed a-sample and the other b-sample. The paper stripes were dried for one hour in darkness. After that, the paper stripes were chromatographed in a glass chamber with a mixture of 4 parts butanol, 1 part

ethanol and 1 part purified water as eluent. The glass chamber was filled with a volume of 15 ml. Now the paper stripes were put vertically in the glass chamber with the running direction against gravity. Run time was 30 minutes. After that, the chromatographed paper stripes were dried for another 30 minutes. This procedure was repeated once. After the paper stripes were completely dry, they were examined under a UV-lamp at 254 nm and the spot at the start point was cut out. To elute the 2-AB-marked oligosaccharides from the paper, the cut spots were put into a Luer-lock syringe with a Millex®-HV-filter screwed onto it. Now 1 ml purified water was filled into the syringes and the syringes were put in the darkness for 10 minutes. Then the syringes were put into 15 ml centrifuge tubes and were centrifuged at 3000 U/min for 5 minutes. The procedure was repeated for three times to wash out the oligosaccharides completely. At the end, there were 3 ml of filtrate in the tubes. Now the a-samples were transferred into 0.5 ml screwing vials and the b-samples into 0.2 ml PCR-tubes. The a-samples were concentrated with water to a volume of 100 µl and the b-samples to a volume of 30 µl.

Before the above procedure was established and standardized, an optimization approach was tried. Because of the relative difficult standardization of paper chromatography, Prozyme® GlycoClean™ S cartridges (Product Code: GKI-4726) were evaluated. The purification efficiency of the cartridges compared to paper chromatography was tested. Purification with the cartridges was performed as described in the manual of the manufacturer. N-glycans of four different samples were prepared under the same conditions except for the purification step after 2-AB-labelling. After purification, the samples were compared with GlycoSepC-chromatography. The loss of 2-AB-label-excess was the efficiency criteria for purification. The results can be seen in table 3.

Table 3: Chromatograms of four different purified samples[10]



[10] *left by GlycoClean™ S cartridge and right the corresponding samples by Whatman® paper chromatography*

As it became obvious from the above chromatograms, the purification efficiency of the paper chromatography regarding 2-AB-label excess was much better than that of the GlycoClean™ S cartridges. The 2-AB-label-peak was much higher after GlycoClean™ S purification in comparison to paper chromatography purification. Therefore, paper chromatography was established although it was harder to standardize.

4.1.11 Digestion of oligosaccharides with sialidase

Charged N-glycans often carry sialic acid residues at their end and belong to the group of acidic glycans as well as glycans with sulphate and phosphate residues. To separate these glycans by their antennary structure and their fucosylation level with the hydrophilic interaction chromatography, these glycans had to be desialylated by an enzymatic procedure. The resulting neutral glycans could then be used for measurements with the Aminophase-HPLC and MALDI-TOF-MS.

The b-samples were digested with sialidase. Therefore 10 µl sialidase buffer and 10 µl sialidase (= 0.1 units) were added to the solution. This mixture was vortexed, centrifuged and incubated for 37^{o}C in a thermo controller for 48 hours. One vessel of sialidase contained 1 unit sialidase in 100 µl, so in total 10 samples could be digested in parallel.

4.1.12 Mixed bed purification

To purify the neutral glycans from enzyme rests and buffer salts, they were chromatographed over a mixed bed consisting of 500 µl anion exchange resin (on the ground) and 500 µl cation exchange resin (on the top), filled in disposable 2 ml polystyrene columns with a frit. The frit of the column was flushed one time with purified water before the resins were pipetted onto the frit. The mixed bed was flushed with 5 x 2 ml purified water. After that, the sample was transferred onto the resins and the flow-through was collected in 15 ml centrifuge tubes. After chromatographing the sample over this mixed bed, the column was rinsed with 1 x 0.5 ml, 1 x 1 ml, 2 x 2 ml of purified water and the eluate was also collected. Afterwards, the eluate was

lyophilized to dryness. Then the sample was transferred to a 0.5 ml screwing vial and concentrated to a volume of 100 µl.

4.1.13 GlycoSepC-HPLC

For quantitative profiling of 2-AB-labelled glycans on the basis of charge, GlycoSepC-HPLC was used. The GlycoSepC-column consisted of a modified weak anion exchanger bound on a polymeric stationary phase with amine functionality. It offered optimized resolution of neutral, sialylated, sulfated or phosphorylated glycans into charge classes (AEX). After the 2-AB-labelled oligosaccharides were separated by the amount of negative charges, the glycans were detected by fluorescence.

Usually not more than 5 peak fractions occured in mammalian derived N-glycan analysis, fractions A0 - A4, which corresponded to the amount of bound sialic acid residues at the end of the oligosaccharide antennae.

From the prepared a-sample solution a 1% (v/v) dilution was prepared. From this dilution, 20 µl were injected for the HPLC-run.

Material:

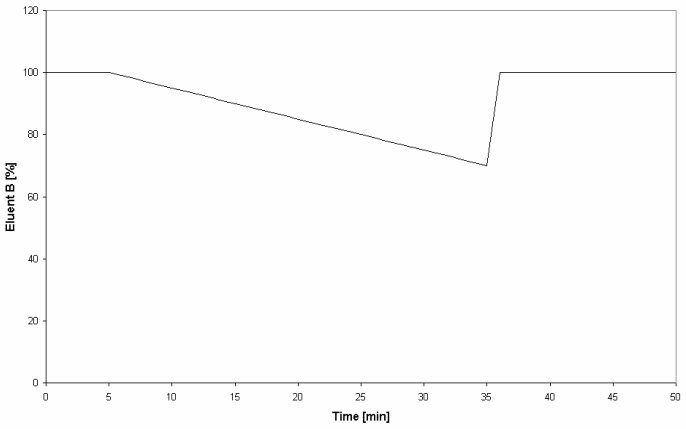
HPLC-device:	QC-HPLC
Software:	Class VP Version 6.12 SP 5
Eluent A:	500mM NH_4Ac pH 4.5 in 20% Acetonitrile (V/V)
Eluent B:	20% Acetonitrile (V/V)
Flow rate:	0.5 ml/min
Chromatography column:	GlycoSep™ C HPLC Column
Specifications:	
Base matrix:	5 µm polymer coated divinyl benzene resin
Derivatization:	DEAE weak anion exchanger

Column dimensions:	4.6 mm * 100 mm
Column volume:	1.7 ml
Typical flow rate:	0.3 – 0.5 ml/min
pH compatibility:	pH 1 to pH 13
Solvent compatibility:	Acetonitrile, methanol, isopropanol Avoid strong oxidants and anion detergents.
Typical buffers:	Water/acetonitrile with ammonium acetate or formate buffer
Column tube:	Stainless steel, Valco compatible end fittings
Maximum Pressure:	3000 psi ≈ 206 bar ≈ 20,6 MPA

Gradient program:

Time [min]	Eluent B [%]
0	100
0(*)	100
5	100
35	70
36	100
50	100

(*): Time of injection



QC-HPLC-settings:

RF-10AXL:

Wavelength:

Excitation wavelength: 330 nm

Emission wavelength: 420 nm

Sensitivity:

Gain: x4

Sensitivity: Medium

Response: 3

Data Acquisition:

Sampling Period: 500 msec

Run Time: 35 min

Oven temperature: 25^{o}C

Auto Injector:

Injection volume: 20 µl

Integration parameters (over the whole run time):

Width: 100

Slope: 25

The above method was named GlycoSepC.met. All following data regarding GlycoSepC-HPLC was based on this method.

4.1.14 Aminophase-HPLC

With the aminophase column, the uncharged N-glycans were separated by their polar interactions with the hydrophilic matrix in an acetonitrile-water-gradient due to their amount of antennae and fucose-residues. Thereby, biantennary glycans eluted at first, followed by triantennary and tetrantennary glycans. The fucosylated glycans eluted after the non-fucosylated glycans with the same antennarity. As external standard, the 2-AB-labelled neutral N-glycans from α_1-acid glycoprotein and from transferrin were used. The injected sample volume was always 20 µl, the 2-AB-labelled N-glycans were analyzed by fluorimetric detection. Identification of the oligosaccharides was performed by the external reference standard and additionally the results were confirmed by MALDI-TOF-MS.

The Aminophase-HPLC was a kind of HILIC. Neutral oligosaccharides were separated by the amount of their monomers in the way that larger molecules were more retented than smaller molecules.

From the prepared b-sample solution a 10% (v/v) dilution was prepared. From this dilution, 20 µl were injected for the HPLC-run.

<u>Material:</u>

HPLC-Device:	QC-HPLC
Eluent A:	15 mM NH_4Ac, pH 5.2
Eluent B:	25% (v/v) 15 mM NH_4Ac (pH 5.2), 75% (v/v) acetonitrile
Flow rate:	1.5 ml/min

Column: Phenomenex® Luna NH2 (4.6 x 150.0 mm, bead volume 3 µm, 100A)

Specifications:

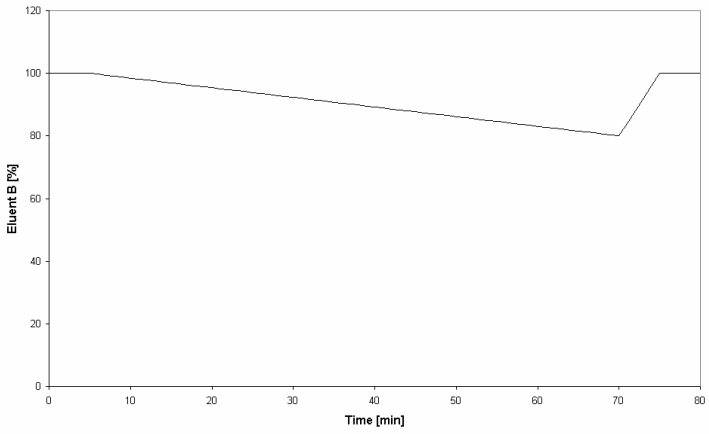
Base matrix:	3 µm spheric particles with a pore size of 100 A
Derivatization:	Endcapping with aminopropyl-groups

Column dimensions:	4.6 mm * 150 mm
Column volume:	2.5 ml
Typical flow rate:	1.5 ml/min
pH compatibility:	pH 1.5 to pH 11
Solvent compatibility:	Acetonitrile, hexane, ethanol, water Avoid strong oxidants and strong acids and bases.
Typical buffers:	Acetonitrile and ammonium acetate solution
Column tube:	Stainless steel, Valco compatible end fittings
Maximum Pressure:	30 MPA

Gradient program:

Time [min]	Eluent B [%]
0	100
5(*)	100
70	80
75	100
80	100

(*): Time of injection



QC-HPLC-settings:

RF-10AXL:

Wavelength:

Excitation wavelength: 330 nm

Emission wavelength: 420 nm

Sensitivity:

Gain: x4

Sensitivity: Medium

Response: 3

Data Acquisition:

Sampling Period: 500 msec

Run Time: 70 min

Oven temperature: 25^{o}C

Auto Injector:

Injection volume: 20 µl

Integration parameters (over the whole run time):

Width: 50

Slope: 5000

The above method was named Aminophase.met. All following data regarding Aminophase-HPLC was based on this method.

4.2 Additional methods

Additional methods were needed to identify unknown glycan structures, as well as to support the validation of the GlycoSepC- and Aminophase-analysis. Because of their character as assisting methods, no effort was undertaken for their validation.

4.2.1 Mass spectrometry analysis (MALDI-TOF-MS)

The matrix-assisted laser desorption ionisation - time of flight - mass spectrometry (MALDI-TOF-MS) allowed a mass determination of molecules to the picomol-level. It was possible to measure molecules up to a molecular weight of 500.000 Da. The MALDI-TOF-method used here worked in the positive ion mode. That was the reason why all samples had to be desialylated (uncharged) before measurement.

In general, it is also possible to measure sialylated (negatively charged) structures, too. Therefore the MS-device has to work in the negative ion mode. Sialylated structures get harder ionised out of the matrix so that the most common MALDI-methods work in the positive ion mode.

To guarantee the most sensitivity possible, the samples should have been desalted before measurement [124].

