Universität für Bodenkultur Wien

Department für Chemie

Abteilung für Biochemie

Vorstand:

O.Univ.Prof. Dipl.-Ing. Dr.techn. Paul Kosma

Betreuerin:

Ao.Univ.Prof. Dipl.-Ing. Dr.nat.techn. Erika Staudacher

O-GLYKANE IN GASTROPODEN

Dissertation

zur Erlangung des Doktorgrades

an der Universität für Bodenkultur Wien

Eingereicht von

Dipl.-Ing. Herwig Stepan

Wien, Januar 2012

Danksagung

Auch wenn man mit Schnecken forscht, kann die Zeit wie im Flug vergehen. Darum soll an diese Stelle kurz innegehalten werden, um allen, in welcher Form auch immer, an dieser Arbeit Beteiligten Dank zu sagen.

Besonderer Dank gilt meiner Betreuerin Erika Staudacher, die mir viele Freiheiten gab und es trotzdem stets verstand mich zu begeistern und zu motivieren, wenn die Ergebnisse bestenfalls im Schneckentempo hereintröpfelten.

Selbstverständlich sei auch Rudolf und Hildegard Geyer, die mich in Gießen in ihr Team aufnahmen und mir damit tolle Ergebnisse am GC ermöglichten, herzlichst gedankt. Für die gute Zusammenarbeit möchte ich mich weiters bei Peter Kaese, Siegfried Kühnhardt, Werner Ulm, Christina Bleckmann, Tina Ulm, Sandra Frank, Kai Maaß und Sebastian Galuska bedanken.

Martin Pabst und Friedrich Altmann möchte ich für die Arbeit am ESI danken, die das Gesamtprojekt zu einem runden Abschluss führte.

Selbstverständlich haben auch mein Beraterteam sowie viele andere, die mir mit Rat und Tat zur Seite standen, einen wesentlichen Beitrag zum Erfolg dieser Arbeit geleistet: Johannes Grillari, Thomas Rosenau, Lukas Mach und Rudolf Krska, sowie Paul Kosma, Falk Liebner, Matthias Schreiner, Athanassios Giannis und Ebrahim Razzazi-Fazeli.

Für die schöne gemeinsame Laborzeit mit lehrreichen Gesprächen und auch viel Spass möchte ich mich bei Ahmed Aufy, Teresa Riegler, Josie Grass, Alba Hykollari, Birgit Schiller, Kathi Paschinger, Iain Wilson und Dudo Rendic bedanken.

Abschließend möchte ich mich für die Unterstützung und Hilfe in allen Lebenslagen bei meinen Eltern bedanken.

Kurzfassung

Glykosylierung ist die häufigste post-translationale Modifikation von Proteinen. Neben anderen Formen von Glykosylierungen spielt O-Glykosylierung eine Schlüsselrolle bei der Proteinstabilität und -funktion und ist an Transport- sowie Erkennungsprozessen beteiligt. Bisher waren keine ausführlichen Daten zur O-Glykosylierung von Gastropoden verfügbar.

In dieser Arbeit wurden hauptsächlich drei Gebiete behandelt: (1) Optimierung des Proteinaufreinigungsverfahrens um saubere O-Glykane aus Schnecken präparieren zu können, (2) Anpassung von Analysemethoden an die speziellen Erfordernisse bei der Aufklärung von Glykanen aus Schnecken und (3) Strukturaufklärung der O-Glykane.

Die Monosaccharid-Analyse mittels HPLC und GC/MS zeigte neben Galaktose, Mannose, Fucose und N-Acetylgalaktosamin auch methylierte Hexosen. 3-*O*-Methyl-Mannose sowie 3- bzw. 4-*O*-Methyl-Galaktose konnten als wesentliche Bausteine von Schnecken-O-Glykanen bestätigt werden. Während alle untersuchten Arten (*Achatina fulica*, *Arianta arbustorum*, *Arion lusitanicus*, *Biomphalaria glabrata*, *Cepaea hortensis*, *Helix pomatia* und *Planorbarius corneus*) 3-O-methylierte Mannose aufweisen, konnten beide Formen der methylierten Galaktose nur in *C. hortensis*, *H. pomatia* und *P. corneus* nachgewiesen werden. In *A. arbustorum* und *B. glabrata* konnte ausschließlich 3-O-methylierte Galaktose gefunden werden, ausschließlich 4-O-methylierte Galaktose wurde in *A. fulica* und *A. lusitanicus* nachgewiesen.

