

Release and analysis of O- and N-linked oligosaccharides from glycoproteins

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Molecular Biotechnology Programme

Uppsala University School of Engineering

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| UPTEC X 07 067 | | Date of issue 2008-01-11 | |
| Author Robert Söderlund | | | |
| Title (English) Release and analysis of O- and N-linked oligosaccharides from glycoproteins | | | |
| Title (Swedish) | | | |
| Abstract Post-translational addition of oligosaccharides is known to modulate the activity, half-life and immunogenicity of proteins. O-linked oligosaccharides were released from glycoproteins by three different forms of alkaline beta-elimination. The released O-glycans were successfully analyzed by high pH anion-exchange chromatography with pulsed amperometric detection, with two column types together providing full coverage of the known range of O-glycan structural diversity. N-linked glycans were released with peptide-N-glycosidase F, derivatized with 9-aminopyrene-1,4,6-trisulfonate and analyzed by capillary electrophoresis with laser induced fluorescence detection. Sequential enzymatic and chemical release of glycans enabled a full O- and N-glycan profile to be derived from the same glycoprotein starting material. | | | |
| Keywords O-glycan, N-glycan, alkaline beta-elimination, HPAEC-PAD, CE-LIF | | | |
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| Project name | | Sponsors | |
| Language English | | Security | |
| ISSN 1401-2138 | | Classification | |
| Supplementary bibliographical information | | Pages 26 | |
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Sammanfattning

Glykaner är kolhydrater som kopplas till förbestämda aminosyror i vissa proteiner via kväve (N-länkade glykaner) eller syre (O-länkade glykaner). Hur många glykaner och vilka typer av glykanstrukturer som kopplas på har visat sig ha stor påverkan på ett proteins funktion och hur länge det kan förbli aktivt t.ex. i blodomloppet. Immunförsvaret kan även reagera på kroppsfrämmande glykaner eller nakna proteinytor som normalt är dolda av kolhydratkedjor. Det är därför viktigt att utveckla metoder för att analysera glykaninnehållet i proteinmaterial för att bättre förstå proteinfunktioner och för att kunna garantera effektiviteten och säkerheten hos proteinläkemedel.

I det här projektet klyvdes N-länkade glykaner loss från protein med hjälp av ett enzym som specifikt katalyserar just denna reaktion. De enzymklyvda glykanerna försågs med en negativt laddad markörmolekyl (APTS) som fluorescerar då den belyses med laserljus, vilket innebar att de kunde separeras och analyseras genom kapillärelektrofores med laserinducerad fluorescens-detektion (CE-LIF).

Eftersom det inte finns något motsvarande enzym för O-länkade glykaner klyvdes dessa loss från proteiner med kemiska metoder som utnyttjar att bindningen mellan glykan och protein är instabil i svagt basiska miljöer. Olika klyvningsstrategier på detta tema har utvecklats och jämförts, bäst resultat uppnåddes med natriumhydroxid som bas och natriumborhydrid för att reducera och därmed skydda de frisatta glykanerna. Nackdelen med denna metod är att de reducerade glykanerna inte kan märkas med den markörmolekyl som möjliggör CE-LIF. En analysmetod för frisatta O-glykaner utvecklades därför baserad på jonbyteskromatografi vid högt pH med pulsamperometrisk detektion (HPAEC-PAD). Genom en kombination av två kolonntyper täcker denna kromatografiteknik det breda spektrum av former som O-glykaner kan anta.

Examensarbete 20p

Civilingenjörsprogrammet Molekylär bioteknik

Uppsala Universitet januari 2007

Contents

| | | |
|----------|---|-----------|
| 1 | Introduction | 2 |
| 1.1 | Glycosylation | 2 |
| 1.1.1 | Basic nomenclature and structure | 2 |
| 1.1.2 | Biological significance | 4 |
| 1.2 | Strategies for analyzing glycosylation | 4 |
| 1.2.1 | Capillary electrophoresis | 5 |
| 1.2.2 | High pH anion exchange chromatography | 5 |
| 2 | Objectives | 6 |
| 3 | Materials and methods | 6 |
| 3.1 | Chemicals | 6 |
| 3.2 | Model proteins | 6 |
| 3.2.1 | κ -casein from bovine milk | 6 |
| 3.2.2 | Bovine fetal serum fetuin | 7 |
| 3.2.3 | Coagulation factor IX from human serum | 7 |
| 3.3 | Glycan release | 7 |
| 3.3.1 | Enzymatic release of N-glycans | 7 |
| 3.3.2 | Chemical release of O- and N-glycans | 7 |
| 3.4 | Glycan modifications | 9 |
| 3.4.1 | Reduction of standards and non-reduced released glycans | 9 |
| 3.4.2 | APTS labelling | 9 |
| 3.4.3 | Enzymatic removal of neuraminic acid | 9 |
| 3.5 | Analysis | 10 |
| 3.5.1 | HPAEC-PAD analysis | 10 |
| 3.5.2 | CE-LIF analysis | 10 |
| 4 | Results | 10 |
| 4.1 | CE-LIF analysis of N-linked glycans | 10 |
| 4.1.1 | Fetuin N-glycans | 10 |
| 4.2 | HPAEC-PAD analysis of large and sialylated glycan species | 12 |
| 4.2.1 | Standards | 12 |
| 4.2.2 | κ -casein and fetuin O-glycans | 12 |
| 4.2.3 | Fetuin N-glycans | 16 |
| 4.2.4 | Total fetuin glycan pools | 16 |
| 4.3 | HPAEC-PAD analysis of neutral mono- and disaccharide glycan species | 17 |
| 4.3.1 | Standards | 17 |
| 4.3.2 | Neuraminidase treated κ -casein and fetuin O-glycans | 19 |
| 4.3.3 | Human coagulation factor IX O-glycans | 20 |
| 5 | Discussion | 20 |
| 5.1 | Comparison and evaluation of chemical release methods | 20 |
| 5.2 | An integrated approach to glycan mapping | 21 |
| 5.3 | Limitations and future prospects | 21 |
| 6 | Conclusions | 22 |
| 7 | Acknowledgements | 22 |
| A | Appendix I: HPAEC elution programs | 25 |
| B | Appendix II: HPAEC-PAD peak area data, κ-casein | 26 |

1 Introduction

1.1 Glycosylation

1.1.1 Basic nomenclature and structure

The fundamental building blocks of carbohydrates are **monosaccharides**; polyhydroxy aldehydes and ketones with the empirical formula $(CH_2O)_n$ with n an integer between 3 and 9. Joining of the aldehyde or keto group with a hydroxyl group creates the closed five or six carbon ring form of monosaccharides that is the most common in solution. Monosaccharide units can be joined by glycosidic bonds to form linear or branched structures referred to as saccharides or **glycans**. A glycosidic bond is described using the spatial orientation of the attached residue (α or β) and the number of the carbons with the attached hydroxyl groups, e.g. an $\alpha(2-3)$ bond. The structure of the major monosaccharide components of glycans are presented in Figure 1. N-acetyl-neuraminic acid and related monosaccharides, interchangeably referred to as **neuraminic** or **sialic acids**, are unique in being sufficiently strong acids to carry a negative charge at physiological pH. [1]

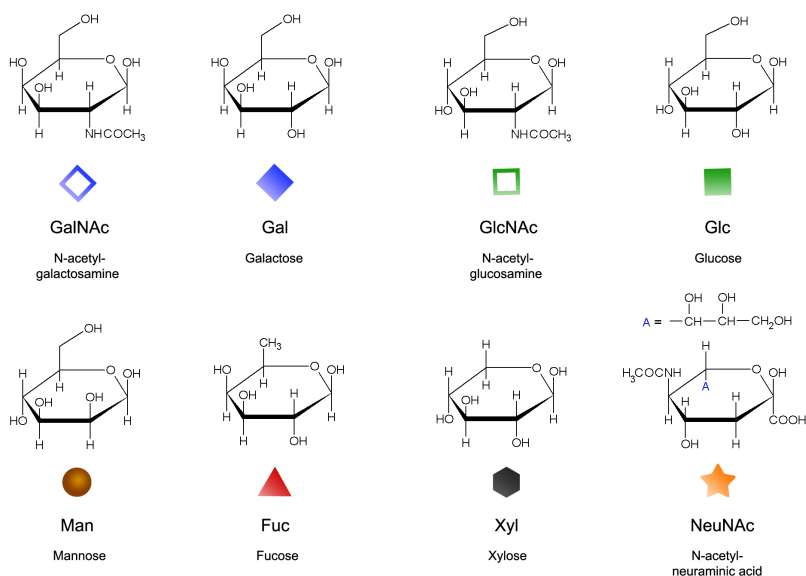


Figure 1: *Monosaccharide components of glycans*, from top to bottom for each monosaccharide: structure, graphic representation in this report, abbreviation, full name.