MALDI-TOF-MS-material:

Device:	Bruker® Biflex™
Matrix:	DHB (2,5-Dihydroxybenzoic acid) solved in ethanole/bistilled water (9:1)
Calibration substance:	Dextran-hydrolysate
Reflector voltage:	20 kV
Acceleration voltage:	19,5 kV

Procedure:

The sample was solved in bidistilled water (ca. 1-20 pmol oligosaccharide/µl) and mixed with the matrix 1:1. 0.5 - 1.0 µl of this sample mixture have been pipetted on the MALDI-target, a carrier plate made of stainless steel with high planarity. After 30 minutes, this mixture has been crystallized at room temperature because of vaporisation of the solvents. After that, this target was put into the high vacuum chamber of the MALDI-device.

Principle:

The sample molecules were desorpted by a nitrogen laser by support of the DHB-matrix, which was able to transfer energy to the molecules. In vacuum the molecules got ionised and built stable Na^+- and K^+-complexes. Now they were accelerated in an electrical field by a ring electrode. The flight direction of the sample molecules was changed in the drift tube by an electrical reflector field (reflectron mode). After passing this field, the molecules moved to the detector where the time of their flight was measured. From the flight-time, the unknown masses of the sample molecules could be calculated. In the Biflex™-device the drift tube had a length of 100 cm. Working in the reflectron mode, the total flight distance was 200 cm. Calibration took place by measuring substances with already known masses, in this case dextran-hydrolysate. In general, MALDI-TOF-MS is only a semi-quantitative method. This is because of different crystallisation behavior of different structures within the matrix. For quantification of the uncharged glycans, the aminophase-HPLC was used.

4.2.2 HPAEC-PAD monosaccharide analysis

Monosaccharide analysis was performed by High pH Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD).

4.2.2.1 Sample preparation for monosaccharide analysis

Material:

Hydrolytic reagent:	4 N Trifluoroacetic acid (TFA)
Vials:	Wheaton® 2.5 ml glass vials with teflon gaskets
Device:	Heating block

Procedure:

For the monosaccharide analysis with HPAEC-PAD, the oligosaccharide samples or whole glycoprotein samples were hydrolysed under aggressive acidic conditions. Therefore the desalted samples were solved in 200 µl of bidistilled water and 200 µl of 4 N TFA was added. Hydrolysis took place in 2.5 ml glass vials with teflon gaskets for 4 hours at 100°C in a heating block. After cooling down the samples to room temperature, an internal standard of 2 nmol fructose and 2 nmol desoxyribose was added to each vial. After that, the samples were concentrated to dryness in a SpeedVac™ to evaporate the volatile TFA. For complete removal of TFA, the samples were washed with 400 µl of bidistilled water and again concentrated in the SpeedVac™. Now the samples could be measured with HPAEC-PAD.

4.2.2.2 HPLC-analysis of monosaccharides

In connection with the above described acidic hydrolysis of oligosaccharides and glycoproteins, HPAEC-PAD can be used for the analysis of the qualitative and quantitative monosaccharide composition of glycans [144]. Thereby, monosaccharides flow through a measurement cell after leaving the chromatography column. In this measurement cell the alteration of the

current at a specific voltage in dependence of the monosaccharide flow is measured. The current changes because of oxidation or reduction of the monosaccharides in the eluate at the surface of an electrode. The pulsation of the applied potential leads to an increased sensitivity and reproducibility and avoids an electrochemical fouling of the electrode.

Material:

Method 1:

HPLC-device:	Dionex® Gradient Pump
	Dionex® Eluent Degassing Module
	Dionex® Addition Pump DQP1
	Dionex® PAD-Detector
	Jun Air Compressor
	Helium 4.6 Messer Griesheim
Software:	Dionex® PeakNet Version 5
Eluent A:	15 mM NaOH
Eluent B:	200 mM NaOH
Eluent C:	100 mM NaOH, 600 mM NaOAc
After-column-mixture:	500 mM NaOH
Flow rate:	1 ml/min
Column:	Dionex® CarboPac™ PA-1 (4.6 x 250 mm)
Pre-Column:	Aminotrap™ and CarboPac™ PA-1 pre-column (4,6 x 50 mm)
Auto injector:	Abimed 231
Injection volume:	50 µl

Gradient program:

Time [min]	Eluent A [%]	Eluent B [%]	Eluent C [%]
0(*)	100	0	0
25	100	0	0
26	0	0	100
36	0	0	100
37	0	100	0
47	0	100	0
48	100	0	0
60=Restart	100	0	0

(*): Time of injection

Method 2:

HPLC-device:	Dionex® Gradient Pump
	Dionex® Eluent Degassing Module
	Dionex® Addition Pump DQP1
	Dionex® PAD-Detector
	Jun Air Compressor
	Helium 4.6 Messer Griesheim
Software:	Dionex® PeakNet Version 5
Eluent A:	2,25 mM NaOH
Eluent B:	200 mM NaOH
Eluent C:	100 mM NaOH, 600 mM NaOAc
After-column-mixture:	500 mM NaOH
Flow rate:	1 ml/min
Column:	Dionex® CarboPac™ PA-1 (4.6 x 250 mm)

Pre-Column: Aminotrap™ and CarboPac™ PA-1 pre-column (4,6 x 50 mm)

Auto injector: Abimed 231

Injection volume: 50 µl

Gradient program:

Time [min]	Eluent A [%]	Eluent B [%]	Eluent C [%]
0(*)	100	0	0
50	100	0	0
51	0	0	100
61	0	0	100
62	0	100	0
72	0	100	0
73	100	0	0
90=Restart	100	0	0

(*): Time of injection

PAD-settings:

Sensitivity: 300 nA

Integration: 0.00 - 540 ms

E1 = 10 mV t1 = 540 ms

E2 = 60 mV t2 = 60 ms

E3 = -60 mV t3 = 60 ms

Standard monosaccharide mixture:

2-Deoxyribose:	2 nmol
D-glucosamine:	1 nmol
D-fructose:	2 nmol
D-galactosamine:	1 nmol
D-glucose:	1 nmol
D-mannose:	1 nmol
L-fucose:	1 nmol
D-galactose:	1 nmol

Procedure:

For each HPLC-run 50 µl were injected by the auto injector. Each sample was determined double. After 5 samples, the external standard with known composition was injected. As internal standard 2 nmol of fucose and 2 nmol of desoxyribose, each of them added to the samples after the acidic hydrolysis, were used. The internal standards were necessary to avoid misinterpretations because of variations of the retention times of the peaks. The internal standards had a retention time before and after the examined monosaccharides so that no disturbing interference took place. With the internal standards relative retention coefficients (r_c) could be calculated [140, 145].

$$r_c = \frac{t_{Rx} \cdot (p_2 - p_1) + p_1 t_{R2} - p_2 t_{R1}}{t_{R2} - t_{R1}}$$

$r_c = relative\ retention\ coefficient$
$t_{Rx} = retention\ time\ of\ component\ x$
$p_1 = first\ standard\ retention\ coefficient$
$p_2 = second\ standard\ retention\ coefficient$
$t_{R1} = retention\ time\ of\ first\ standard$
$t_{R2} = retention\ time\ of\ second\ standard$

The retention coefficients of the two standards were arbitrary. For calculations in this study, 1 was chosen for p_1 and 2 was chosen for p_2.

For the quantitative determination of monosaccharides, the internal standard 2-deoxyribose was used to calculate response factors (r_f) of the different monosaccharides.

$$r_f = \frac{A_{St} \cdot C_S}{C_{St} \cdot A_S}$$

$r_f = response\ factor$
$A_{St} = s\tan dard\ area$
$C_{St} = s\tan dard\ concentration$
$A_S = sample\ area$
$C_S = sample\ concentration$

In this study two different methods for monosaccharide separation were used. This was due to the fact that it was not possible to resolute all monosaccharides in a quality that quantification of them was possible with only one gradient. The relative retention coefficient profiles showed that two methods were necessary to resolute all monosaccharides completely (Figure 27). Therefore a gradient with 15 mM NaOH and a gradient with 2.25 mM NaOH were established.

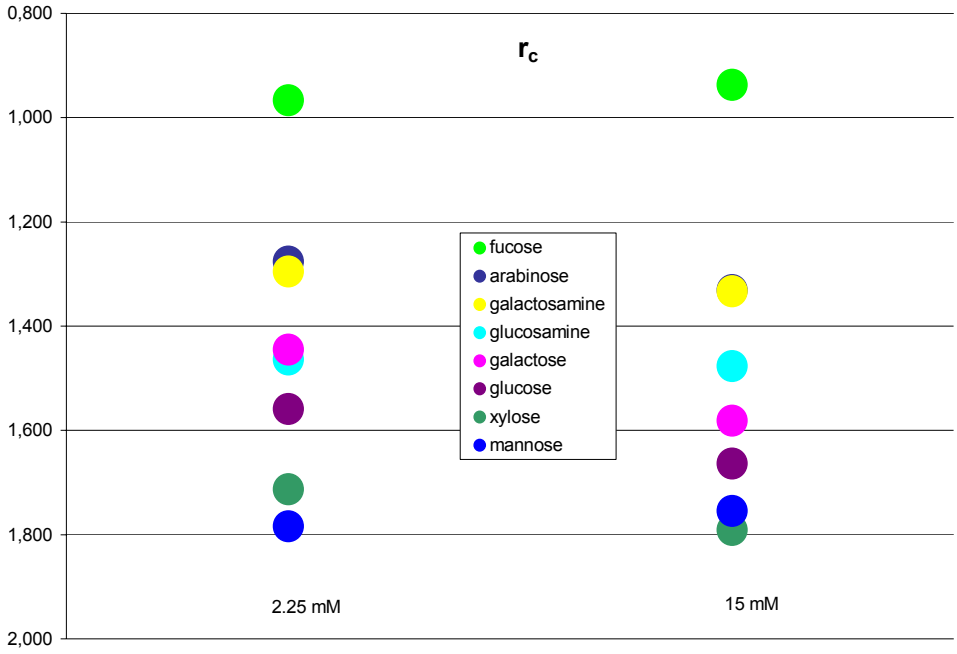
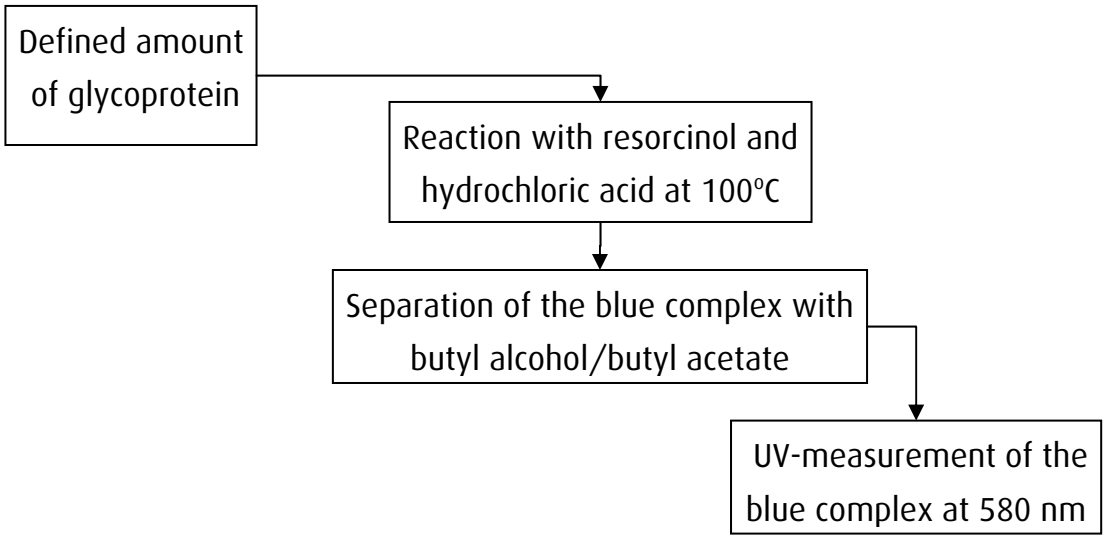


Figure 27: Profile of relative retention coefficients of different monosaccharides in HPAEC-PAD-analysis with two gradients

4.2.3 Colorimetric method of Svennerholm

For the absolute quantification of sialic acids of a glycoprotein sample (mol sialic acids / mol glycoprotein), a colorimetric method based on the monograph 1316 - concentrated Erythropoietin solution Ph. Eur. was established and slightly modified (scheme below).