Die Strukturaufklärung wurde mittels PGC-LC-ESI/MS und GC/MS Bindungsanalyse durchgeführt. Für das Screening verschiedener Arten mittels PGC-LC-ESI/MS wurden Schneckenproteine mittels SDS-PAGE aufgereinigt und die Glykane direkt nach der Abspaltung durch β-Elimination aus dem Gel eluiert. So war es möglich eine „Kernstruktur“ zu finden, die, bestehend aus einem proteingebundenen N-Acetylhexosamin und zwei weiteren methylierten Hexosen, um weitere Hexosen, Methyl-Hexosen oder Fucose vergrößert sein kann. Diese „Kernstruktur“ war zugleich die am häufigsten gefundene O-Glykan-Struktur in *A. fulica*, *A. lusitanicus* und *H. pomatia*. *B. glabrata* und *C. hortensis* zeigten eine um eine bzw. zwei Hexosen verlängerte Struktur als Haupt-O-Glykan. Lediglich *P. corneus* wies eine unmethylierte Struktur bestehend aus einem N-Acethylhexosamin und vier Hexosen als Hauptstruktur auf.

Zuletzt wurde die „Kernstruktur“ von *A. lusitanicus* mittels GC/MS Bindungsanalyse als zwei terminale 4-O-Methyl-Galaktosen, die über ein 3,6-disubstituiertes N-Acetylgalaktosamin an das Protein gebunden sind, entschlüsselt.

Schlüsselwörter: Schnecke, Gastropode, O-Glykan, Methylierung

Abstract

Glycosylation is the most frequent post-translational modification of proteins. Among other forms of glycosylation, O-glycosylation has an important role on protein stability and function and is also involved in transport and recognition processes. So far, no extensive data on the O-glycosylation potential of gastropods has been available.

Throughout this work, mainly three working areas have been processed: (1) modification of the protein purification protocol to adapt it specifically for the separation of pure O-glycans from snail derived material, (2) method adaption of analytical protocols to suit the special needs in gastropod glycosylation analysis and (3) final structural elucidation of O-glycans.

Monosaccharide analysis on HPLC and GC/MS has revealed the frequent occurrence of galactose, mannose, fucose, N-acetylgalactosamine and methylated hexoses, namely 3-*O*-methyl-mannose as well as 3- and 4-*O*-methyl-galactose, as major constituents of snail O-glycans. While all investigated species (*Achatina fulica*, *Arianta arbustorum*, *Arion lusitanicus*, *Biomphalaria glabrata*, *Cepaea hortensis*, *Helix pomatia* and *Planorbarius corneus*) contain 3-*O*-methyl-mannose, only *C. hortensis*, *H. pomatia* and *P. corneus* have both forms of methylated galactose. *A. arbustorum* and *B. glabrata* were found to contain 3-*O*-methyl-galactose only, while *A. fulica* and *A. lusitanicus* contain 4-*O*-methyl-galactose only.

Structural elucidation was performed using PGC-LC-ESI/MS and GC/MS linkage analysis. For the glycan screening of multiple species via PGC-LC-ESI/MS an in-gel release method was applied. Therefore proteins are purified using SDS-PAGE and glycans are subsequently released via β-elimination from the protein and eluted from the gel. Using this approach, it was possible to determine a “core” structure consisting of a protein bound N-acetylhexosamine elongated by two methylated hexose residues which can be further modified with additional hexose, methyl-hexose or fucose residues. This structure was found to be the most abundant O-glycan in *A. fulica*, *A. lusitanicus* and *H. pomatia*. *B. glabarata* and *C. hortensis* have an elongated “core” with one or respectively two additional hexoses as most abundant structure. Only *P. corneus* displays a non-methylated glycan consisting of one N-acetylhexosamine and four hexoses.

Finally, the “core” structure of *A. lusitanicus* was determined by GC/MS linkage analysis as a 3,6-disubstituted N-acetylgalactosamine residue linked to the peptide backbone and elongated by two 4-*O*-methyl-galactoses.