Covalent linkage of carbohydrates to other classes of molecules creates **glycoconjugates**. The focus here will be on the enzymatic modification of amino acids in peptide chains to create **glycoproteins**. Glycans attached to proteins are classified in two major groups: **N-linked** (attached to nitrogen in asparagine residues) or **O-linked** (attached to oxygen in threonine or serine). The unique properties of O- and N-glycans will be discussed in the next sections. There is no exact template in the genome or elsewhere that pre-determines certain glycan structures, neither is there any major proofreading activity controlling the product. This results in variable structures where the same glycosylation site on two proteins translated simultaneously can end up occupied by quite different glycans, a phenomenon referred to as glycosylation **microheterogeneity**. When a site is completely unoccupied on a fraction of proteins this is considered **macroheterogeneity**. A

given makeup of site occupancy and glycan structures creates a **glycoform** of the protein. It is not uncommon for relative amounts of glycoforms to be highly tissue- and time dependent, reflecting the expression and subcellular localization of glycosyltransferases and glycosidases as well as the protein's expression rate and rate of transportation through the ER/golgi apparatus. [2]

N-linked glycans Presence of the consensus peptide sequence Asn-(any amino acid except proline)-Ser/Thr triggers the co-translational addition of a dolichol-oligosaccharide precursor molecule in the ER. Once attached, all precursors undergo a series of set monosaccharide trimmings and additions. This constant phase is followed by diversification by further modifications in the golgi. As a consequence of the structure of the precursor, N-glycans are branched and usually consist of seven monosaccharide units or more. Depending on the enzymatic milieu, simple repeated addition of mannose can create a **high-mannose** type N-glycan. Alternatively, addition of GlcNAc and further extension of branches creates **complex** type N-glycans commonly classified by the number of substituted branches or **antennae**. N-glycans with at least one branch of each kind (high mannose, complex) are considered **hybrid** type. Common modifications to complex type N-glycans include addition of fucose to a core GlcNAc and terminal addition of neuraminic acid. [1] Some example structures are presented in Figure 2.

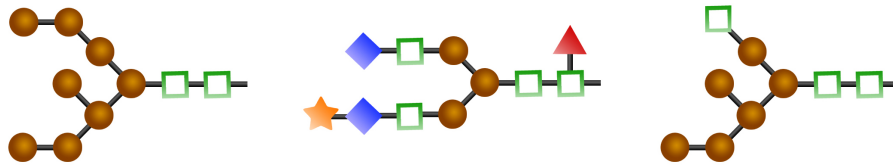


Figure 2: *Examples of N-glycan structures*, from left to right high-mannose type, complex type, hybrid type.

O-linked glycans No consensus sequence for O-linked glycosylation has been determined, but sites tend to cluster in regions rich in Ser, Thr and Pro residues. Addition of monosaccharides is step-wise without a composite precursor, creating structures that range from simple neutral monosaccharides to large branched oligosaccharides with several charged neuraminic acid residues. The most common form of O-glycosylation is initiated by addition of a GalNAc residue to the fully translated and folded protein, and is referred to as **mucin-type**. Alternative forms initialized by addition of glucose, N-acetylglucosamine or fucose are less common. Mucin type O-glycans are classified as having eight **core structures** of one to three monosaccharides, with the core 1 structure of Gal β (1-3)GalNAc the most widespread. [3] Examples of O-glycan structures are given in Figure 3.

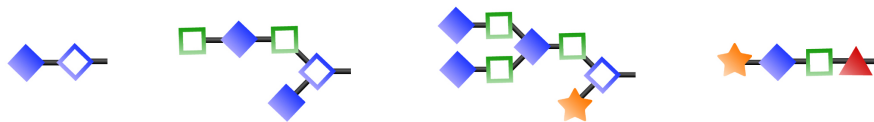


Figure 3: *Examples of O-glycan structures*, from left to right core 1 mucin-type, core 2 mucin type, core 3 mucin type, fucose-linked EGF-domain type.

1.1.2 Biological significance

There is increasing evidence of the diverse and critical roles of glycosylation to the properties and function of proteins. Deficiencies in the glycosylation machinery have been proven causative in diseases, and therapeutic proteins expressed in different cell systems have produced inconsistent pharmacological and pharmacokinetic properties. A few key examples of known relations between glycosylation and protein function will be covered to illustrate this.

Specific activity In some cases, absence of a certain glycan will result in a partial or complete loss of biological activity. For instance, Fc receptor affinity has been proven to be 100x higher for IgG antibodies carrying N-glycans without core fucose, with resulting increases in Fc-receptor mediated immune functions in vivo [4]. One way glycans affect the functionality of proteins is by altering the tertiary structure, with interaction between glycans and solvent as a likely mechanism. Mucins carry clusters of O-linked glycans that maintain their rod-shaped or filamentous structure, with deglycosylation resulting in the protein collapsing into a globular form [5].

Serum half-life, stability and aggregation Full glycosylation of a protein with neuraminic acid in particular can extend serum half-life by a number of incompletely understood mechanisms including blocking access to protease cleavage sites and increasing stability. For example, Amgen has successfully released a glycoengineered form of EPO with three extra N-glycosylation sites and a three times longer serum half-life [6]. Sensitivity to denaturation by heating is higher for the deglycosylated forms of bovine pancreatic ribonuclease, granulocyte colony stimulating factor and other proteins [3]. The hydrophilic nature of carbohydrates increases the solubility of a glycosylated protein while decreasing aggregation. For example, lower neuraminic acid content of the O-glycans of IgA increases aggregation and is a possible cause of IgA nephropathy, a disorder where deposits of antibodies impair kidney function [3].

Certain terminal glycan motifs will decrease the half-life of a protein by triggering receptor-mediated uptake and elimination. Thus, a glycoprotein with exposed galactose or N-acetylglucosamine binds to asialoglycoprotein receptors in hepatocytes and is eliminated. Exposed mannose residues will be recognized and cleared by liver endothelial cells and macrophages carrying mannose receptors. The distribution of these receptors has been used by Genzyme to specifically target recombinant glucocerebrosidase to macrophages by in vitro removal of terminal residues in complex N-glycans. [6]

Immunogenicity Differences in enzyme repertoire between species can result in immune responses to therapeutic proteins from non-human sources. For instance, roughly 1 % of the circulating IgG antibodies of a healthy non-allergic human are specific for Gal- α (1-3)-Gal, a modification common in non-primate mammals. Proteins produced by insect cells carry immunogenic core α (1-3) fucose and plants produce complex type N-glycans with high content of fucose and xylose that provoke strong IgE-mediated response. Additionally, full glycosylation of a protein can mask an immunogenic antigen on the protein surface. [7]

1.2 Strategies for analyzing glycosylation

Characterization of glycosylation can be performed with two basic strategies: analysis of glycans while attached to proteins or peptides, or analysis of pools of glycans cleaved and purified from the protein material. The first strategy commonly aims to identify distribution between glycoforms, determine site location and occupancy or locate antigenic motifs, but mass spectrometric methods have increasingly proven effective in directly identifying the structure of glycans while attached to peptides. Separating carbohydrate conjugates from their carrier proteins means loss of glycoform

and site information, but in return efficient separation based on charge, monosaccharide composition and linkage isomerism is possible. Commonly full or sufficient information on glycosylation makeup can only be acquired by integrating several approaches. Various strategies and methods have been extensively reviewed recently [8]. The work described here is in line with the second of the previously mentioned strategies, with sequential release of N- and O-glycans. This approach is inspired by recent work by Morelle and Michalski [9], who released and separated glycan pools for mass spectrometric analysis. Here we will employ electrophoretic and chromatographic techniques to analyze the released material, these will be briefly described in the following sections.

1.2.1 Capillary electrophoresis

Acceleration of charged species when influenced by an electric field is used to separate compounds in various forms of **electrophoresis**, where the movement of any given species will be determined by charge and mobility in the separation medium. In **capillary electrophoresis (CE)**, the separation is performed in a narrow tube to minimize convection and diffusion. The high surface to volume ratio of a thin capillary also permits the use of high voltage since cooling is efficient, and in total CE allows high resolution separation with short analysis times. [10] In the applications used here, the so-called electroosmotic flow created by buffer ions attracted to charges in the capillary wall is reduced to almost zero by using a coated capillary. Sample is injected by dipping one capillary end in the sample vial and applying either pressure or voltage, both capillary ends are then submerged in buffer reservoirs and a voltage is applied to these to start the migration of the analytes. The analytes are detected by e.g. UV absorption or laser induced fluorescence as they pass a window where the protective outer layer has been stripped to create a light path through the transparent capillary walls. Non-charged species can be separated more efficiently if derivatized with a charged tag. We here employ 9-aminopyrene-1,4,6-trisulfonate (APTS) which confers not only an extra three negative charges at most analysis pHs, but also is a fluorophore enabling sensitive laser induced fluorescence (LIF) detection. [11]

1.2.2 High pH anion exchange chromatography

Liquid chromatography generally separates analytes by their distribution between a liquid mobile phase and a solid stationary phase, with higher affinity for the stationary phase translating to longer elution time. In **ion exchange** columns, ionic analytes are attracted to charged sites in the stationary phase. Gradient elution can be achieved by adding an increasing concentration of displacing ions or by changing the mobile phase pH in order to change the charge of analytes. [12] The most efficient form of liquid chromatography application for carbohydrates is based on strong anion exchange resins that are stable at high pH, allowing exploitation of the weakly acidic properties of most carbohydrates. The technique is referred to as **high performance (or pH) anion exchange chromatography (HPAEC)**. As in most liquid chromatography setups, a guard column loaded with the same resin as the main column can be used to extend the column lifetime. Detection is based on **pulsed amperometry (PAD)**, with the mobile phase passing a gold surface that undergoes a three-stage voltage cycle. The current created by oxidation of analytes close to the electrode surface in the first stage corresponds to the detected signal. In the second stage, a higher potential is applied to fully oxidize the gold to gold oxide in order to clean the electrode, and finally the oxide is reduced back to gold to ready the detector for a new cycle. Since the voltage is optimized for the oxidation of carbohydrates, detection is fairly specific, sensitivity is high and no derivatization is required. [13]