Material:

Glass test tubes:

12 x 100 mm, Schott, Product-No. 261301101

Glass test tube plugs:

Sigma-Aldrich, Product-No. Z100730

Glass chamber with silicon oil M100:

Roth, Product-No. 4025.1

Resorcinol:

Aldrich, Product-No. 398047

Copper(II)-sulfate:

Sigma, Product-No. C7631

Hydrochloric acid:

VWR Merck, Product-No. 1.00317.1000

Butyl acetate:

Aldrich, Product-No. 27068-7

Butyl alcohol:

VWR Merck, Product-No. 8222622500

Purified water:

Milli-Q

N-acetyl-neuraminic acid:

Fluka, Product-No. 01398

Heater

Photometer:

Amersham Biosciences, Ultrospec 4300 pro (qualified)

Cuvettes:

1.8 ml volume, 10 mm optical layer thickness

Lyophilisator:

ISMATEC, Ser. No. 215106

Producing of reagents and preparation of the sample:

As reagents resorcinol reagent R and a mixture of butyl alcohol / butyl acetate (1:4, v/v) were needed.

Resorcinol reagent R was produced of 80 ml hydrochloric acid R1, 10 ml resorcinol solution R and 0.25 ml copper(II)-sulfate solution R as described in the Ph. Eur..

Hydrochloric acid R1 (c = 250 g/l HCl) was produced of 70 g concentrated hydrochloric acid and was diluted with purified water to 100 ml. Resorcinol solution R was an aqueous solution with 20 g/l resorcinol. Copper(II)-sulfate solution was also an aqueous solution with a concentration of 25 g/l.

The resorcinol reagent R had to be produced at least 4 hours before usage and was storable for one week at 2-8^{o}C.

A calibration curve was created of an aqueous parent solution with a concentration of 0.3 mg/ml (0.97 µmol/ml) N-acetyl-neuraminic acid (M_R = 309 g/mol). This parent solution was now diluted to the following concentrations: 0.26 mg/ml (0.84 µmol/ml), 0.22 mg/ml (0.71 µmol/ml), 0.18 mg/ml (0.58 µmol/ml), 0.14 mg/ml (0.45 µmol/ml), 0.10 mg/ml (0.32 µmol/ml), 0.06 mg/ml (0.19 µmol/ml), 0.02 mg/ml (0.06 µmol/ml) and 0.00 mg/ml (0.00 µmol/ml) (Figure 28).

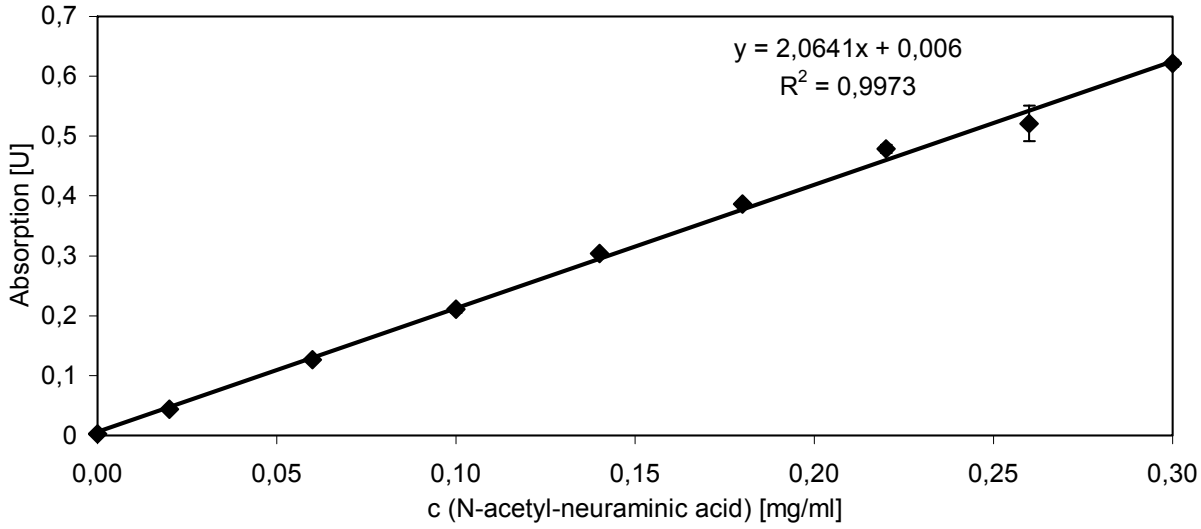


Figure 28: Calibration curve of the colorimetric sialic acid determination by Svennerholm

The concentration of the measured glycoprotein sample was so defined, that the resulting sialic acid concentration was within the calibration curve. The calculated amount of glycoprotein was then lyophilized and afterwards dissolved in 100 µl water.

Performing the colorimetric reaction:

A triple repetition of each sample including standards was performed. Every sample and standard solution was filled in 12x100 mm glass test tubes and 1.0 ml resorcinol reagent R was added. After sealing the glass test tubes with plugs, they were incubated in a glass chamber of silicon oil standing on a heating plate at 100^{o}C for 30 minutes. After incubation, the samples were cooled down in crushed ice. After that, 2.0 ml of a butyl alcohol butyl acetate mixture (1:4, v/v) were added to the samples. Both phases were energetically mixed and were stood still until phase separation took place. The above phase contained the blue coloured complex which was created during the reaction. 1 ml of the above phase were pipetted in the cuvettes and absorption was measured at 580 nm by the photometer.

Evaluation of results:

By usage of the calibration curve, the amount of sialic acid per sample was determined and the average of three measurements was calculated. The calculated amount of N-acetyl-neuraminic acid was also representative for N-glycolyl-neuraminic acid because the absorptions of the coloured complexes of both acids were nearly identical at 580 nm [146-148].

4.3 Data evaluation

There were two possible ways to interpret the resulting chromatograms produced by GSC- and AP-chromatography. The first was to normalize all peaks to 100% and then to quantify all peak areas relative to each other. The second approach was to calculate the absolute peak areas of all peaks and to quantify all peaks absolute to each other.

4.3.1 Relative quantification of oligosaccharides

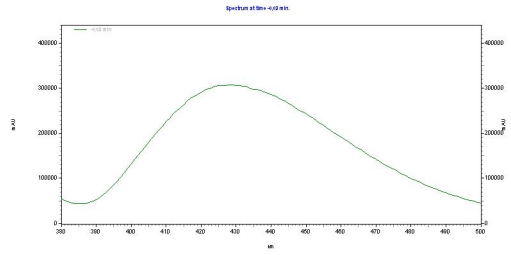
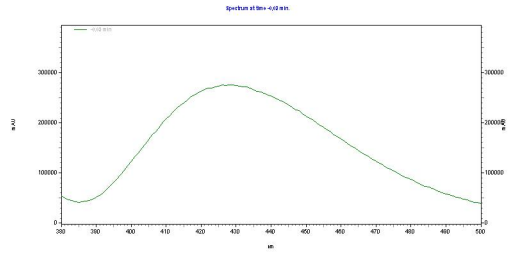
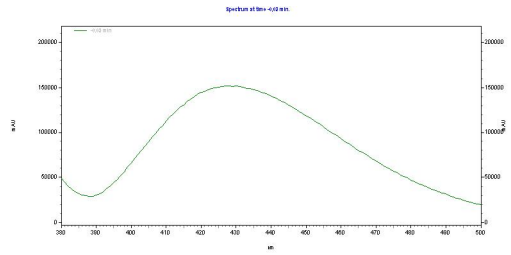
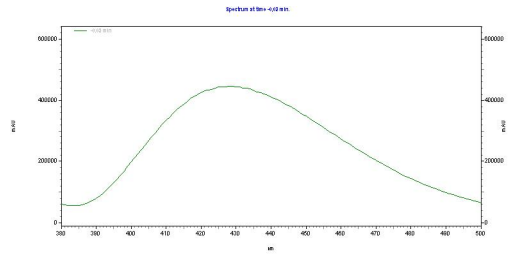
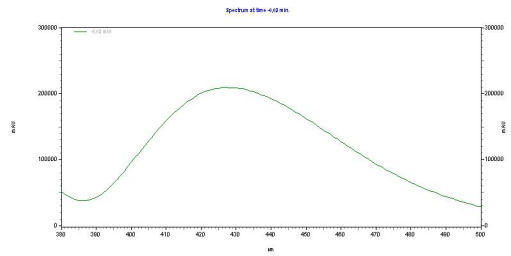
Before relative quantification could be performed, some crucial aspects of the method had to be evaluated, e.g. if a selective loss of oligosaccharides occurred during sample preparation and if the response factors of all labelled

oligosaccharides were comparable. Therefore a recovery study and a response factor determination of oligosaccharides were performed. The second critical point was the reproducibility of the results. Therefore a five time repetition of a GlycoSepC- and an Aminophase-analysis was performed.

4.3.1.1 Determination of response factors

An important criteria that this method really worked, was the examination of the response factors of each glycan fraction. Therefore emission spectra of the different GlycoSepC-peaks of CHO-GP-N-glycans were examined (Table 4). Emission spectra were taken during normal HPLC runs. When a peak occured, the pumps were stopped and the emission spectra were taken at comparable peak intensities. The spectrum scans were performed between 300 nm - 500 nm. The wavelength with the maximal emission (λ_{max}) was calculated from 380 nm - 500 nm and is shown in the right column in the table below (Table 4). Excitation took place at 330 nm.

Table 4: Emission spectra of CHO-GP-GlycoSepC-peaks (A0 - A4)

	CHO-GP-A0: λ_{max} = 428 nm
	CHO-GP-A1: λ_{max} = 428 nm
	CHO-GP-A2: λ_{max} = 428 nm
	CHO-GP-A3: λ_{max} = 428 nm
	CHO-GP-A4: λ_{max} = 428 nm

As result, it became obvious that all labelled oligosaccharides had the same maximal emission wavelength and therefore the same response factors. The above method of glycan quantification was correct. For electrochemical detection systems (PAD, PED, etc.), this would not have been possible because of different responses of the analytes at the electrochemical detector [107].

4.3.1.2 Determination of recovery

To determine the recovery of the oligosaccharides during the sample preparation procedure, several methods were used including UV-spectroscopy, the sialic acid determination by Svennerholm and HPAEC-PAD-monosaccharide analysis. The combination of the different methods was necessary because not all methods were able to quantify the glycans in each status of the preparation procedure (Figure 29).