Keywords: snail, gastropod, O-glycan, methylation

Table of content

1 Introduction

1.1 Glycobiology

According to the Oxford English Dictionary glycobiology is defined as “the branch of science concerned with the role of sugars in biological systems.” Although the importance of glycobiology in basic research, medicine, biotechnology and, for example, pharmaceutical production is generally accepted today, this specific research field at the border of carbohydrate chemistry and biochemistry is relatively new. The term glycobiology has been introduced only in the late 1980s when its broad influence on other related fields such as molecular genetics, protein chemistry, cell biology, developmental biology, physiology and medicine was discovered [1]. While early discoveries in this field date back to the nineteenth century, the big uprising of molecular biology in the 1960s and 1970s was dominated by research on DNA and proteins and the knowledge on carbohydrate moieties lagged far behind. This of course can be attributed to their high complexity and therefore difficult structural elucidation. While one specific linkage type is used to form linear polymers from nucleotides and amino acids to generate DNA, RNA or protein molecules, a monosaccharide can be connected via α or β linkage to various positions of another one. It can be calculated that from three different nucleotides or amino acids only six different trimers can be generated, while three different hexoses could theoretically generate 27648 unique trisaccharides [1] and the nine monosaccharides commonly found in humans could be assembled into more than 15 million tetrasaccharides [2]. Nevertheless today a big number of different glycoconjugates are known. Glycoconjugates such as glycolipids, lipopolysaccharides, glycopeptides or glycoproteins are known as molecules consisting of a glycone (carbohydrate-part) that is covalently linked via a glycosidic bondage at the reducing sugar end to an aglycone (non-carbohydrate-part).

1.2 Types of glycosylation

So far, many different kinds of glycosylation have been elucidated. Research has focused on human glycosylation – natural occurring glycans as well as congenital disorders of glycosylation (CDG) or glycans that are related to diseases such as cancer. However, the elucidation of the glycosylation pattern of other vertebrates and invertebrates is an emerging field.

Figure 1 gives a short overview of various different kinds of glycosylation that are found in humans. Even though also other forms are occurring, the depicted ones are the most

abundant ones. Apart from N-glycans (a) bound to asparagine residues of the protein backbone via a typical core structure consisting of two N-acetlyglucosamine and three mannose residues and O-glycans (b) which will be described in detail later, also glycosphingolipids (c), GPI-anchors (d) and glycosaminoglycans (e+f) are important structures containing carbohydrates. While glycosphingolipids consist of carbohydrates linked to the lipid ceramide, GPIs are glycans covalently bound to phosphatidylinositol. Glycosaminoglycans (GAG) may occur as free chains (e.g. hyaluronan) or linked to proteoglycan core proteins (e.g. chondroitin sulfate or heparin sulfate). One GlcNAc residue bound to cytoplasmic or nuclear proteins is considered as an additional form of glycosylation [3].