2 Objectives

The aim of the project described in this report was primarily to develop methods to study O-linked glycosylation of glycoproteins. This requires the development of chemical methods to release glycans, as well as the creation of analytical protocols using HPAEC-PAD to separate and to some extent identify released glycan species from model proteins. Additionally, existing methods for N-glycan analysis using enzymatic release and CE-LIF analysis were to be improved on and integrated with O-glycan methods to enable the full glycan mapping of proteins with both O- and N-linked glycans. In brief, the aim of this work is to:

- Implement and evaluate reductive and non-reductive β -elimination methods for chemical release of O-linked oligosaccharides from glycoproteins
- Develop methods for HPAEC-PAD analysis of O-linked oligosaccharide alditols
- Improve existing methods for enzymatic release and CE-LIF analysis of N-linked oligosaccharides from glycoproteins
- Integrate methods for O- and N-glycosylation analysis to enable full glycan mapping of proteins carrying both types of modifications

3 Materials and methods

3.1 Chemicals

Sodium cyanoborohydride in tetrahydrofuran, κ -casein from bovine milk, sodium borohydride, borane-ammonia complex, 28% ammonium hydroxide solution, acetic acid, hydrochloric acid and Dowex 50 W x 8 H⁺-form were purchased from Sigma Aldrich. Glycan standards galacto-N-biose, 3 α ,4 β ,3 α galactotetraose, and 3'-N-acetylneuraminy-N-acetyllactoseamine sodium salt as well as disaccharide standards lactose and maltose were also acquired from Sigma Aldrich, while monosaccharide standards were purchased from Supelco and disaccharide standard sucrose from Merck. 0.1M Sodium hydroxide solution, Carbohydrate Separation Gel Buffer-N, 9-aminopyrene-1,4,6-trisulfonate (APTS) and glucose ladder standard were acquired from Beckman-Coulter. Fetuin from fetal bovine serum and α 2-3,6,8,9-neuraminidase (*Arthrobacter ureafaciens*, recombinant in *E. coli*) were purchased from Calbiochem, and PNGaseF with NP40 and incubation buffer from New England BioLabs. Finally, model proteins human coagulation factor IX (Nanotiv) was supplied by Octapharma. All water used was produced by a Millipore MilliQ185 system and had a resistance of 18.2M Ω cm.

3.2 Model proteins

Method development and evaluation in this study has been performed on a panel of commercially available proteins selected to represent the spectrum of carbohydrate structures likely to be encountered on glycoproteins of interest.

3.2.1 κ -casein from bovine milk

κ -casein is the major glycoprotein component of milk, and carries only O-glycan sites clustered at Ser 141 and Thr 131,133,135,142. Only three of these sites have been described to be significantly glycosylated [14]. The O-glycans present on non-colostrum bovine κ -casein have been determined as primarily a disialylated core 1-structure and two structural isomers of monosialylated core 1 [15]. Since it lacks N-glycans and is available in large quantities this protein represents an excellent model to develop methods for O-glycan release and analysis.

3.2.2 Bovine fetal serum fetuin

Fetuin is a protein of the cystatin superfamily, present in large amounts in mammalian fetal serum with levels decreasing in adulthood. Fetuin is N-glycosylated at Asn 99, 156, 176, and O-glycosylated at Thr 280 and Ser 271,282,341 [16]. The N-glycans consist primarily of triantennary complex type carrying three or four neuraminic acid residues, with $\alpha(2-3)/\alpha(2-6)$ neuraminic acid linkage isomers of both of these dominating species [17]. The main components of the O-glycan makeup are identical to those of κ -casein [18]. Fetuin is utilized here as a model for N-glycan analysis as well as the problem of isolating O- and N-linked glycans from the same protein material.

3.2.3 Coagulation factor IX from human serum

Human coagulation factor IX is both N- and O-glycosylated, carrying complex-type N-glycans and O-glycans of mucin type, but also specific O-glycosylations only found on EGF-domains in a handful of proteins. One of these is a fucose-linked tetrasaccharide containing a single terminal neuraminic acid, while the other consists of glucose extended by one or two xylose residues [19]. The presence of the second of these glycan types makes factor IX a suitable model for the analysis of small, neutral glycans.

3.3 Glycan release

3.3.1 Enzymatic release of N-glycans

Specific cleavage of N-glycans was achieved by incubating 100 μ l of a 1mg/ml glycoprotein solution in MilliQ water with 10 μ l 10% Np40 and 3 μ l PNGaseF (corresponding to an activity of >1500U with units as defined by manufacturer) at 37°C for 17 hours. To ascertain full release, some samples received an additional 10 μ l 10x glycoprotein denaturing buffer containing 5% SDS and 0.4M DTT and were heated to 100°C for 10 minutes prior to incubation. To remove SDS, these samples were passed through SepPakC18 cartridges (Waters Milford, MA) after incubation. In cases where the proteins were to undergo O-glycan cleavage, 3 volumes of ice cold ethanol were added and the samples cooled to -20°C for two hours to precipitate proteins. The precipitated proteins were collected by centrifugation 10 min at 14krpm, and the pellet was resuspended in more cold ethanol and the precipitation procedure repeated to wash away remaining N-glycans. Both supernatants were pooled and dried on a Savant SpeedVac Plus (Thermo Waltham, MA) to collect the released N-glycans, and the dried pellet passed on to O-glycan release protocols.

3.3.2 Chemical release of O- and N-glycans

The glycosidic bond joining carbohydrates to amino acids in glycoproteins is labile under mild alkaline conditions and tends to break down by the process of **alkaline β -elimination**. This enables a simple means of non-enzymatic release of intact, reducible carbohydrate moieties of both N- and O-linked types. Unfortunately, the released glycans will undergo further, unwanted elimination reactions referred to as **peeling**, and are also susceptible to alkali-catalyzed isomerization reactions. As a result, the released glycans must somehow be conserved in a base-stable form. The traditional way of achieving this is to carry out the release reaction in a reducing environment by the addition of sodium borohydride, which will transform the released glycans to a base stable sugar-alcohol or **alditol** form. This effectively obstructs peeling and isomerization, but has the distinctive disadvantage of rendering the released material unsuitable for derivatization, limiting the available strategies for analysis. As an alternative, the release can be carried out in the presence of excess ammonium carbonate. This will temporarily preserve the released glycans as stable glycosylamine-carbonate conjugates, and once the release reaction

is completed boric acid is added restoring the glycans to the desired reducible form. Since this method produces reducible carbohydrates, it is referred to as a non-reductive release method. In total, two reductive release methods based on sodium hydroxide/sodium borohydride and ammonia-borane complex/ammonium hydroxide solution as well as a single non-reductive method based on ammonium hydroxide solution/ammonium carbonate will be considered here.

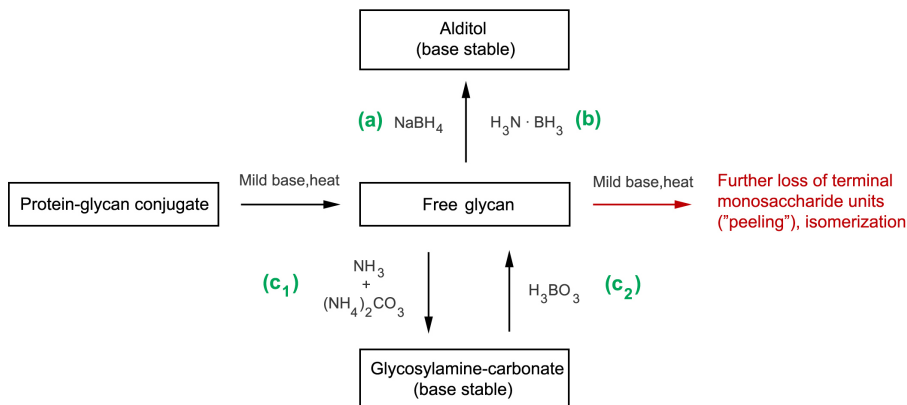


Figure 4: Three strategies to protect glycans released by alkaline β -elimination, once the glycan is released it is susceptible to unwanted peeling and isomerization reactions, and must be transformed to a base-stable form. Addition of a reducing agent (in this case either (a) sodium borohydride or (b) ammonia-borane complex, depending on release method used) can irreversibly convert the glycan to a base stable sugar-alcohol form (alditol). Alternatively, in the non-reductive method excess ammonium carbonate transforms the glycan to a base-stable glycosylamine intermediate (c_1) that can be returned to the reducible form by addition of boric acid (c_2) once the incubation is complete.

Sodium hydroxide release Glycans were released by alkaline β -elimination by a modified version of the protocol described by Carlson [20]. 200 μ g of glycoprotein was incubated in 0.075M sodium hydroxide and 1M sodium borohydride for 17h at 45°C in a screw-cap vial. The reaction was interrupted by addition of 3-4 drops of glacial acetic acid. Sodium hydroxide based β -elimination is an old and frequently used method, and the literature reports highly variable experimental conditions. Given this, reaction conditions were initially optimized for the factors sodium hydroxide concentration (levels 0.05-0.075-0.1M) and incubation time (levels 14-17-20h) to maximize yield with a response surface modelled on a 2^3 experimental design in Minitab 13.2 [21]. The area of the first and best separated O-glycan species released from fetuin, analyzed by separation program 1 with HPAEC-PAD as described below, was used as response. No replicates were created in this exploratory analysis. The created response surface indicated good yields in the high concentration/short time range, but since incubation times <17h were considered inconvenient further experiments were carried out with 17h incubation and 0.1 or 0.075M NaOH. Finally 17h/0.075M was chosen as standard operating conditions as a compromise between yield and convenience.