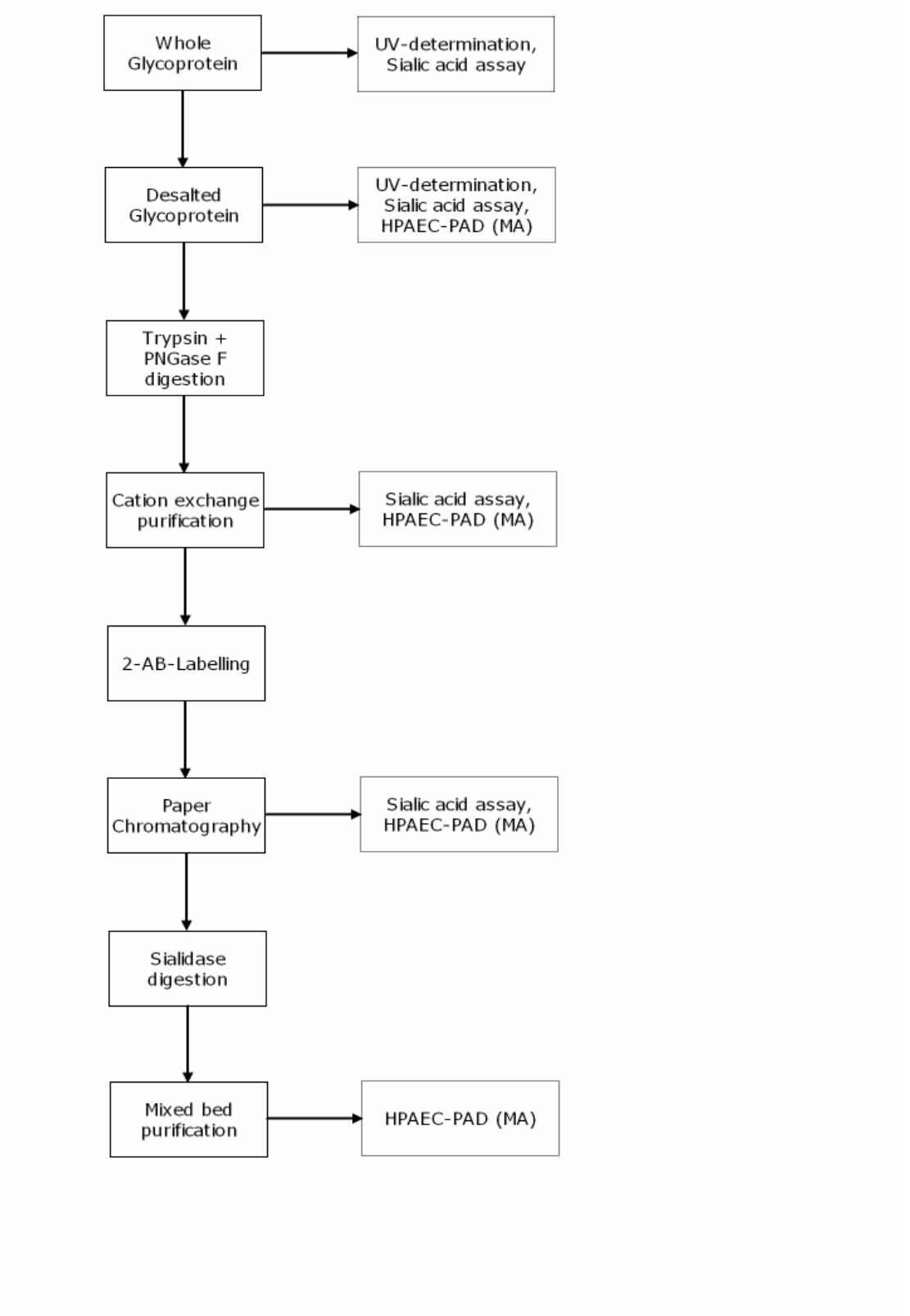


Figure 29: Scheme for the recovery determination during the glycoanalytical sample preparation[11]

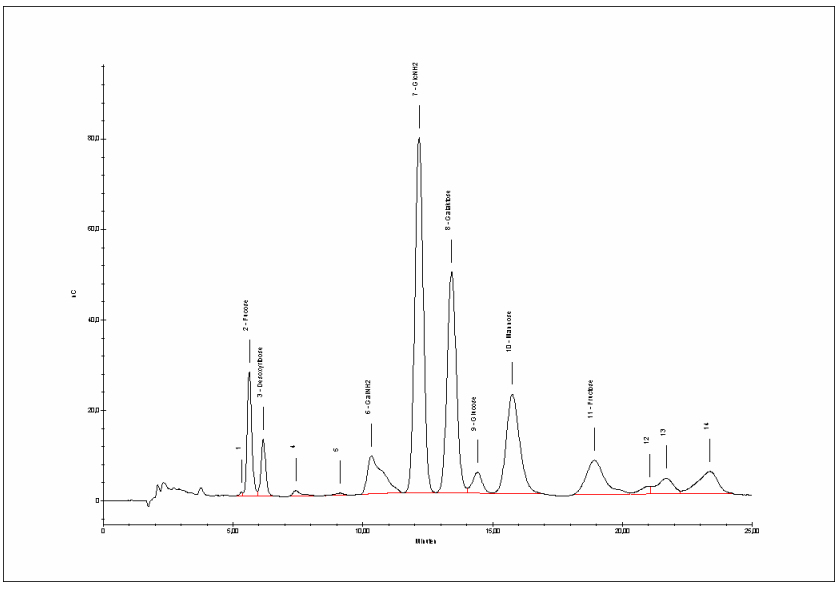
[11] *HPAEC-PAD (MA) = HPAEC-PAD monosaccharide analysis*

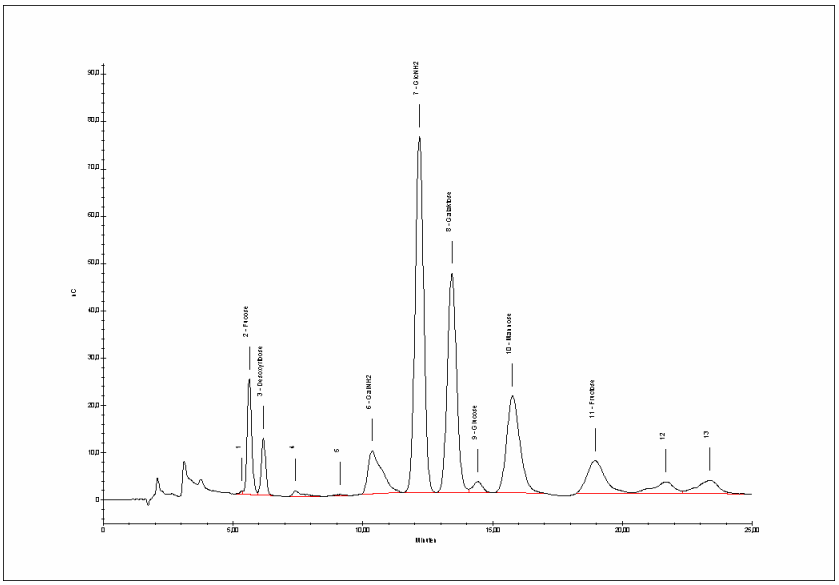
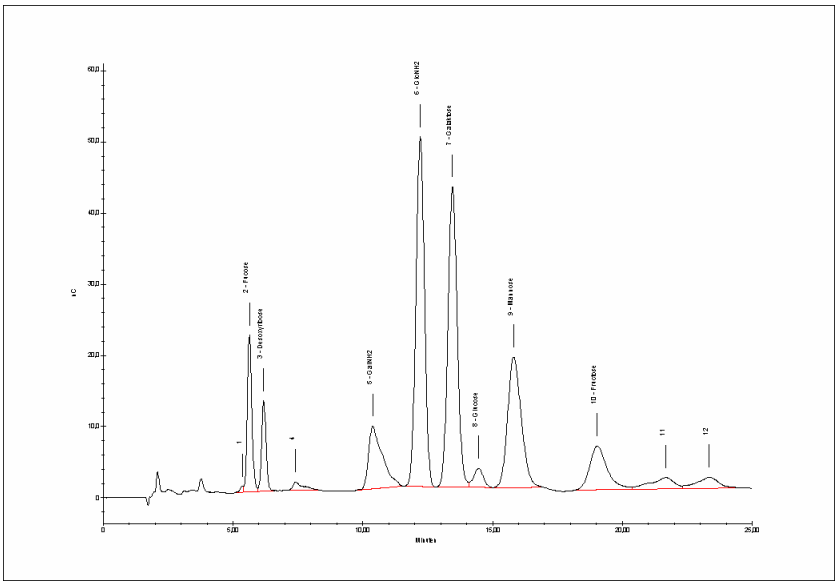
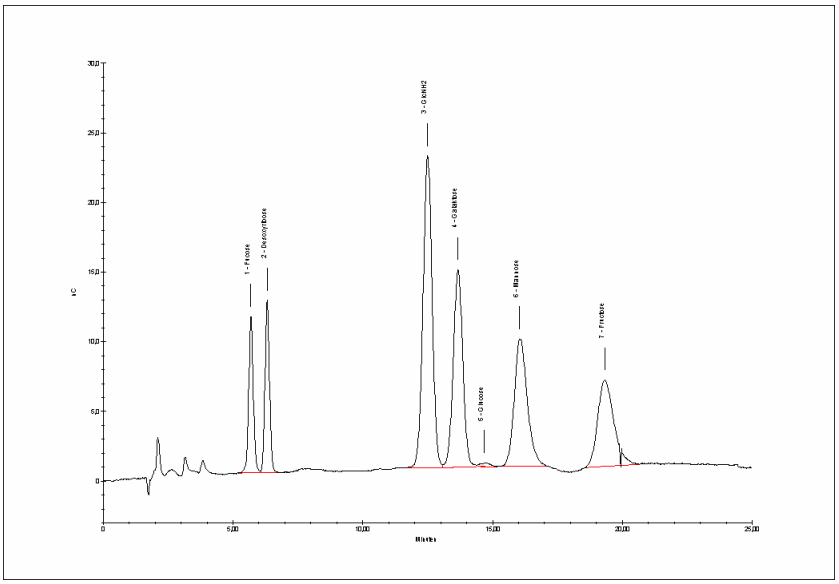
For the desalting step, the recovery of the whole glycoprotein could be calculated by measuring UV-absorptions at 280 nm. Values were calculated by averaging three measurements.

Untreated glycoprotein [AU]:	**Desalted glycoprotein [AU]:**	**Recovery [%]:**
0.427	0.369	87

HPAEC-PAD monosaccharide analyses are shown below for each of the preparation steps (Table 5). For calculating the recovery for the prepared N-glycans, the fucose peak area was chosen for quantification. This monosaccharide is primarily present in CHO-N-glycans as core-fucose and not in the average CHO-O-glycan-structure (further explanations below). Three repetitions of each HPLC-run were performed. The HPAEC-PAD-MA had a RSD of 1% and was therefore the most precise of the three methods.

Table 5: Chromatograms of HPAEC-PAD monosaccharide analyses at different times during sample preparation

	Desalted glycoprotein (A)

	After trypsin and PNGase F digestion (B)
	After 2-AB-Labelling (C)
	After sialidase digestion (D)

The SA-assay data were recorded as UV-absorptions of the coloured Svennerholm-complex and together with the protein determination and the HPAEC-PAD-analyses, recovery values were calculated.

The results of this study are shown below (Figure 30).

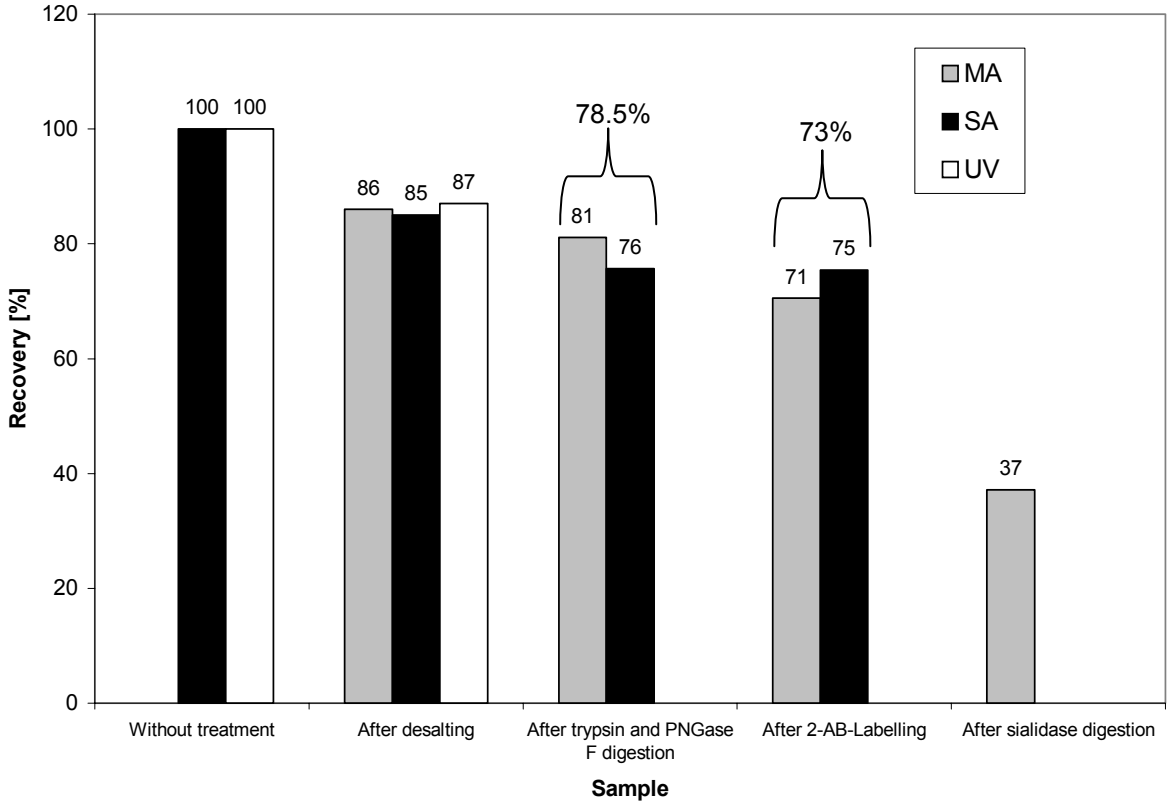


Figure 30: Total loss of oligosaccharides during sample preparation[12]

As mentioned in the Pharmacopoiea, the method of Svennerholm has an estimated relative standard deviation of 10%. Considering this low precision when comparing the results of the different methods, the recovery values confirmed each other. As it becomes obvious from the data above, the highest loss of oligosaccarides occured after the sialidase digestion and in the mixed bed purification (Loss of 48% = 100% - 37/71*100%; loss factor of 1.9 = 71%/37%).