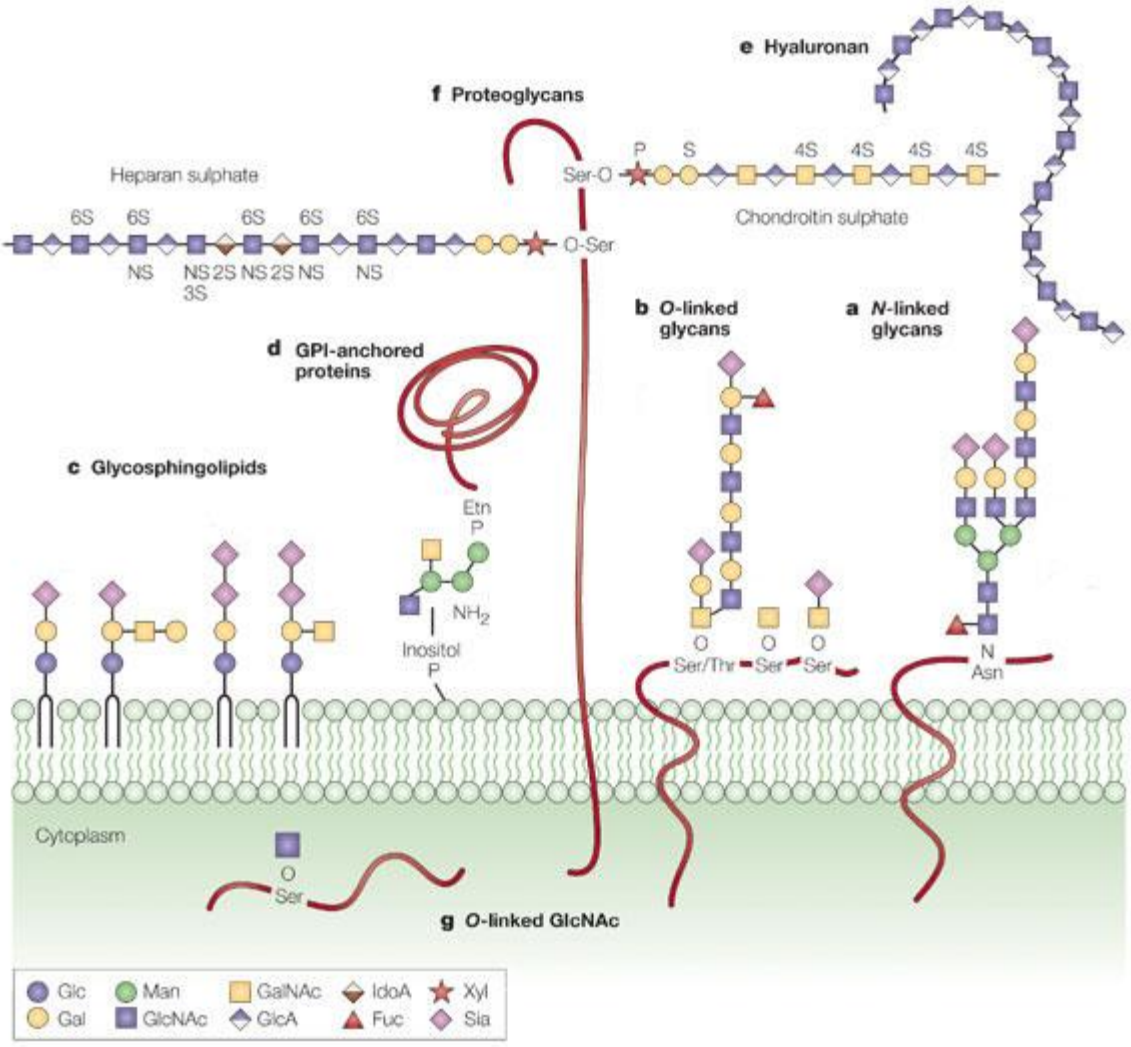


Figure 1 - Overview of different forms of glycosylation in humans (modified from Fig. 1 in [3]). [Iduronic acid (IdoA); glucuronic acid (GlcA)]

1.3 Biological functions of glycans

To gain some understanding of the importance of glycans in general, a brief overview on various glycan functions seems adequate. Since an enormous diversity of glycans has been developed in all kinds of organisms, it is not surprising that only a rather small number of glycans has been attributed to a defined function so far. Moreover this undertaking is made even more difficult by the fact that a given glycan can play different roles in different organisms or tissues at different stages of development. While glycan interactions are crucial for the development, growth or even survival of the synthesizing organisms, elucidation of all glycan functions is still an ongoing work. In the past, several different approaches have been employed: prevention of initial glycosylation or glycan chain elongation, alteration of glycan processing, enzymatic or chemical deglycosylation of completed chains or genetic elimination of glycosylation sites [1]. Unfortunately, often results gained from *in vitro* and *in vivo* studies differ significantly; alterations which don't entail any changes in cell culture are lethal to intact organisms. Conservation of glycan structures across species is also ambiguous. The N-glycan core structure has been found in all eukaryotes but outer-chain glycosylation varies vastly. Obviously one structure cannot be assigned to one universal role across species but similar functions are fulfilled by different glycans in different species.

While biological roles of glycans are often overlapping, they still can be divided into two large groups: structural/modulatory roles on the one hand side, and the specific recognition of glycans by other molecules on the other hand side [4].

1.3.1 Structural/modulatory roles of glycans

One general, undisputed function of glycans is their use as a physical barrier. While various prokaryotic cells use polysaccharide coats, all eukaryotic cells are covered by a dense glycan array. This so called glycocalyx is composed of glycans attached to matrix molecules, such as proteoglycans, and has protective as well as organizational functions. On glycoproteins for example, glycans hide the underlying polypeptide chain from recognition by proteases or antibodies.

Apart from this structural function, glycans also fulfill a number of modulatory functions. It is well known that the addition or removal of glycan structures in the endoplasmatic reticulum (ER) is an important control mechanism to ensure the proper folding of newly synthesized polypeptide chains. Correct glycosylation is a prerequisite for some proteins to exit the ER, while misfolded and incorrectly glycosylated proteins are transferred to and recycled in the proteasomes. Another function of glycans is the tuning of protein functions. Acting as an on-off switch, glycans can obviously be used to inhibit early interactions of proteins that are

synthesized in the same cell. More often tough, protein functions are not completely dependent on glycosylation but are somehow fine-tuned. The addition of sialic acids to the neural cell adhesion molecule (NCAM) acts as a good example. While NCAM usually mediates the homophilic binding between neural cells, this function is down regulated during embryonic development or in other states of neural plasticity. Polysialic acid chains act more or less as a spacer which physically separates the cells and therefore inhibit homophilic binding [1, 5].