Reductive ammonia release An alternative release protocol similar to the one developed more recently by Huang et al. [22] was tested. In this case, reductive β -elimination is performed

in a 5mg/ml solution of ammonia-borane complex in 28% ammonium hydroxide, with 200 μ g of glycoprotein in 200 μ l solution. The reaction is incubated for 21h at 45°C in screw-cap vials. After neutralization with 3-4 drops of acetic acid the samples are dried on SpeedVac and reconstituted in 100 μ l of water.

Non-reductive ammonia release Non-reductive ammonia release was performed essentially as described by Huang and co-workers [23]. Briefly, 200 μ g of glycoprotein was dissolved in 200 μ l of 28% ammonium hydroxide solution saturated with ammonium carbonate at room temperature. An additional excess of 100 μ g of solid ammonium carbonate was added to each reaction, and the samples were incubated at 60°C for 40 hours. Ammonium carbonate and -hydroxide were removed by repeated SpeedVac evaporations of 300 μ l amounts of water until no salt was visible in the tubes. Each of the dried samples was dissolved in 10 μ l of 0.5M boric acid and incubated at 37°C for 30 minutes. After the incubation, boric acid was removed by five evaporations of 300 μ l methanol and the samples dissolved in 100 μ l of MilliQ water.

Ion exchange purification of samples To remove proteins, peptides and other contaminants, samples were purified by passing through Dowex 50W x 8 H⁺-form ion exchange resin. The resin was prepared by mixing and decanting twice with 3M NaOH, neutralized by repeated decanting with water, repeating this process with 3M HCl and finally decanting three times with 5% acetic acid. The resin can be stored for extended periods in this state. A pasteur pipette was plugged with a small ball of glass wool and packed up to the pipette constriction point with the resin while 5% acetic acid was flowing through. The end of the pipette was extended with a 2cm length of rubber tube to reduce flow rate and facilitate stopping of the flow when necessary. The column was washed with 4ml 5% acetic acid, sample was added and roughly 1.5ml of eluent was collected while eluting with 5% acetic acid. Initially up to four ml were collected, but all or most of the analytes were found to elute in the first ml (data not shown). The eluents were dried on SpeedVac to remove acetic acid. Samples initially released by reductive methods were finally purified from borate by SpeedVac drying with five additions of 5% acetic acid in methanol.

3.4 Glycan modifications

3.4.1 Reduction of standards and non-reduced released glycans

To render carbohydrate standards and glycans released enzymatically or by non-reductive chemical cleavage comparable to the glycans released by reductive means, these samples were incubated in 1M sodium borohydride solution at 45°C for 17h. The samples were desalted by the same ion exchange method described earlier.

3.4.2 APTS labelling

To make cleaved glycans detectable by laser induced fluorescence, derivatization with 9-aminopyrene-1,4,6-trisulfonate (APTS) was carried out. Glycans released from 100 μ g of glycoprotein were dried on SpeedVac and mixed with 3 μ l 1M sodium cyanoborohydride in tetrahydrofuran and 3 μ l of a solution made by dissolving 5mg of APTS in 48 μ l 0.9M citric acid. The reaction mixture was incubated at 37°C for 17h, and was stopped by the addition of 200 μ l MilliQ water.

3.4.3 Enzymatic removal of neuraminic acid

Glycan pools cleaved from 100 μ g of fetuin or κ -casein were dissolved in 97 μ l of MilliQ water. 3 μ l of α 2-3,6,8,9-neuraminidase (corresponding to an activity of >15mU with units as defined by

manufacturer) was added and the 100 μ l reactions were incubated at 37°C for 3h. Negative control samples were incubated with enzyme solution replaced by 3 μ l of MilliQ water.

3.5 Analysis

3.5.1 HPAEC-PAD analysis

High pH anion exchange chromatography with pulsed amperometric detection was performed using an ICS2500 Ion Chromatography System (Dionex Co. Sunnyvale, CA) with CarboPac PA-200 (for oligosaccharide alditols >2 monosaccharide units and charged oligosaccharide alditols) and CarboPac MA-1 (for mono- and disaccharide alditols) columns with corresponding guard columns. For glycan analysis on the PA-200 column, elution program 1 was utilized, while monosaccharide analysis on the MA-1 was performed with program 2 and disaccharide analysis with program 3 (See Appendix I). The column was equilibrated by three blank injections at the start of each sample batch analyzed. To protect the column and detector, all samples and standards intended for HPAEC-PAD analysis were deproteinized on Dowex 50W x 8 H⁺-form ion exchange resin as described earlier as well as subjected to 0.2 μ m filtration.

3.5.2 CE-LIF analysis

Capillary electrophoresis with laser induced fluorescence detection was performed on a Beckman P/ACE 5510 system with a 488nm argon-ion laser module (Beckman-Coulter Fullerton, CA). APTS-derivatized samples were pressure injected during three seconds into a Beckman 50 μ m x 47 cm N-CHO capillary (effective length 41 cm) and separated during 20 minutes at 20kV in a Carbohydrate Separation Gel Buffer-N supplied by the same manufacturer. The capillary was washed by high pressure rinse for two minutes with water and eight minutes with separation buffer prior to each run.

4 Results

4.1 CE-LIF analysis of N-linked glycans

4.1.1 Fetuin N-glycans

PNGaseF released N-glycans released by PNGaseF were analyzed as APTS conjugates using CE-LIF. As expected, κ -casein samples produced no peaks since this protein lacks N-glycans. Four replicate fetuin samples produced electropherograms similar to what was expected from literature, [17] with peaks corresponding primarily to two linkage isomers each of tri- and tetrasialylated triantennary complex glycans. All chromatograms from this section are presented in Figure 5.

To test the effect of full denaturation on release efficiency, four samples were heated in the presence of SDS before the enzyme treatment. This produced extra peaks, the dominating of which was also present in a control sample with only APTS-incubated SDS-buffer. Removal of SDS by passing the samples through SepPakC18 hydrophobic interaction cartridges removed the extra peaks completely leaving the original pattern. Replicate variation in both peak area and ratios was negligible in these experiments. The results indicate that enzymatic release of N-glycans from fetuin was not improved by SDS/heat denaturation, and since the SDS/SepPakC18 process reduced overall yield by roughly 60% the pre-treatment was excluded from further fetuin N-glycan experiments. As a safeguard the full program should be used at least once for each new protein investigated to ensure denaturation does not influence release efficiency.

Additionally, one fetuin N-glycan sample treated with α 2-3,6,8,9-neuraminidase was analyzed. All major peaks in the original pattern are lost in good accordance with a protein carrying glycans

with a high content of terminal neuraminic acid. The main peak in the new pattern is likely to represent a triantennary complex glycan without neuraminic acid, while minor peaks may represent the presence of glycans carrying one or more branched neuraminic acids that are resistant to enzymatic action.