[12] UV-analysis (UV), Sialic acid assay (SA) and HPAEC-PAD monosaccharide analysis (MA)

The above results could also be used to find out if any selective losses occurred during the sample preparation procedure. From step A to C GalNAc was found in the chromatograms. This was due to O-glycans from CHO-GP. Regarding the sample preparation procedure, the O-glycans should have been separated from the N-glycans in the cation-exchange purification step because they were not cleaved in the PNGase F-digestion and were still bound on peptide-residues. Within the cation exchange purification step, the peptide residues should have been bound to the cation exchange resin at pH 3-4. But because of special CHO-GP-protein characteristics (IEP = 3; lots of acidic, charged peptides after trypsin digestion), this did not work properly and the O-glycopeptides stayed with the N-glycans. Just in step 4, the anion exchange resin from the mixed bed could separate the negatively charged O-glycopeptides from the neutral N-glycans, so that GalNAc (1 mol) could not be found any more. 1 mol galactose also decreased in step four significantly. Imaging the average O-glycan structure -GalNAc-Gal-SA of mammalian cells [149], this is understandable. The apparent loss of 1 mol GlcNH2 in step C could be explained by the labelling procedure. The 2-aminobenzamide was bound to the reducing end of 1 mol GlcNAc. After acidic hydrolysis, the resulting 2-AB-GlcNH2 could not react electrochemically at the PAD-detector, because no reducing aldehyde group was available. Examining the monosacharide ratios of the individual preparation steps, it could be shown that no selective loss of monosaccharides occured (Table 6). The average structure of CHO-GP was a triantennary complex structure, confirming the GlycoSepC-, the Aminophase- and the MALDI-TOF-MS-results (Figure 31).

Table 6: Examination of selective losses with monosaccharide ratios

Monosaccharide ratio	Sample preparation step
Average N-glycan structure: Mannose 3.00 ≈ 3 GlcNH2 5.14 ≈ 5 Fucose 1.26 ≈ 1 Galactose 2.94 ≈ 3 Average O-glycan structure: Galactose 1.00 ≈ 1 GalNAc 0.77 ≈ 1	Desalted glycoprotein (A)
Average N-glycan structure: Mannose 3.00 ≈ 3 GlcNH2 5.56 ≈ 5 Fucose 1.25 ≈ 1 Galactose 3.16 ≈ 3 Average O-glycan structure: Galactose 1.00 ≈ 1 GalNAc 0.85 ≈ 1	After trypsin and PNGase F digestion (B)
Average N-glycan structure: Mannose 3.00 ≈ 3 GlcNH2 3.85 ≈ 4 Fucose 1.17 ≈ 1 Galactose 2.99 ≈ 3 Average O-glycan structure: Galactose 1.00 ≈ 1 GalNAc 0.85 ≈ 1	After 2-AB-Labelling (C)
Average N-glycan structure: Mannose 3.00 ≈ 3 GlcNH2 4.02 ≈ 4 Fucose 1.29 ≈ 1 Galactose 2.92 ≈ 3 Average O-glycan structure: Galactose 0.00 ≈ 0 GalNAc 0.00 ≈ 0	After sialidase digestion (D)

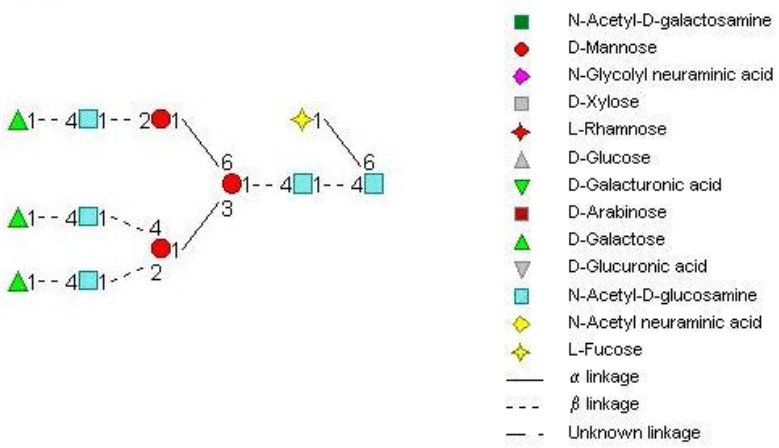


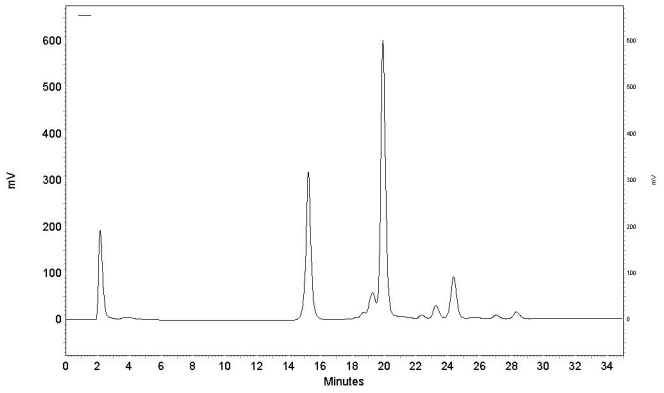
Figure 31: Average CHO-GP-N-glycan structure based on HPAEC-PAD monosaccharide analyses

No unexplainable selective loss of oligosaccharides could be detected. Relative quantification was possible.

4.3.1.3 Reproducibility of the analytical methods

Reproducibility of the methods was examined by performing a five time repetition of GlycoSepC- and Aminophase-analysis with CHO-GP as reference protein on one concentration level (Table 7 + 8).

Table 7: Five time repetition of GlycoSepC-analysis

	CHO-GP-sample: GSC-1

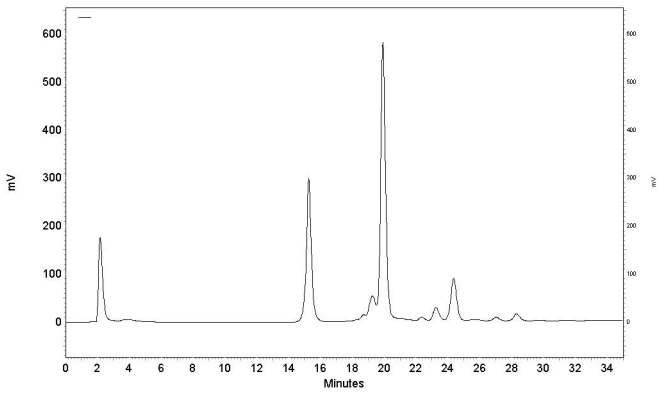
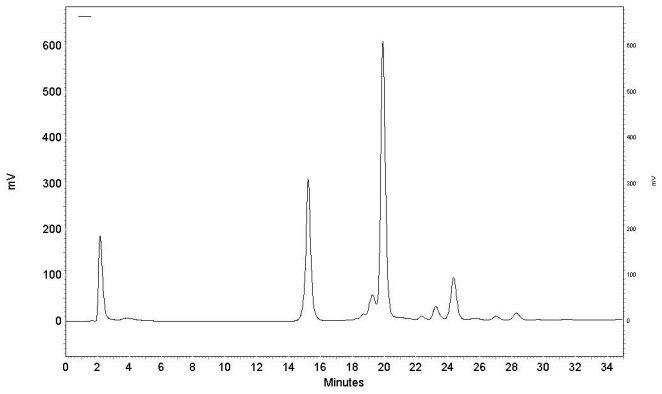
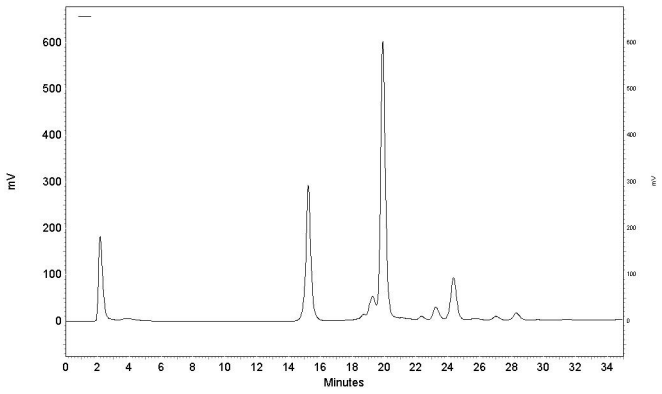
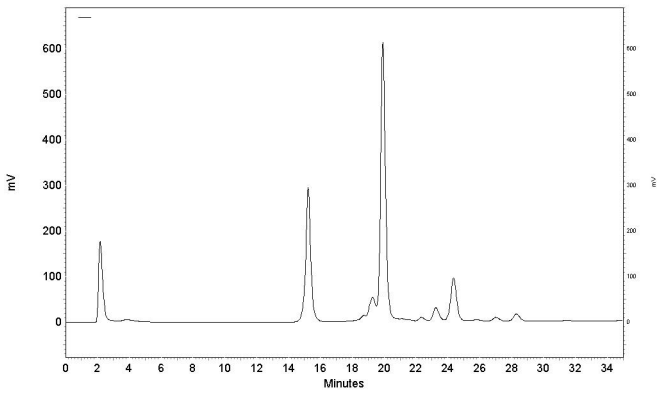
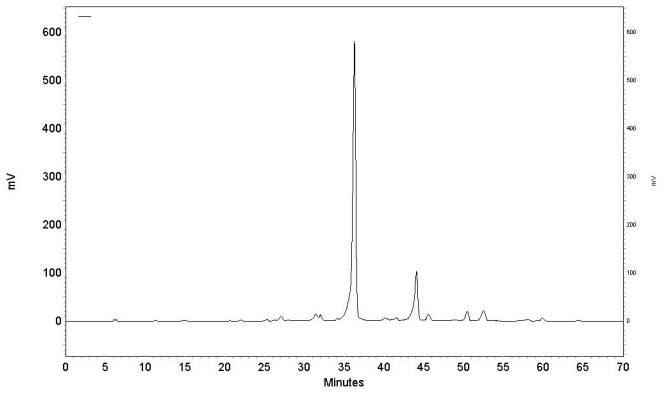
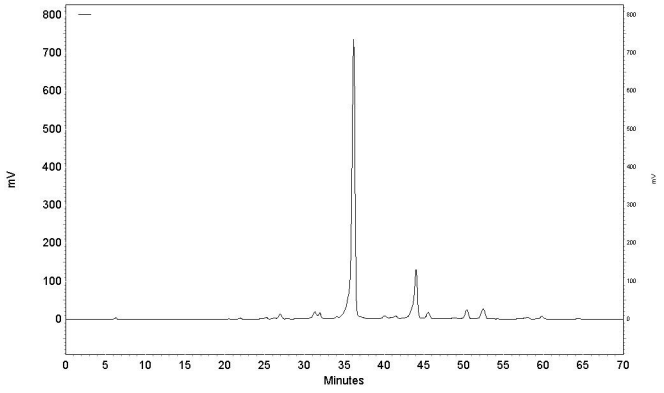
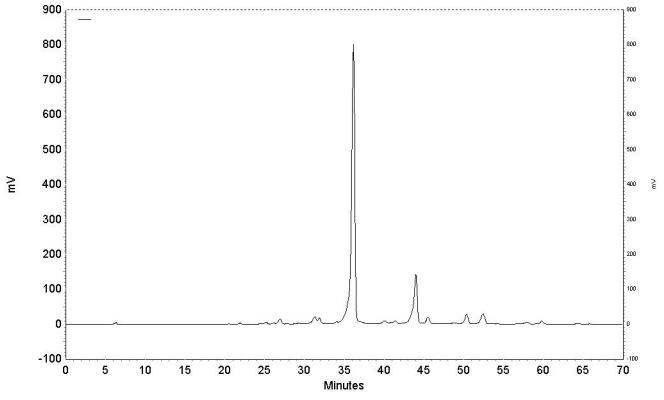
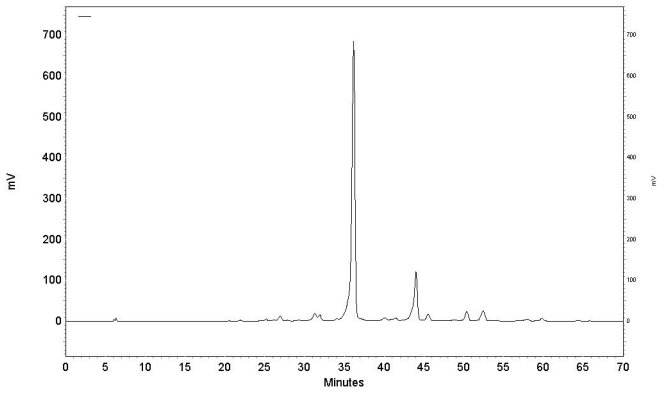
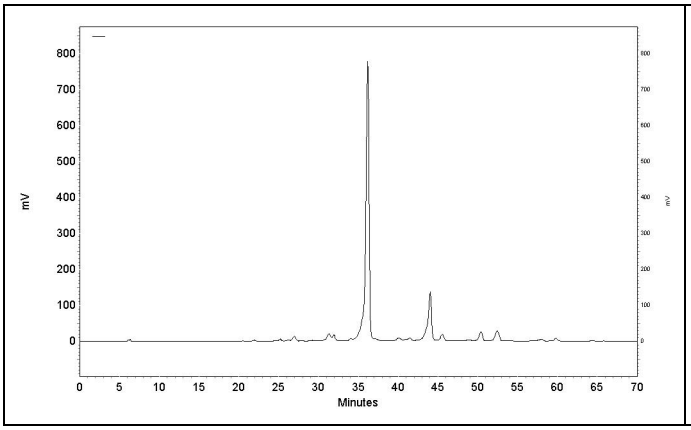
	CHO-GP-sample: GSC-2
	CHO-GP-sample: GSC-3
	CHO-GP-sample: GSC-4
	CHO-GP-sample: GSC-5