1.3.2 Specific recognition of glycans

The second, large group of glycan functions can be united by the fact that various glycan structures act as recognition point for other molecules, most commonly glycan-binding proteins (GBP). While intrinsic GBPs recognize glycans from the same organism, extrinsic GBPs detect glycan structures from other organisms. Intrinsic GBPs usually play a role in cell-cell or cell-matrix interactions or recognition of extracellular molecules. The selectin family of adhesion molecules acts as a good example. It mediates numerous interactions between blood cells and vascular cells after the recognition of specific glycan structures. Although it has to be mentioned that very often a combination of glycan and protein structure of the ligand is detected. P-selectin which is known to bind to sialyl Lewis^x structures, also requires a sulfated tyrosine residue at the amino-terminal end of P-selectin glycoprotein ligand-1 to reach high affinity.

In contrast to that, microbial adhesins or agglutinins are considered as extrinsic GBPs. A vast variety of viruses, bacteria and parasites uses glycans for recognition of host cells, and many plant and bacterial toxins target glycans as well.

It seems feasible that the interaction of the two classes of GBPs (extrinsic/intrinsic) has been one of the driving forces in the development of more complex glycan structures [6]. Multiple cycles of changes in the glycosylation pattern of the host-cell to circumvent recognition from pathogens, followed by continuous adaption of microbes, could explain complex structures found especially on mucosal surfaces or secreted mucins, areas of frequent microbial contact. By adding glycan structures that are recognized by parasites to soluble glycoproteins (e.g. secreted mucins), the attention of toxins or pathogenic microorganisms is lead away from vulnerable cell membranes. Contrary, some microbial pathogens try to decorate their outside with host-glycan structures as an act of molecular mimicry to evade the defense mechanisms of the immune system [1, 5].

1.4 Structure and biosynthesis of O-glycans

Unlike N-glycans which have a common core structure consisting of two N-acetylglucosamine and three mannose residues, and which can only be attached to an asparagine residue of the protein backbone, O-glycans show a broad variety of core structures and attachment to the protein is also not restricted to one single amino acid. O-glycans can be attached to the protein via the hydroxyl group of serine (Ser), threonine (Thr), tyrosine (Tyr), hydroxylysine (Hyl) or hydroxyproline (Hyp). While there are algorithms available on the internet to predict O-glycosylation sites [7], so far no general consensus sequence has been found [8, 9]. Considering only O-glycan structures found in humans, so far seven different types have been found [10]. Apart from mucin-type O-glycans, which are the most common form in humans, glycosaminoglycans (GAG) as well as O-linked galactose, mannose, glucose, fucose and N-acetylglucosamine are discriminated. Non-mammalian O-glycosylation even includes O-linked arabinose or N-acetylfucosamine [11]. The whole variety of different peptide-sugar bonds that are occurring in O-glycans is depicted in Figure 1.

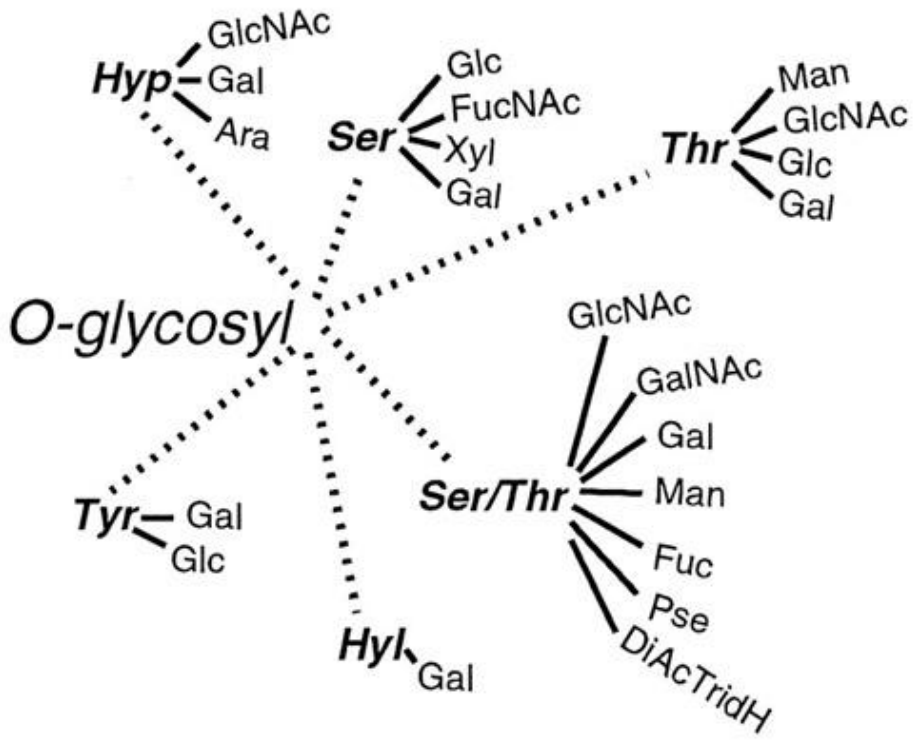


Figure 2 - Heterogeneity of various peptide-sugar bonds occurring in O-glycan structures (modified from Fig. 1 in [11]). [Pseudaminic acid (Pse); 2,4-diacetamido-2,4,6-trideoxyhexose (DiAcTridH); N-acetylfucosamine (FucNAc)]