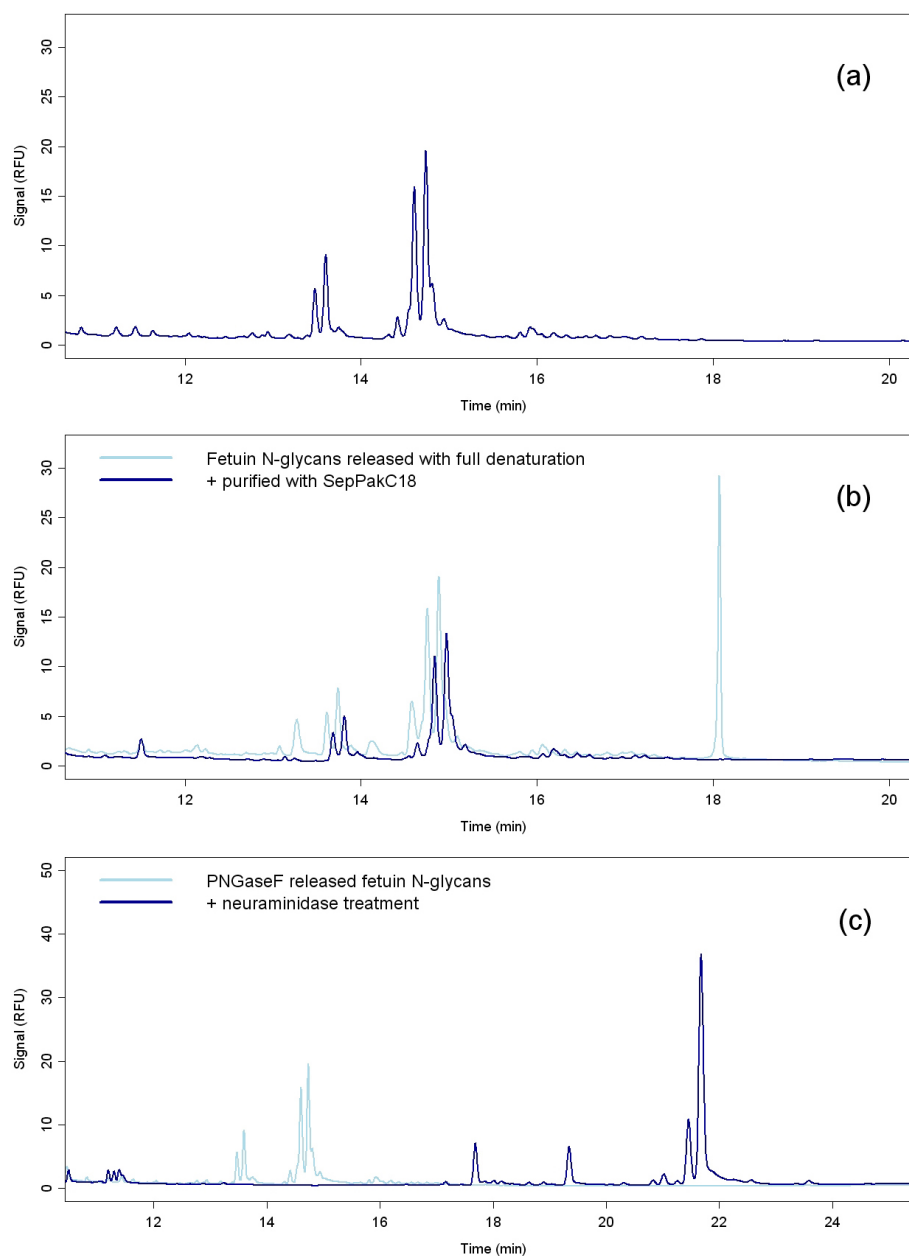


Figure 5: *CE-LIF analysis of PNGaseF released and APTS labelled N-glycans from fetuin*, a) standard conditions, b) heated in SDS/DTT denaturation buffer prior to PNGaseF release +/- purified on SepPakC18 c) standard conditions +/- treated with alpha2-3,6,8,9-neuraminidase to remove terminal neuraminic acid residues.

Non-reductive ammonia released N-glycans cleaved from fetuin by non-reductive ammonia release were analyzed by the same method. The generated peak pattern did not differ significantly from the PNGaseF-released samples indicating that the release method successfully cleaved N-glycans and protected them from peeling reactions, see Figure 6. However, chemically released samples produced less clean electropherograms with more baseline noise and minor peaks. Release efficiency was in the same order of magnitude as for enzymatic release.

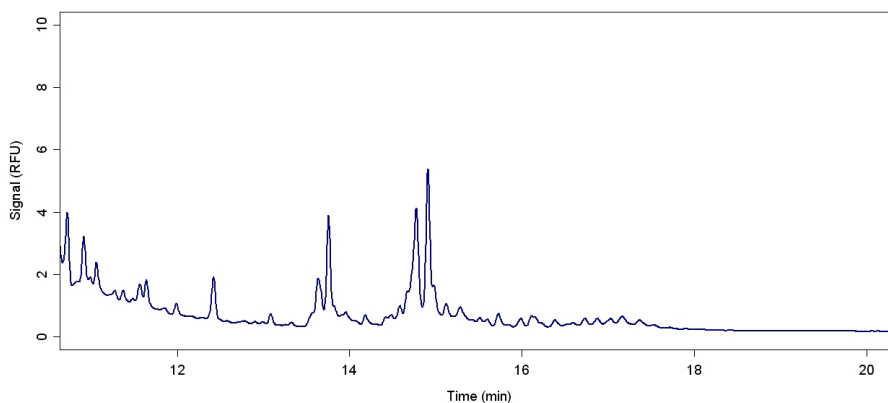


Figure 6: *CE-LIF analysis of N-glycans from fetuin released by ammonium carbonate in ammonium hydroxide solution (non-reductive release).*

4.2 HPAEC-PAD analysis of large and sialylated glycan species

4.2.1 Standards

A number of standards were analyzed using a CarboPac PA200 HPAEC- column using program 1. As a simple size marker, a reduced glucose ladder consisting of mono- to more than 20-mers of glucose was analyzed. By comparing these to elution times for reduced disaccharide (maltitol) and trisaccharide (raffinitol) standards, the number of monosaccharide units represented by each ladder peak was identified. It was clear that while trisaccharides and structures consisting of up to 30 glucose units could readily be separated, disaccharides eluted too early to be detected in any biological sample with high levels of contaminants. Due to drift in elution time between runs and run batches, glucose ladder was later injected in every batch to facilitate comparison. To enable identification of monosialyl disaccharide O-glycans, a 3'-N-acetylneuraminy-N-acetylactosamine standard was also analyzed (see Figure 7). Free NeuNAc standard sample was analyzed since it represented an expected product of neuraminidase cleavage experiments, the second expected fragment consisting of the core 1 -structure was also run as a standard but found to elute too early for meaningful results to be obtained on this column type.

4.2.2 κ -casein and fetuin O-glycans

Sodium hydroxide released Once a release protocol had been optimized as described in the materials and methods section, de-N-glycosylated fetuin and κ -casein were analyzed with both samples producing two major peaks (designated A and B). κ -casein chromatograms from all three release methods are presented in Figure 9. The fetuin samples additionally contained

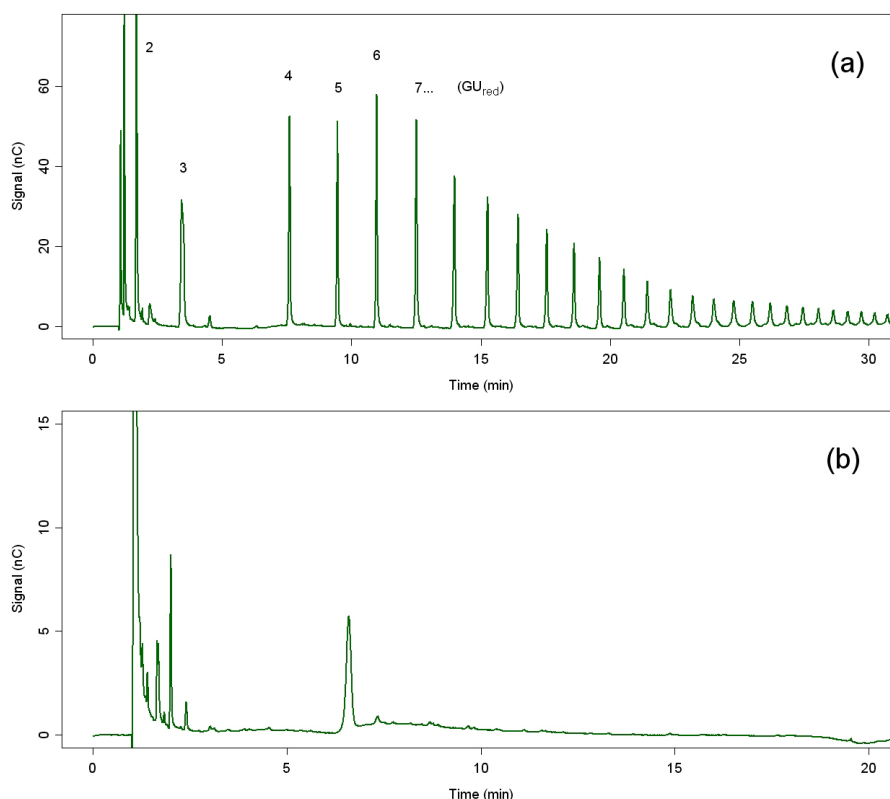


Figure 7: *Sodium borohydride reduced standards separated on CarboPac PA200 column (program 1), (a) Reduced glucose ladder standard shows good separation in the range 3 to 30+ monosaccharide units. Established ladder measured in (reduced) 'glucose units', scale established by maltitol (≈ 2 GU_{red}) and raffinitol (≈ 3 GU_{red}) spiking. (b) Reduced 3'-N-acetylneuraminyl-N-acetylglucosamine standard provides approximate elution time for small monosialyl O-glycans.*

remaining traces of what could later be identified as N-glycans, as evident in Figure 8. Peaks were tentatively identified as follows: sodium hydroxide released O-glycans from both proteins were treated with neuraminidase, which removed both main peaks while producing a single peak that was identified by spiking with standard as free NeuNAc, the full results of the neuraminidase experiment is presented in Figure 13. Peak A has an elution time highly similar to that of 3'-N-acetylneuraminyl-N-acetylglucosamine, a standard with similar structure to the linear monosialyl core 1 O-glycan expected from literature to be abundant on this protein. Hence, peak A is likely NeuNAc- β (2-3)-Gal- β (1-3)-GalNAc, Gal- β (1-3)- (NeuNAc- β (2-6))-GalNAc or a mixture of both, while peak B corresponds to NeuNAc- β (2-3)-Gal- β (1-3)-(NeuNAc- β (2-6))-GalNAc.

Given the many manual steps involved, high intersample variability was a possible problem with this release method. To assess this, four further replicate κ -casein samples were cleaved and analyzed (without the PNGaseF steps). Reassuringly, intersample variability was low with a RSD of 1% for the area of peak A and 3% for the area of peak B. Full data is presented in Appendix II.