Table 8: Five time repetition of Aminophase-analysis

	CHO-GP-sample: AP-1
	CHO-GP-sample: AP-2
	CHO-GP-sample: AP-3
	CHO-GP-sample: AP-4

	CHO-GP-sample: AP-5

As it got obvious from the results above, both methods seemed to have good reproducibility. Due to the good resolution of the peaks, integration of the chromatograms could be performed without any problems.

Because of these good method characteristics, this analytical approach was chosen for the regular batch-consistency quality control at ProBioGen.

4.3.2 Strategies for absolute quantification of oligosaccharides

For absolute quantification, it was necessary to determine the loss of oligosaccharides within the analytical scheme (further details above).

4.3.2.1 Absolute quantification of charged oligosaccharides by combination of methods (GlycoSepC-HPLC and Svennerholm's method)

As mentioned in the introduction, besides the analyses of the microheterogeneity of glycan structures, it is also very important to get information about the macroheterogeneity of glycoproteins. Therefore, the methods GlycoSepC-HPLC and the colorimetric method by Svennerholm described above were combined. By evaluating relative peak areas of GlycoSepC-chromatograms and total sialic acid amounts from the Svennerholm method, it was now possible to calculate an estimation of the total amount of glycans per mol glycoprotein. Because of the restriction to N-glycans within the sample preparation procedure, an important assumption

for this absolute quantification approach was that the glycoprotein contains only N- and no O-glycosylation. In this study, the two model proteins CHO-GP and CHO-Mab were not practical for this kind of analysis. CHO-GP was also an O-glycosylated protein and CHO-Mab was very low sialylated. So a theoretical approach is shown here.

Estimates for a glycoprotein:

- glycosylation sites: 5
- Svennerholm results: 8.49 mol sialic acid/mol protein
- GlycoSepC results:

A0	A1	A2	A3	A4	∑
0.073	**0.211**	**0.541**	**0.135**	**0.041**	**1**

Result of GlycoSepC-HPLC (relative quantification without A0):

A1	A2	A3	A4	∑
0.228	**0.583**	**0.145**	**0.043**	**1**

That was an average distribution of sialylated structures on each glycosylation site. Combined with the Svennerholm result and this average distribution, the total amount of SA-glycan (sialylated glycan) [mol] per glycoprotein [mol] could be calculated.

$$8.49\left[\frac{mol\ SA}{mol\ protein}\right] = 4\left[\frac{mol\ SA}{mol\ SA-glycan}\right] * 0.043 * x\left[\frac{mol\ SA-glycan}{protein}\right] +$$
$$3\left[\frac{mol\ SA}{mol\ SA-glycan}\right] * 0.145 * x\left[\frac{mol\ SA-glycan}{protein}\right] +$$
$$2\left[\frac{mol\ SA}{mol\ SA-glycan}\right] * 0.583 * x\left[\frac{mol\ SA-glycan}{protein}\right] +$$
$$1\left[\frac{mol\ SA}{mol\ SA-glycan}\right] * 0.228 * x\left[\frac{mol\ SA-glycan}{protein}\right]$$

$$\Leftrightarrow 8.49\left[\frac{mol\ SA}{mol\ protein}\right] = 2.001 * x\left[\frac{mol\ SA}{mol\ protein}\right]$$

$$\Leftrightarrow \underline{\underline{x = 4.24}}$$

As we knew now the total amount of sialylated glycan per mol glycoprotein, it was possible to quantify the neutral fraction (A0) in mol NSA-glycan (not-sialylated glycan) per mol protein by regarding the A0-fraction of the GlycoSepC-chromatogram. This was further correlated with the total amount of sialylated glycan per mol glycoprotein.

$$\frac{0.073}{(1-0.073)} = \frac{x}{4.24}$$

$$\Leftrightarrow x = 4.24 * \frac{0.073}{0.927}$$

$$\Leftrightarrow \underline{\underline{x = 0.33}}$$

The total amount of glycan per mol glycoprotein was then:

$$x\left[\frac{mol\ glycan}{mol\ protein}\right] = 4.24\left[\frac{mol\ SA-glycan}{mol\ protein}\right] + 0.33\left[\frac{mol\ NSA-glycan}{mol\ protein}\right]$$

$$\underline{\underline{= 4.57\left[\frac{mol\ glycan}{mol\ protein}\right]}}$$

Together with the knowledge of the available glycosylation sites of the glycoprotein, it was now possible to calculate the glycosylation site occupancy of the protein.

$$x[\%\ site\ occupancy] = \frac{Found\ amount\ of\ glycan / mol\ protein}{Theoretical\ amount\ of\ glycan / mol\ protein}$$

$$= \frac{4.57\left[\frac{mol\ glycan}{mol\ protein}\right]}{5.00\left[\frac{mol\ glycan}{mol\ protein}\right]} = \underline{\underline{91.4\%}}$$

In the example above, a glycosylation site occupancy of 91.4% would be the result.

4.3.2.2 Absolute quantification by use of an external standard

Because of the result that the labelled oligosaccharides had all the same response factors (see 4.3.1.1), it was investigated if it would be possible to find an external standard substance which could be used for absolute quantification. The external standard substance had to fulfill the following requirements:

- Access to weighable amounts of the substance
- Good characterized substance (certificate of a manufacturer)
- Same response factors as the oligosaccharide analytes (λ_{max} = 428 nm)

Because of the very difficult access to weighable amounts of natural occurring N-glycans (in most cases N-glycans are produced by fractionation and lyophilisation in µg amounts; www.prozyme.com, www.ludger.com), different mono- and disaccharides and the label 2-aminobenzamide itself were evaluated.

Material:

2-Aminobenzamide:

Sigma, Product-No. A8,980-4

Glucose:

Dextrose, anhydrous, granular, EP, BP, USP, JP, Product-No. D-8066, SIGMA

Lactose:

β-Lactose, minimum 99% total lactose, Product-No. L3750, SIGMA

Maltose:

Maltose-monohydrate, >99% purity, Product-No. 1.05912.0025, VWR Merck

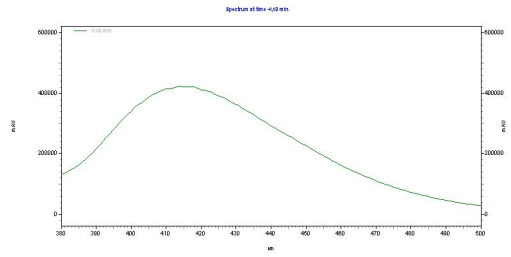
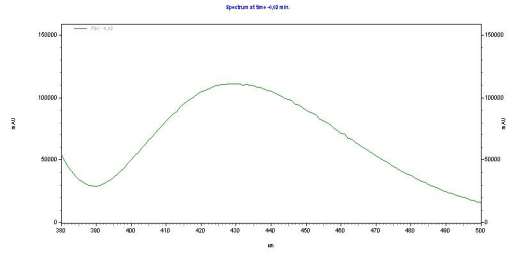
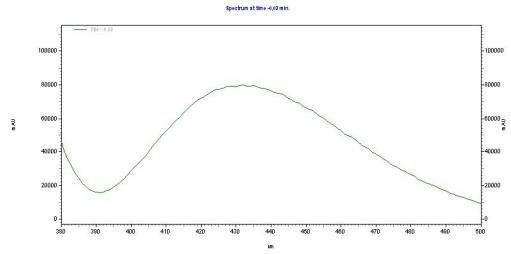
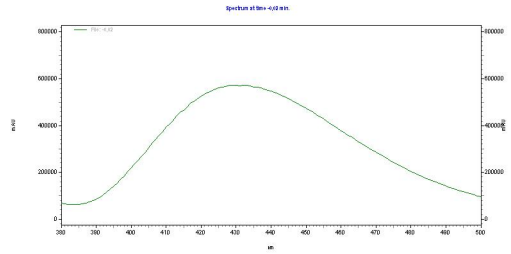
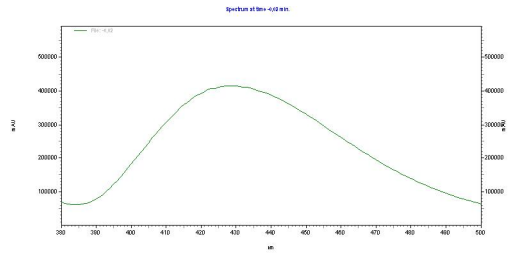
N-Acetyl-glucosamine:

N-acetyl-D-glucosamine, minimum 99% powder, Product-No. A8625, SIGMA

Method:

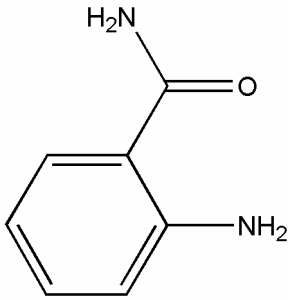
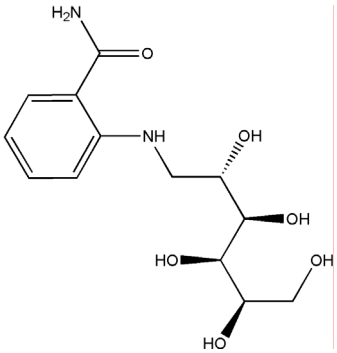
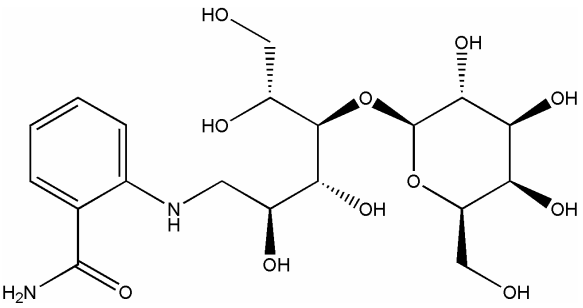
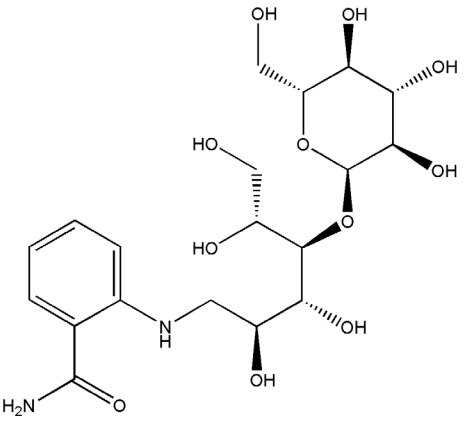
Solutions of ca. 1 mg/ml of the substances above were produced, lyophilized and labelled with 2-aminobenzamide. Labelling excess was separated by paper chromatography. After elution from the paper stripes, samples were lyophilized and individually diluted to get an optimal response at the detector. Resulting emission spectra are shown in Table 9.

Table 9: Emission spectra of 2-AB and its labelled saccharides to find an external standard

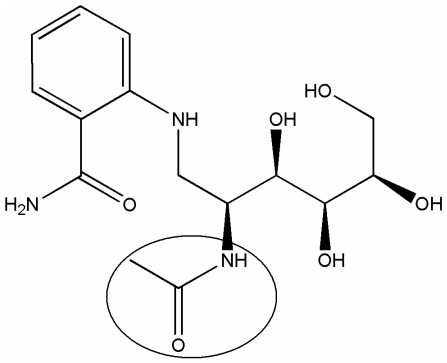
	2-AB: λ_{max} = 414 nm
	2-AB-glucose: λ_{max} = 430 nm
	2-AB-lactose: λ_{max} = 432 nm
	2-AB-maltose: λ_{max} = 432 nm
	**2-AB-GlcNAc:** **λ_{max} = 428 nm**

As it became obvious from the results above, there was only one substance which met all of the requirements above. This was N-acetyl-D-glucosamine. Although N-acetyl-glucosamine is quite similar to the other tested saccharides regarding its structure, the major difference between them lays in the N-acetyl-substitution in position 2 (Table 10).

Table 10: Structures of 2-aminobenzamide and labelled saccharides and the corresponding λ_{max}[13]

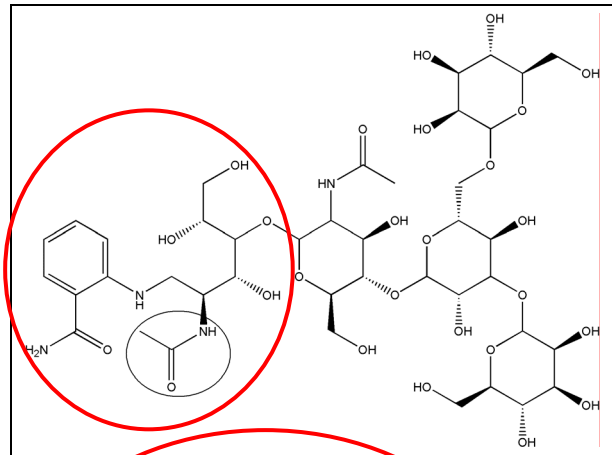
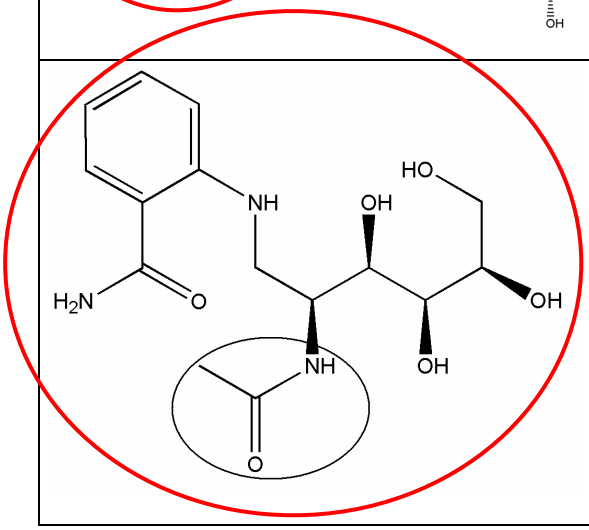
	2-AB: λ_{max} = 414 nm
	2-AB-glucose: λ_{max} = 430 nm
	2-AB-lactose: λ_{max} = 432 nm
	2-AB-maltose: λ_{max} = 432 nm

[13] *black circle = N-acetyl-group*

	**2-AB-GlcNAc:** **λ_{max} = 428 nm**

When the labelled core-N-glycan is compared with 2-AB-GlcNAc, the structural similarities of both molecules become obvious (Table 11).

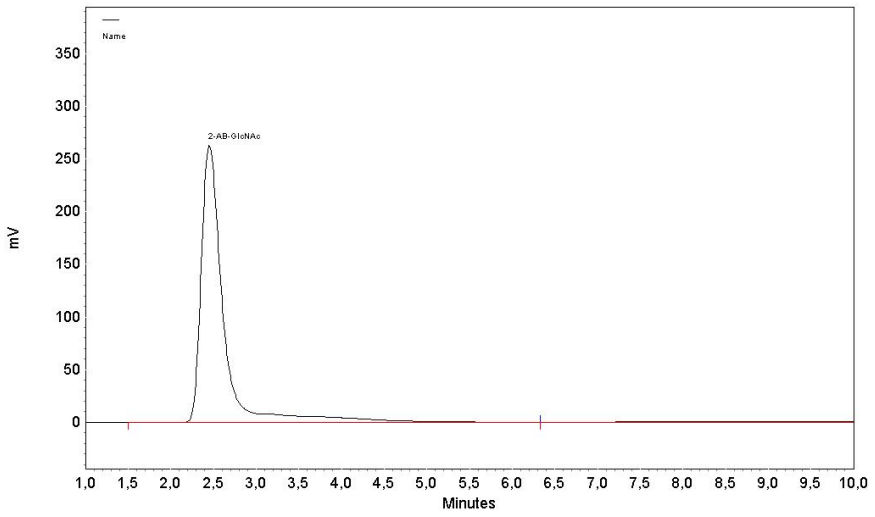
Table 11: Structural similarities between 2-AB-Core and 2-AB-GlcNAc[14]

	**2-AB-Core**
	**2-AB-GlcNAc**

[14] *red circle = same structural components*

For quantification with the external standard a calibration curve was created (Table 12 and Figure 32). As method GlycoSepC.met was used to be able to quantify glycan fractions directly from GlycoSepC-chromatograms.

Table 12: Chromatogram of 2-AB-GlcNAc for a calibration curve

	2-AB-GlcNAc: 16.5 pmol

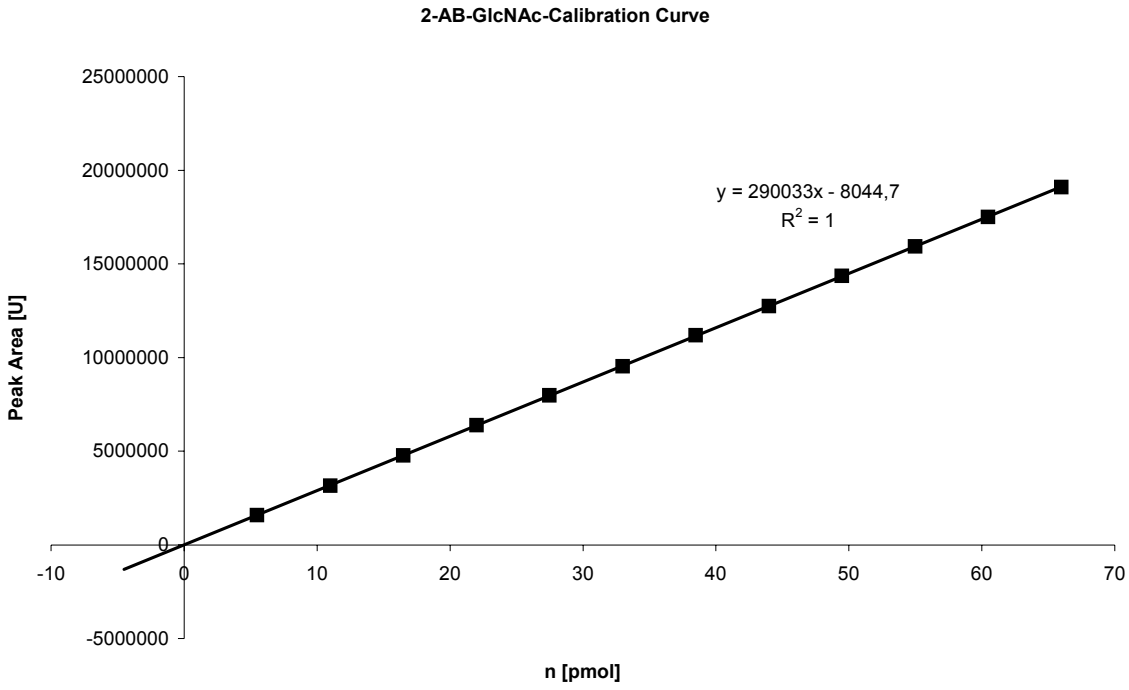
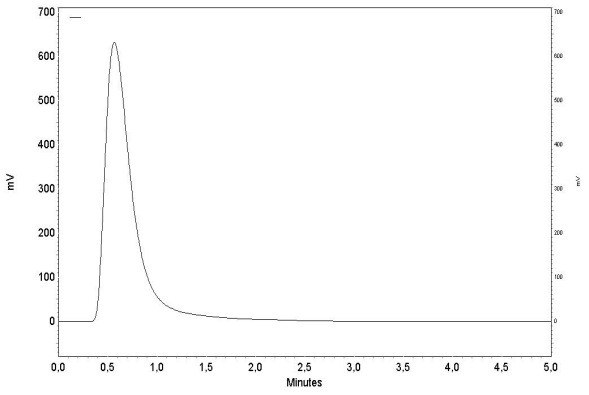
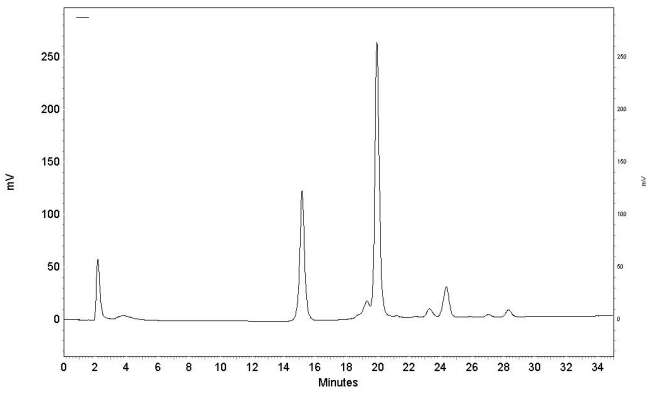


Figure 32: Absolute quantification by 2-AB-GlcNAc-calibration curve

4.3.2.2.1 Absolute quantification of charged N-glycans

For the calculation of an unknown amount of N-bound oligosaccharides on a glycoprotein, some correction factors had to be implemented in the calculations. The first resulted from the loss of 2-AB-GlcNAc in the paper chromatography purification step for the separation of 2-AB-label excess, the second was due to the GlycoSepC-column loss during sample analysis and the third was the loss during sample preparation of the glycoprotein before its glycans were 2-AB-labelled. The loss in paper chromatography was examined in the recovery study together with the loss in trypsin and PNGase F digestion as well as the cation exchange purification step (see also 4.3.1.2 Determination of recovery). Based on these results, a loss of 7.5% for paper chromatography was estimated. The column loss was examined by a five time repetition run of a CHO-GP-sample without and with the GlycoSepC-column. The total peak area was calculated and the difference between the two runs represented the column loss (Table 13). The average GlycoSepC-column loss was calculated to be 3.3% (loss factor = 100%/96.7% = 1.03).