1.5 Mucin-type O-glycans

So far, mucin-type O-glycans are the best investigated subtype. By definition, they are made up of an N-acetylgalactosamine residue which is α-linked to a serine or threonine of the polypeptide chain and which is then further elongated by N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) or galactose (Gal) residues to form one of the eight mucin-type core structures depicted in Figure 2.

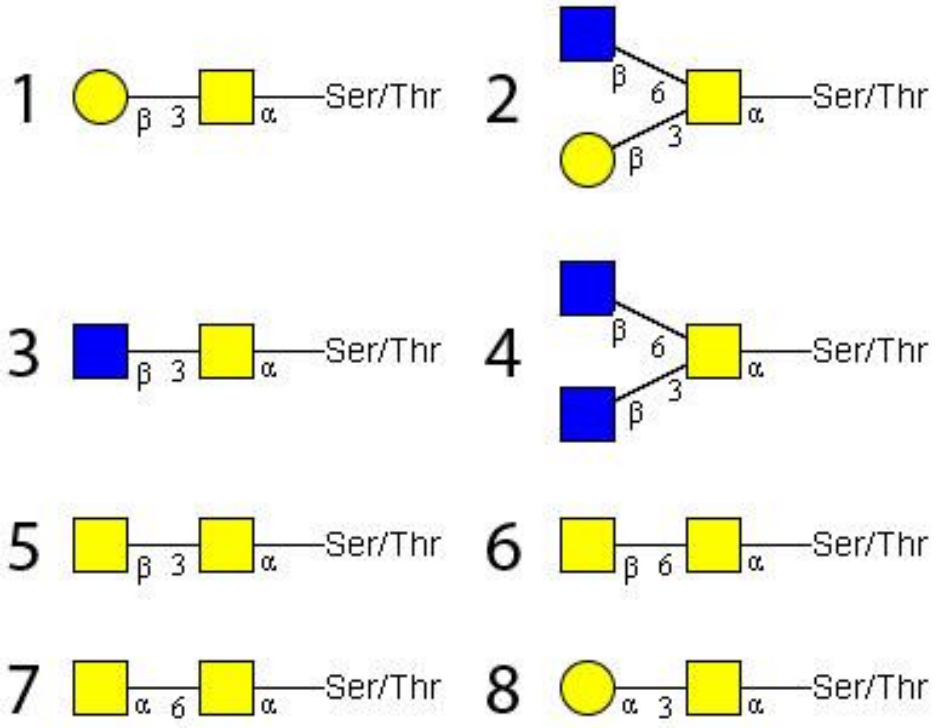


Figure 3 - Core structures 1-8 of mucin-type O-linked glycans.

This type of glycosylation not only occurs in mammals but is evolutionary conserved and found down to certain types of fungi [12, 13]. Unlike at the beginning of N-glycan biosynthesis there is no oligosaccharide transferred from a dolichol precursor but a single GalNAc is added to the hydroxyl group of the amino acid (Ser or Thr). This is initiated by an UDP-GalNAc:polypeptide N-acetylgalactosaminyl transferase (GalNAcT, EC 2.4.1.41) and results in the generation of GalNAc(α1)-Ser/Thr which is known as Tn-antigen. The location of this step may depend on the type of GalNAcT and is still disputed. It may range from the ER, an intermediate ER-Golgi compartment to the Golgi apparatus [14]. So far, there are reports of 15 distinct mammalian GalNAcTs and homology searches revealed the potential for 20 human and 18 mouse isoforms [15]. Nine functional transferases have been found in *Drosophila* [13, 16, 17] and five in *Caenorhabditis elegans* [18]. They all are type II transmembrane proteins composed of a catalytic region, a variable length stem region, a

hydrophobic transmembrane region and a short N-terminal cytoplasmic tail [15]. Differences in GalNAcT isoforms arise in their substrate preferences and while some are able to act on unmodified substrates, others need glycosylated substrates ("glycopeptides transferases") [19-22]. The addition of further monosaccharide residues is believed to take place in the Golgi apparatus [14]. While core 1 (Tn antigen) is built by addition of a Gal in a β1-3 linkage by core 1 β1-3 galactosyltransferase, β1-3 N-acetylglucosaminyltransferase is responsible for the generation of core 3. Further elongation of core 1 and 3 is catalyzed by β1-6 N-acetylglucosaminyltransferases. So far, three isoforms have been found that are capable of transforming core 1 to core 3, and one of them is also responsible for the formation of core 4 form the core 2 precursor [23].