Ammonia released Ammonia released κ -casein O-glycan samples in triplicate were analyzed with program 1, and produced the same two main peaks as NaOH release. However, peak ratios

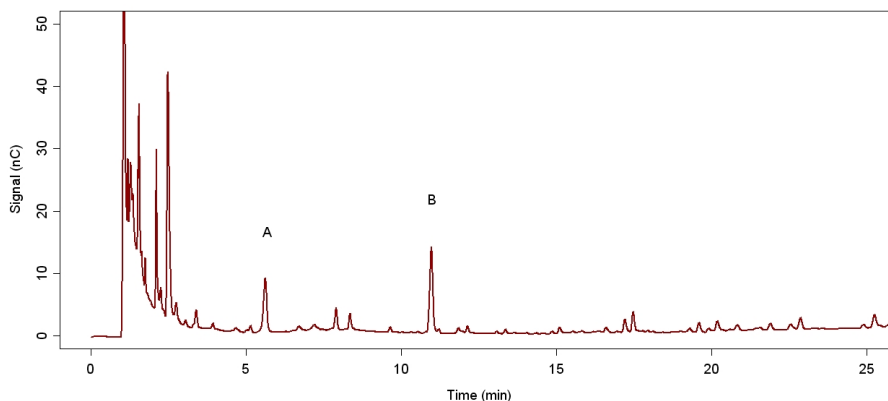


Figure 8: *O*-glycans released from fetuin by sodium hydroxide + sodium borohydride release, following *N*-glycan removal by PNGaseF treatment and precipitation. Note evidence of remaining *N*-glycan derived peaks, particularly in the timeframe 15-25min.

were significantly different with the yield of peak B markedly lower when ammonia was used. (See Appendix II) To ensure that the reaction time was sufficient, triplicate samples each of 18, 24 and 41 hour incubation were produced. Regardless of time, peak ratio was in the same range as that for sodium hydroxide release in this experiment, although total yield was still poor. Intersample variability was comparable to sodium hydroxide only after 42h incubation (Figure 9), with RSD 1.8% for the area of peak A and 1.92% for the area of peak B. It is unclear why the first sample batch produced inconsistent results, but it bears pointing out that the intersample variability was also substantially higher in this exploratory run.

There was also concern that the pH adjustment after incubation could cause selective yield decrease for disialyl glycan species in the ion exchange purification step. To address this, samples receiving no acetic acid and excessive amounts (an additional 5 drops) of acetic acid were created. The amount of acid added had no major impact on yield or peak ratio. (data not shown)

Non-reductive ammonia released κ -casein *O*-glycans released by the non-reductive ammonia protocol, although not primarily intended for HPAEC-PAD analysis, were analyzed under the same conditions for comparison. Elution times were compared to the unreduced 3'-*N*-acetylneuraminy-*N*-acetylactosamine standard to identify the equivalents of peak A and B. The only two replicates analyzed produced dissimilar peak ratios compared to both reductive methods described, see Figure 9 (full data in Appendix II).

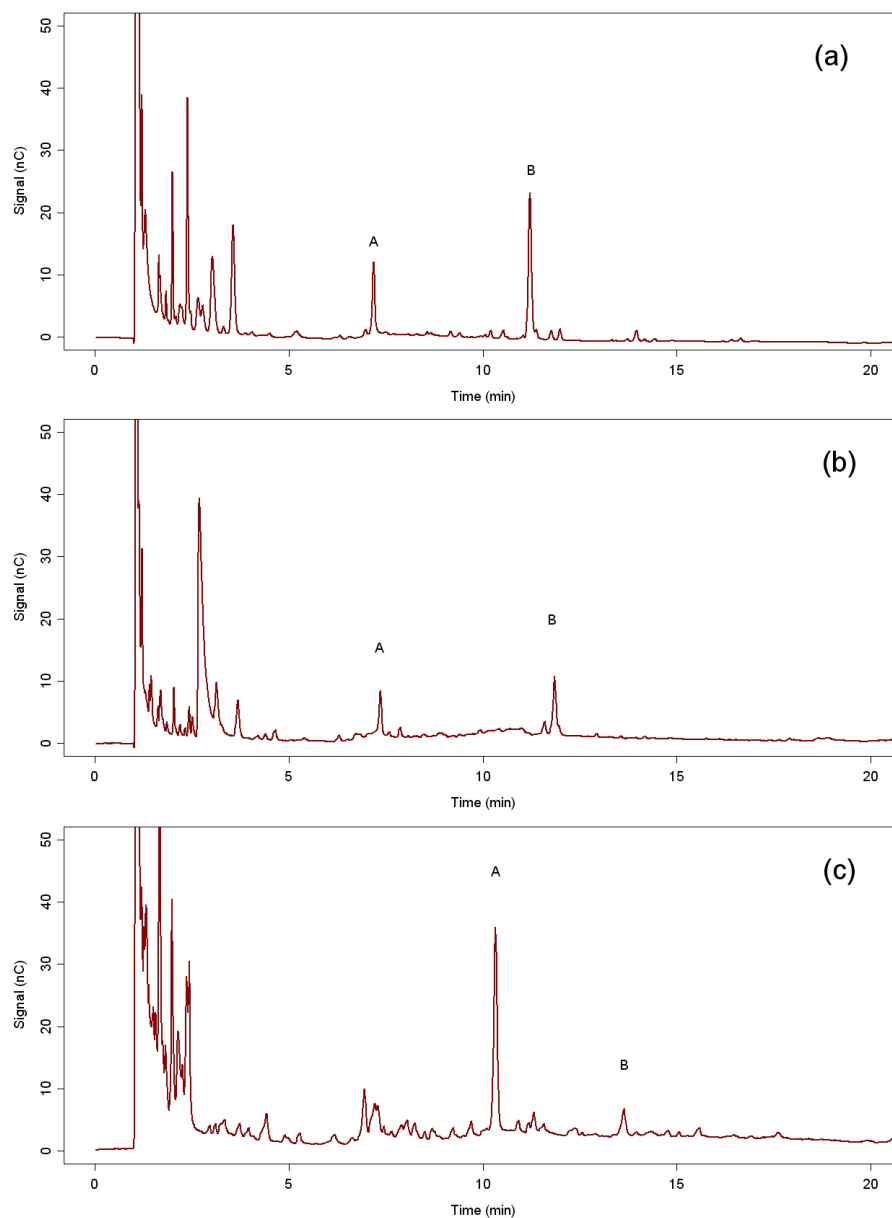


Figure 9: *O*-glycans released from κ -casein by chemical methods and analyzed by HPAEC-PAD on a CarboPac200 column (program 1), (a) sodium hydroxide + sodium borohydride release, (b) ammonia-borane complex in ammonium hydroxide solution 42h, (c) ammonium carbonate in ammonium hydroxide solution (non-reductive release). Note differences in yield and peak ratio, elution times in (c) are different since the peaks represent non-reduced glycans more strongly retained on the column.

4.2.3 Fetuin N-glycans

Glycan pools liberated from fetuin by PNGaseF were reduced to render them comparable to O-glycan alditols and separated by the same elution program (1). Similar results to the CE-LIF analysis of the same material was achieved with predominantly tri- and tetrasialylated triantennary structures, notably HPAEC enables separation of the $\alpha(2-3)$ and $\alpha(2-6)$ linkage isomers under these conditions (Figure 10). In contrast with CE-LIF, distinctive peaks corresponding to mono- and disialyl N-glycan species were present in these runs. Comparison of patterns and elution times allowed the identification of several late-eluting peaks from the fetuin O-glycan analysis as N-glycan material incompletely removed. Peak identities are based on manufacturer information [24]. Comparison to other runs performed in-house indicates that the substantial baseline drift during the runs is likely to be the result of NP40 non-ionic detergent carried over from the PNGaseF release protocol. Analysis of non-reduced N-glycan pools from the same protein was also carried out, and produced the same peaks eluting substantially later. This is in agreement with the general principle that retention decreases on HPAEC when the terminal of a carbohydrate is reduced to alditol form [25].

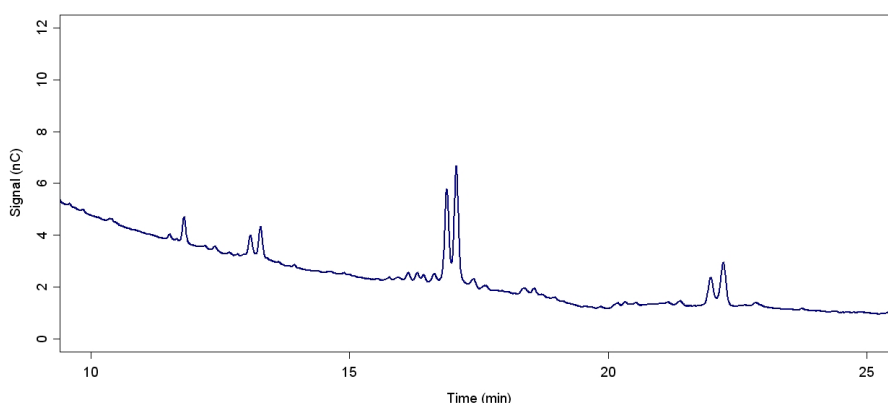


Figure 10: *Fetuin N-glycans released by PNGaseF and analyzed by HPAEC-PAD on a CarboPac200 column, glycans were reduced with sodium borohydride prior to analysis to facilitate comparison with O-glycan profile and protect from base-catalyzed transformation.*

4.2.4 Total fetuin glycan pools

To assess glycan integrity following chemical release methods, total glycan pools released from fetuin by sodium hydroxide and non-reductive ammonia release were analyzed and compared to fetuin glycans released by PNGaseF. Sodium hydroxide released samples suffered from significant decay of released glycans producing numerous sub-peaks for each pair of linkage isomers, with the peak pattern of O-glycans obscured by N-glycan-derived peaks (Figure 11). It is clear that this release protocol is unsuitable for simultaneous release of N- and O-glycans.