Table 13: GlycoSepC-column loss determination

HPLC-run without column:	HPLC-run with column:
	

The third discussed loss factor (sample-preparation-loss-factor) was calculated to be 100% / 73% = 1.37 (see also 4.3.1.2).

Using the general linear equation

$$y = b \times x + a\,,$$

the following formula was now used to calculate the absolute amount of glycans

$$x = \frac{y - a}{b} \times f \times g \times h\,,$$

where the variables were defined as

$$\begin{aligned}
&x = amount\ of\ \ glycan\ in\ \ pmol \\
&y = \ peak\ area\ in\ units \\
&b = slope\ of\ \ the\ calibration\ curve \\
&a = y - \mathrm{int}\, ercept \\
&f \ = \ paper - chromatography - \ factor = 0.93 = 73\% / 78.5\% \\
&g = column - loss - \ factor = 1.03 \\
&h = sample - \ preparation - loss - \ factor = 1.37
\end{aligned}$$

.

The calculation above could be simplified to

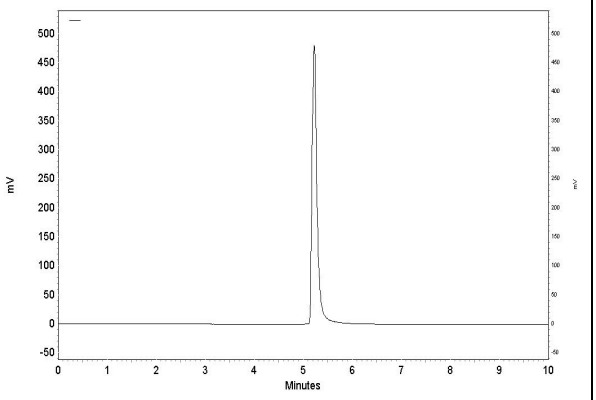
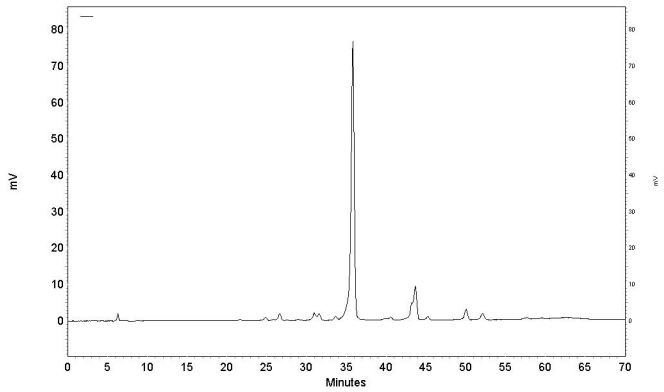
$$x = \frac{y - a}{b} \times 1.3$$

4.3.2.2.2 Absolute quantification of uncharged N-glycans

To establish an absolute quantification of the uncharged glycan structures, the following approach could be applied. The 2-AB-GlcNAc GlycoSepC-calibration curve was used. But the loss during sialidase digestion, mixed bed purification and Aminophase-HPLC had also to be considered in the calculations. Therefore the sample-preparation-loss-factor increased from 1.37 to 100% / 37% = 2.70.

The column loss was also important for the Aminophase-HPLC. The average loss was 2.5% (five time repetition) (Table 14) and the resulting correction factor therefore 1.03, too.

Table 14: Aminophase-column loss determination

HPLC-run without column:	HPLC-run with column:
	

The equation for calculating an unknown concentration of oligosaccharides by an external standard could now be adapted to

$$x = \frac{y-a}{b} \times f \times g \times h\,,$$

where the variables were defined as

$$\begin{aligned}
x &= amount\ of\ \ glycan\ in\ \ pmol \\
y &= \ peak\ \ area\ in\ units \\
b &= slope\ of\ \ the\ calibration\ curve \\
a &= y - \mathrm{int}\, ercept \\
f &= \ paper - chromatography - \ factor = 0.925 \\
g &= column - loss - \ factor = 1.03 \\
h &= sample - \ preparation - loss - \ factor = 2.70
\end{aligned}$$

.

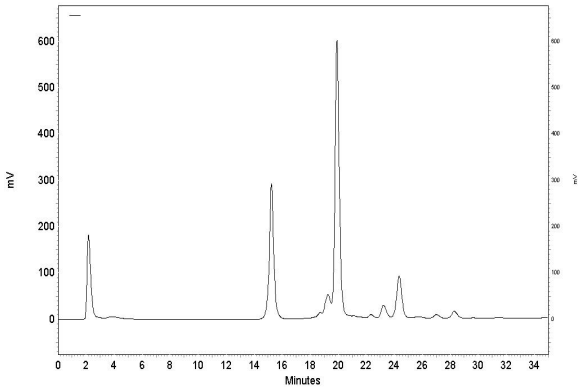
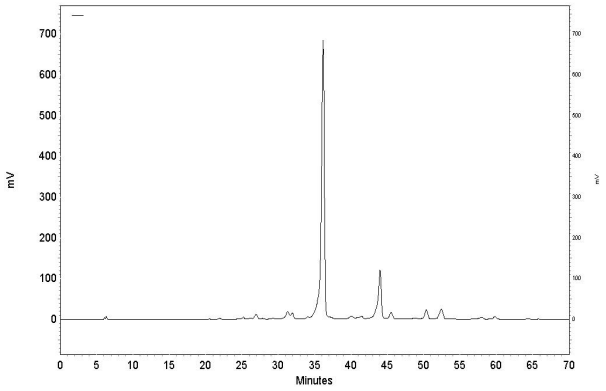
The equation above could then be simplified to

$$x = \frac{y-a}{b} \times 2.6\,.$$

There was one critical aspect which had to be examined more detailed. When the total peak areas of the GlycoSepC-chromatograms were compared with the total peak areas of the Aminophase-runs (Table 15), it was expected that the Aminophase-chromatograms showed five times higher total peak

areas than the GlycoSepC-chromatograms. This was due to the fact that the injected sample amount for Aminophase-runs contained half of the oligosaccharides from GlycoSepC-analysis and a 10% solution instead of the 1% solution for GlycoSepC-analysis. An average loss factor of 4.8 (five time repetition) was therefore calculated. Compared to the recovery study by HPAEC-PAD-monosaccharide analysis, this was a recovery rate of 21% instead of 52% (see Figure 30) calculated by HPAEC-PAD.

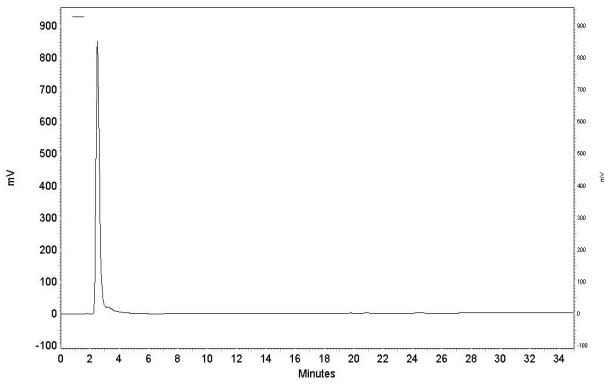
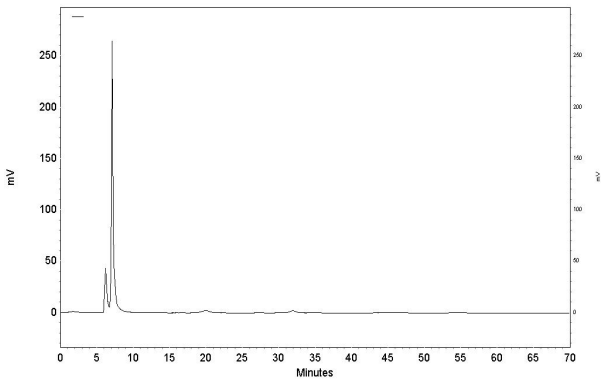
Table 15: Loss determination during Aminophase-analysis-sample-preparation[15]

	
Found total peak area [AU]: 30514047	Found total peak area [AU]: 31213110 Expected peak area [AU]: 152570235 Factor: 4.8

This observation could be explained by the different flow rates of the two HPLC-methods. When the same concentration of 2-AB-GlcNAc was injected in a GlycoSepC-HPLC-run and in an Aminophase-HPLC-run, the peak intensity (total peak area) in the Aminophase-run was 2.9 times lower than that in the GlycoSepC-run. This explained exactly the difference of the two measured loss factors (4.8 - 1.9 = 2.9) (Table 16).

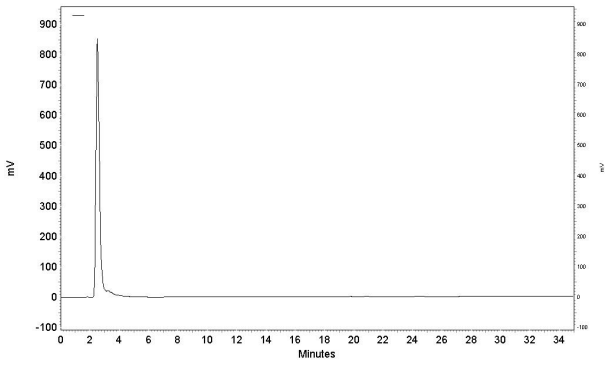
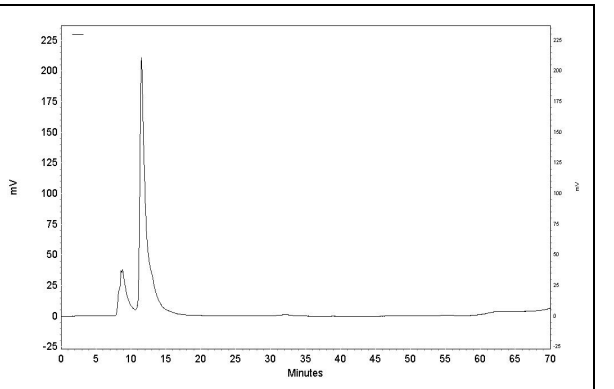
[15] *Injected Aminophase-samples were concentrated five times higher than the GlycoSepC-samples*

Table 16: Measurement of the same amount of 2-AB-GlcNAc with GlycoSepC- and Aminophase-HPLC[16]

	
<u>GlycoSepC-flow rate:</u> 0.5 ml/min Total Peak Area [AU]: 15365217	<u>Aminophase-flow rate:</u> 1.5 ml/min Total Peak Area [AU]: 5251004 Area factor: 2.9

This was due to the flow rate that was three times higher in the Aminophase-HPLC (1.5 ml/min versus 0.5 ml/min) compared to the GlycoSepC-HPLC. Detection time was three times shorter and so the total peak area decreased to a third. When the Aminophase-flow rate was reduced to 0.5 ml/min, total peak areas were nearly equal (Table 17). Differences resulted from integration variability of the peaks.

Table 17: Same amount of 2-AB-GlcNAc with GSC- and AP-HPLC

	
<u>GlycoSepC-flow rate:</u> 0.5 ml/min Total Peak Area [AU]: 15365217	<u>Aminophase-flow rate:</u> 0.5 ml/min Total Peak Area [AU]: 15791764

[16] *Total peak area was strongly influenced by the flow rate of the HPLC-method.*