1.6 Specific functions of O-glycans

One of the most evident functions of O-glycans is their significant contribution to the stability of a glycosylated protein. Since O-glycans are closer to the peptide backbone than N-glycans, they are more efficient in perturbing the secondary structure of a protein [14]. Therefore, they are able to confer protease and heat resistance. As a result O-glycans are frequently found on secreted and membrane-bound proteins as well as in areas of frequent microbial contact. Obviously, the attached carbohydrate reduces the susceptibility of a polypeptide to degradation by simply blocking access of the protease [24]. Other studies, for example, show that one single O-linked sugar protects the granulocyte colony stimulating factor against polymerization at 37 °C and against heat denaturation [25] or that mainly O-glycans are contributing to the protein stability of the gluco-amylase of *Aspergillus awamori* [26].

Another consequence of O-glycosylation is their influence on protein conformation and tertiary structure. Typically, sites of O-glycosylation are not spread evenly but are found clustered in sequences where Ser and Thr comprise 25-40% of the sequence. Since hydrophilic interactions and steric hindrance of the carbohydrate sidechains are stronger forces than the hydrophobic interactions that usually promote protein folding, peptide chains with many attached sugars tend to show a "bottle brush"-like structure. Mucins that are known for their high O-glycosylation potential therefore show specific rheological and hydrodynamic properties [27, 28]. The radius of gyration (R_g) is a statistical average value to describe the solution volume occupied by a certain molecule. It is influenced by the length of the amino acid chain and the flexibility of the chemical bonds linking the residues [29]. It has been shown that while deglycosylated as well as denatured approximately have the same R_g, mucins have a 2.5 to 3-fold greater R_g. This is mainly attributed to the chain stiffening effect

of the first GalNAc residue added [30]. Together with the molecular weight of about 10^7 Da, these two properties lead to very large molecules that are roughly equivalent to the size of small bacteria or cell organelles. At physiological concentrations, stable intertangled networks are formed which build up a viscoelastic gel [29]. As a result, mucin-rich fluids of the respiratory or gastrointestinal tract, for example, are highly viscous and serve as a physical barrier. Lubrification of mucosal surfaces and of the surface of the eye are other well known effects [14]. It has been shown that mucins secreted on the human ocular surface show characteristic O-glycosylation (Tn antigen and sialyl-Tn antigen) [31]. The tear film which is responsible for hydration and lubrication as well as clearance of particles or pathogens, contains excessive amounts of α(2,6)-linked sialic acid [32].

1.7 O-glycans in non-mammalian animals

Not surprisingly, the largest set of data on O-glycans is concerned with structures from mammalian origin. While there is a quite some data on human O-glycans available, the research on non-mammalian O-glycans is still at its beginning. Apart from common model organisms such as *Caenorhabditis elegans* or the fruitfly *Drosophila melanogaster*, most knowledge concentrates on species that are related to human health such as parasites.