The same peak duplication phenomenon applied to the non-reductive ammonia released pool, although quality was clearly better in this case. Given that identical samples appear far more intact when derivatized and analyzed with CE-LIF, the extended period under basic conditions in the analysis itself is likely part of the problem. This is also evident when comparing HPAEC-PAD chromatograms for enzyme released N-glycans analyzed in reducible and alditol

form, with the non-reduced sample producing significant side-peaks. The likely cause is Lobry de Bruyn - Alberda van Enkenstein-transformation, a base-catalyzed reaction that can interchange carbohydrate stereoisomers. Specifically, N-acetyl-glucosamine has been shown to spontaneously convert to N-acetyl-mannosamine under basic condition [26]. Since alditols are immune to LdB-AvE-transformation, it is clear that in cases where extended analysis times are needed and significant concentrations of alkaline employed, carbohydrate samples should be reduced to alditols prior to HPAEC analysis. (See also [13])

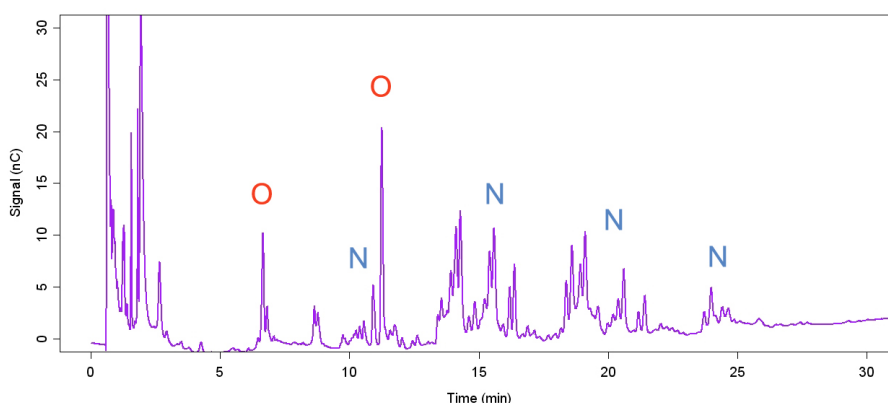


Figure 11: *Sodium hydroxide/sodium borohydride release of total fetuin glycan pool analyzed by HPAEC-PAD (program 1), severe decay of N-glycans is evident in this chromatogram, partially obscuring the more intact O-glycan peaks. Compare to enzyme-released N-glycans (Figure 10) and O-glycans separately released from fetuin (Figure 8).*

4.3 HPAEC-PAD analysis of neutral mono- and disaccharide glycan species

4.3.1 Standards

To expand the range of this analytical approach to cover small, neutral O-linked glycans, further HPAEC-PAD experiments were carried out using a CarboPac MA1 column. Using a gradient elution program (program 2, see Appendix I) similar to the one recommended by the manufacturer [27], a standard mix of the monosaccharides commonly present in mammalian O-glycans (fucose, GalNAc, GlcNAc, xylose, glucose, galactose, mannose) reduced to the alditol form were successfully separated. Using an isocratic elution approach with 700mM NaOH (program 3), three reduced disaccharide standards (lactose, maltose, sucrose) could readily be separated with a shorter analysis time. Extending the time at max NaOH concentration in the monosaccharide program could easily produce a composite program for the simultaneous analysis of mono- and disaccharides.

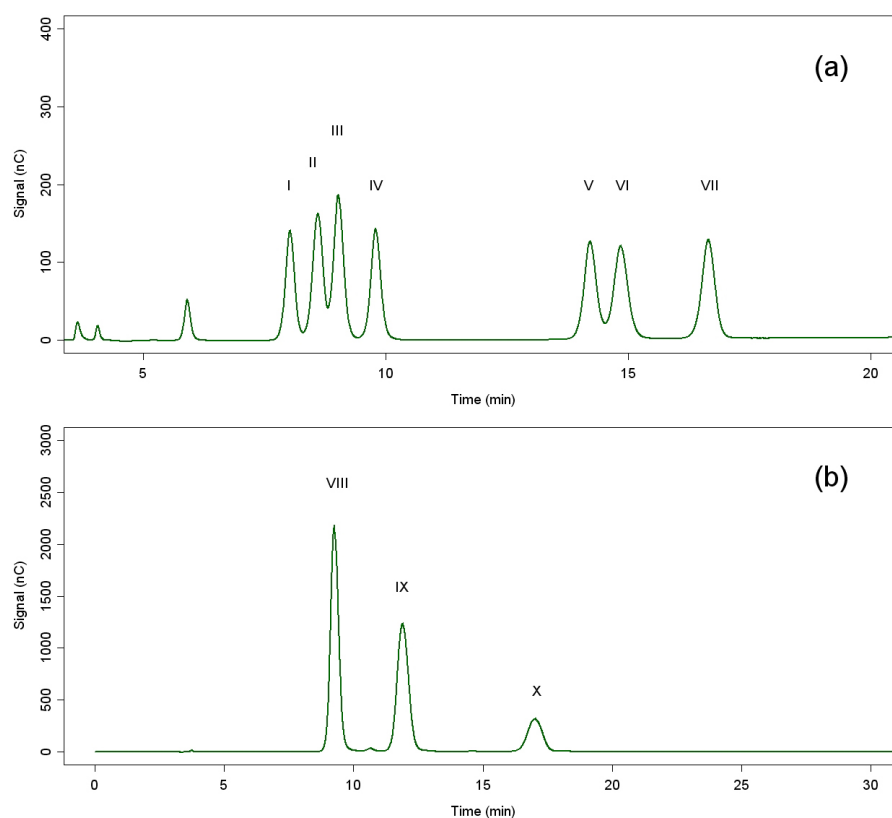


Figure 12: *Mono- and disaccharide alditol standards analyzed by HPAEC-PAD on a CarboPac MA1 column, (a) monosaccharide alditols eluted with program 2, (b) disaccharide alditols eluted with program 3. Standard identities as follows: I fucitol, II GalNAcol, III GlcNAcol, IV xylitol, V galactitol, VI glucitol, VII mannitol, VIII lactitol, IX maltitol, X sucritol.*

4.3.3 Human coagulation factor IX O-glycans

Finally, human coagulation factor IX was utilized as a demonstration of applied profiling of small, neutral O-glycans. Glycans cleaved from 200 μ g of protein material by reductive sodium hydroxide release were analyzed with both mono- and disaccharide elution programs as described above. The results indicate the presence of trace amounts of monosaccharides, although this could be due to contamination, as well as significant amounts of two species eluting within the same timeframe as reduced disaccharide standards with the first with elution time between lactitol and maltitol and the other between maltitol and sucritol (Figure 14). Literature predicts the presence of the disaccharide Xyl-Glc as well as a trisaccharide (Xyl₂)-Glc this protein [19], but no standards are available to verify peak identities.

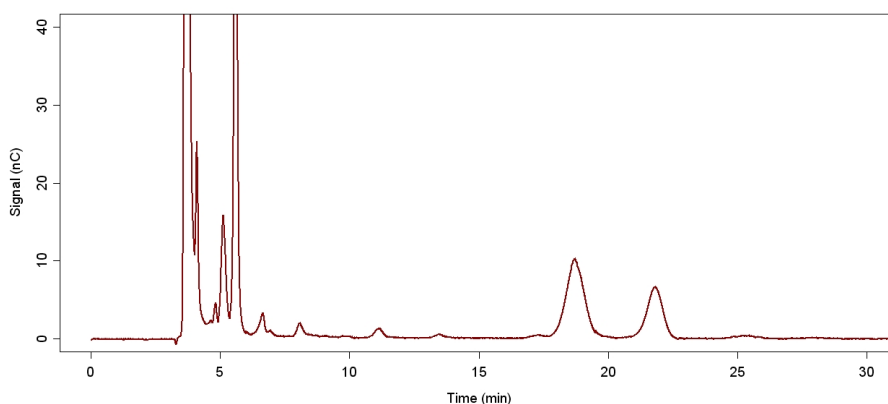


Figure 14: *Human coagulation factor IX O-glycans released by sodium hydroxide + sodium borohydride treatment and analyzed by HPAEC-PAD on a CarboPac MA1 column, Elution by program 3, two major peaks representing likely neutral disaccharides.*

5 Discussion

5.1 Comparison and evaluation of chemical release methods

With both reductive release methods tested, intersample variability was low for individual peak areas and peak ratio once the methods had been implemented a few times. However, the yield was substantially lower for ammonia release, and 42 hour-incubations were necessary to even get close to the kind of peak areas achievable by 17h of sodium hydroxide release. Both methods displayed tendencies of batch variability regarding peak ratio. This problem could be resolved simply by more practice in the execution of these far from trivial protocols, or could be somehow inherent in the methods used. In either case, the addition of an internal standard at some stage in the process to monitor loss and degradation of glycan material seems prudent, the O-glycan-like 3'-N-acetylneuraminy-N-acetylactoseamine standard used in this study would be a prime candidate for such a role. The use of internal standards would also enable semi-quantitative comparison of glycan profiles between different glycoproteins and analysis batches.

Overall, for standard analysis the classic sodium hydroxide/sodium borohydride seem to be the most efficient reductive release method. The reductive release method based on ammonia is

supposedly more efficient in protecting glycan integrity [22], so it can not be ruled out that although the total yield is lower, ammonia release produces a slightly better description of the glycan profile. If this is a concern ammonium release from increased amounts of protein starting material would also be a viable option.

Non-reductive release as implemented here is a labor-intensive and time-consuming method. The initial incubation takes a full 40 hours, and post-incubation processing is extensive. This method also requires addition of a fix amount of solid to each sample, which is particularly tedious. The final result is decent yield of peak A in our κ -casein model, but very low quantities of peak B. There is also far more baseline noise and minor peaks compared to both reductive methods, creating a cluttered chromatogram where minor peaks would be hard to detect. Glycan material released by non-reductive methods is suboptimal for HPAEC analysis due to the risk of base-catalyzed transformation during separation. On the other hand, glycan material released by this method is proven here to be readily derivatized with APTS and analyzed on CE-LIF. Thus, non-reductive ammonia release would be a useful method in cases where for some reason enzymatic release of N-glycans is not desired. With the addition of a suitable capillary electrophoresis O-glycan separation protocol, non-reductive chemical release could also serve as a complement to enzymatic release for the full profiling of N- and O-glycans on CE-LIF.

5.2 An integrated approach to glycan mapping

The primary objective of this project was the development of a functional method for O-glycan analysis, a task primarily performed using the only O-glycosylated protein κ -casein. With the approach presented here, the wide span of O-glycan structure is effectively covered by β -elimination-based chemical release followed by the use of two types of HPAEC column. Intersample variability is very low, enabling detection of small discrepancies in glycosylation between two samples.

Integration of N- and O-glycan analysis is desirable, since full analysis of glycans released from the same protein material is likely to give a better perspective on the glycosylation profile than two separate protocols using different starting material. Since chemical release methods to some extent liberate both N- and O-linked glycans, enzymatic release is also necessary to classify the products as N- or O-linked. Using fetuin as a model, sequential release and separate analysis of N- and O-glycans is achieved here, although with too few replicates to fully evaluate the usefulness of the method. Total starting material consumption for such a full glycosylation analysis amounts to no more than 300 μ g per replicate sample.

Several types of exoglycosidases are commercially available, but neuraminidase treatment represents a highly cost-effective method in profiling glycans. The strong acidic character of these monosaccharide residues is particularly important to the biological modulation of protein function by an attached glycan. Charged residues will also dominate retention when glycans are analyzed, both in HPAEC and CE methods. As a consequence, comparison of chromatograms and electropherograms before and after removal of neuraminic acid by enzymatic release can provide key information on glycan characteristics with a minimum of effort.

5.3 Limitations and future prospects

Edward Tarelli recently demonstrated that reductive β -elimination utilizing ammonia under conditions similar to those employed here only efficiently cleaved O-glycans linked to one of six sites on IgA1 glycopeptides. Results for sodium hydroxide-based release were inconclusive [28]. This might indicate that the efficiency of chemical release is dependent on peptide sequence, and although differences in release efficiency are less of a concern for comparative glycan mapping, the potential of totally resistant sites is troubling. More data is likely to be published on this subject.

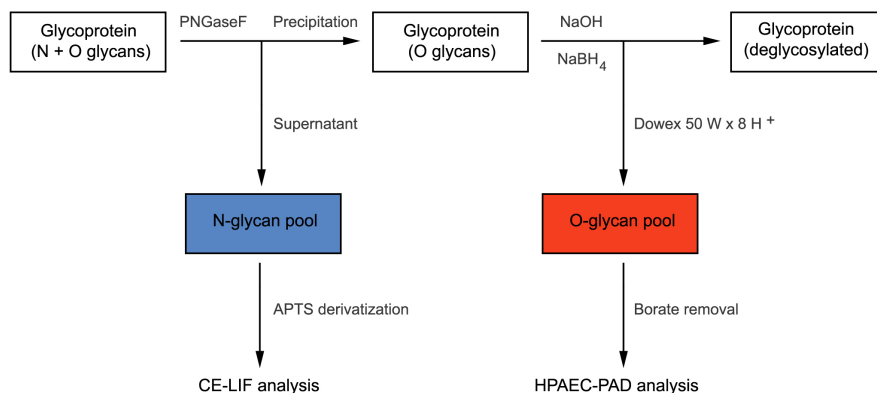


Figure 15: *An integrated approach to profiling of both N- and O-linked oligosaccharides from glycoproteins*

Finally, ethanol-based separation of PNGaseF released N-glycan material and glycoproteins failed completely for one batch of fetuin samples during this project, while producing unsatisfactory results for a second. The development of improved means of separating glycan and protein material with intact recovery of both has high priority in the future improvement of this method.

6 Conclusions

With the proposed strategy, complete profiling of the O- and N-linked oligosaccharides present on a glycoprotein can be achieved from $\approx 300\mu\text{g}$ of starting material as demonstrated on commercially available model proteins. Sequential enzymatic and chemical release allows isolation of N- and O-glycan pools for subsequent analysis with capillary electrophoresis and high pH anion exchange chromatography, respectively.

7 Acknowledgements

First of all I would like to thank my supervisor Akbar Ansari for invaluable support. I would also like to thank the rest of the Glycans and Proteins group at Biovitrum, in particular Björn Garpefjord, Sergei Kuprin and Alona Pavlova. Finally thanks to everyone who backed me up during the project, at Uppsala University and elsewhere, and the people who reviewed and helped improve this text.

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A Appendix I: HPAEC elution programs

Program 1

Eluent A 100mM NaOH

Eluent B 100mM NaOH, 250mM NaAc

Flow 0.50 ml/min

Time program [min]:

0 min Inject 19 μ l, Detector on, Detector autozero, A = 100%, B = 0%

5 min A = 100%, B = 0%

60 min A = 0%, B = 100%

65 min Detector off, A = 100%, B = 0%

75 min A = 100%, B = 0%, End

Program 2

Eluent A MilliQ water

Eluent B 700mM NaOH

Flow 0.40 ml/min

Time program [min]:

0 min Inject 19 μ l, Detector on, Detector autozero, A = 86%, B = 14%

5 min A = 86%, B = 14%

30 min A = 0%, B = 100%

45 min Detector off, A = 86%, B = 14%

50 min A = 86%, B = 14%, End

Program 3

Eluent A MilliQ water

Eluent B 700mM NaOH

Flow 0.40 ml/min

Time program [min]:

0 min Inject 19 μ l, Detector on, Detector autozero, A = 0%, B = 100%

60 min Detector off, A = 0%, B = 100%, End

PAD waveform (used for all programs):

data collection rate=2, reference electrode= AgCl

0 msec potential 0.10

0.20 msec potential 0.10, begin integration

0.40 msec potential 0.10, end integration

0.41 msec potential -2.00

0.42 msec potential -2.00

0.43 msec potential 0.60

0.44 msec potential -0.10

0.50 msec potential -0.10

B Appendix II: HPAEC-PAD peak area data, κ -casein

NaOH red

| | Peak A | Peak B | Ratio A/B |
|-------------|---------|--------|-----------|
| Replicate 1 | 1,63 | 3,93 | 0,41 |
| Replicate 2 | 1,66 | 3,88 | 0,43 |
| Replicate 3 | 1,63 | 3,74 | 0,44 |
| Replicate 4 | 1,64 | 3,70 | 0,44 |
| | | | |
| Average | 1,64 | 3,81 | 0,43 |
| StDev | 0,02 | 0,11 | 0,01 |
| RSD% | 0,99732 | 2,8947 | 2,9550354 |

Ammonia red

21h, first experiment

| | Peak A | Peak B | Ratio A/B |
|-------------|---------|--------|-----------|
| Replicate 1 | 1,37 | 1,20 | 1,14 |
| Replicate 2 | 1,37 | 1,50 | 0,91 |
| Replicate 3 | 1,46 | 1,26 | 1,16 |
| | | | |
| Average | 1,40 | 1,32 | 1,07 |
| StDev | 0,05 | 0,16 | 0,14 |
| RSD% | 3,71154 | 11,836 | 12,68234 |

16h

| | Peak A | Peak B | Ratio A/B |
|-------------|--------|--------|-----------|
| Replicate 1 | 0,57 | 0,96 | 0,59 |
| Replicate 2 | 0,64 | 1,16 | 0,55 |
| Replicate 3 | 0,58 | 1,11 | 0,52 |
| | | | |
| Average | 0,60 | 1,08 | 0,56 |
| StDev | 0,04 | 0,10 | 0,04 |
| RSD% | 6,3451 | 9,6672 | 6,4398753 |

24h

| | Peak A | Peak B | Ratio A/B |
|-------------|---------|--------|-----------|
| Replicate 1 | 0,62 | 1,28 | 0,48 |
| Replicate 2 | 0,50 | 0,99 | 0,51 |
| Replicate 3 | 0,53 | 1,01 | 0,52 |
| | | | |
| Average | 0,55 | 1,09 | 0,50 |
| StDev | 0,06 | 0,16 | 0,02 |
| RSD% | 11,3545 | 14,814 | 4,0003277 |

42h

| | Peak A | Peak B | Ratio A/B |
|-------------|--------|--------|-----------|
| Replicate 1 | 0,83 | 1,65 | 0,50 |
| Replicate 2 | 0,86 | 1,66 | 0,52 |
| Replicate 3 | 0,84 | 1,71 | 0,49 |
| | | | |
| Average | 0,84 | 1,67 | 0,50 |
| StDev | 0,02 | 0,03 | 0,01 |
| RSD% | 1,8113 | 1,921 | 2,6689903 |

Ammonia nonred

| | Peak A | Peak B | Ratio A/B |
|-------------|---------|--------|-----------|
| Replicate 1 | 5,75 | 0,84 | 6,85 |
| Replicate 2 | 3,18 | 0,62 | 5,13 |
| | | | |
| Average | 4,47 | 0,73 | 5,99 |
| StDev | 1,82 | 0,16 | 1,21 |
| RSD% | 40,7002 | 21,31 | 20,26914 |