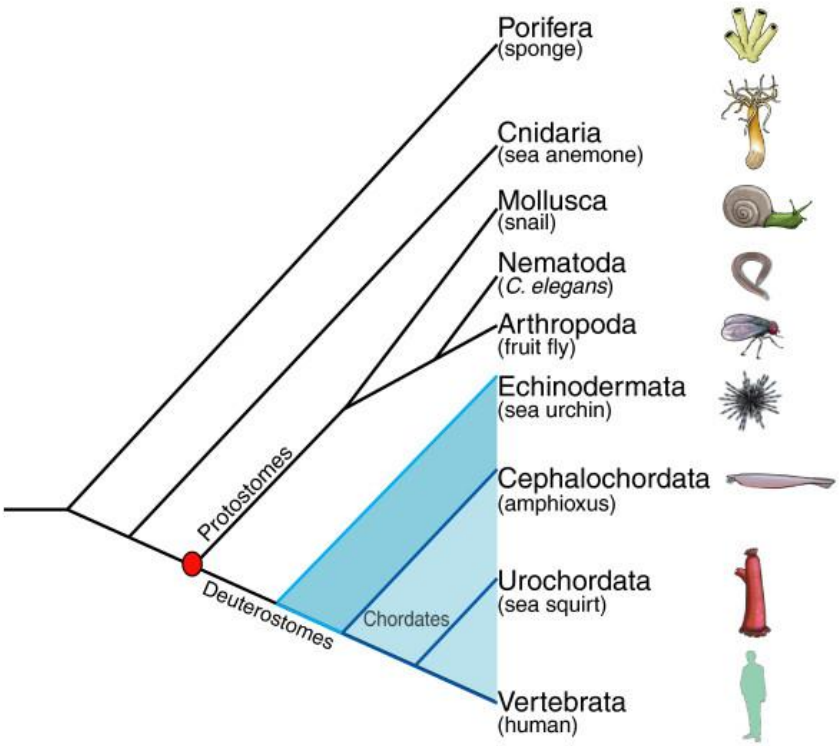


Figure 4 - Philogenetic tree of selected metazoans. The protostomes/deuterostomes split is indicated by a red circle (Fig. 1 in [33]).

As pictured in Figure 3, mollusca, nematoda and arthopoda are among the most prominent aminals of the protostome lineage. From the phylum of nematoda, *C. elegans* is the best known species in terms of O-glycosylation capacity. It was found that O-glycans from *C. elegans* either build up on a type 1-core or a core based on Gal(β1-6)[Gal(β1-3)]GalNAc. After that, a trisaccharide block consisting of GlcUA(β1-3)Gal(β1-3)GalNAc is frequently attached which further gets elongated by (β1-6)Glc and/or (β1-4)Glc residues [34]. Additionally, O-glycans were found that are attached to the peptide via a GlcNAc residue. Various structures based on this innermost sugar have been also described in *Trypanosoma cruzi* where it usually gets elongated by Gal residues [35, 36]. However, single O-linked GlcNAc units are occurring as well. This, for example, has also been reported for *Schistosoma mansoni*, *Plasmodium falciparum* or *Trypanosoma brucei* [37-39]. Apart from all that *C. elegans* is also capable of building structures including (β1-2)-linked fucose and 2-O-methylated fucose. This kind of methylated fucose has also been found in *Toxocara canis* and *T. cati* [40].

The phylum of arthopoda is further divided into chelicerata (e.g. spiders), crustacea (e.g. shrips and lobsters), myriapoda (e.g. centipedes and millipedes) and hexapoda (insects). One of the most obvious common features of this phylum is the chitin exoskeleton. Considering insects, *Drosophila melanogaster* has been found to exhibit rather simple structures. Studies on embryos have shown the occurrence of mucin-type core 1 structures only, studies on S2 cells showed the T-antigen as well [41, 42]. However, a modification of mucin-type glycans with fucose or glucuronic acid is also possible and other forms of O-glycosylation such as O-GlcNAc, O-mannose or O-fucose can be detected in addition [43]. More complex O-linked glycans have been recently described in wasp (*Vespula germanica*) and hornet (*V. cabro*). Interestingly, wasp mucins can carry charged glycans with a phosphoethanolamine group [44]. This could act as a replacement for the sialic acid or sulfate groups that are frequently found on mucin-type glycans and which confer a negative charge to the glycan. Especially, the occurrence of sialic acid in insects has been controversially discussed and seems to be a rare modification that is limited to glycoproteins associated with the nervous system [45]. However, apart from this unusual modification wasp glycans are typically based on mucin-type core 1 or 2, while hornet glycans consist of core 1 elongated by galactose and fucose residues.

The phylum of cnidaria includes, for example, sea anemonae and jellyfish (*Aurelia aurita*). Recently the O-glycosylation of jellyfish Q-mucin has been elucidated. The structures found are consisting of GalNAc as well as GalNAc modified by a phosphate or 2-aminoethylphosphonate (AEP) group at C6 [46].

In the lineage of deuterostomes the phylum of vertebrata is the best elucidated one in terms of O-glycosylation. Apart from many data available on mammals such as human, chicken and mouse (*Mus musculus*), information on frogs and zebrafish has been collected. *Danio rerio*, the zebrafish, exhibits the two structures Fuc(α1-3)GalNAc(β1-4)[Neu5Ac(α2-3)]Gal(β1-3)GalNAc-itol and Fuc(α1-3)GalNAc(β1-4)[Neu5Gc(α2-3)]Gal(β1-3)GalNAc-itol as most abundant O-glycans. In addition these structures may be oligosialyated in early developmental stages [47]. Concerning the O-glycans in frogs, egg-jelly mucins from *Xenopus laevis* have been analyzed. Here, the sequence Fuc(α1-2)Gal(β1-3)[Fuc(α1-2)]Gal(β1-3) which is commonly found in amphibians, is characteristic for one of the two main O-glycan families. The other group of O-glycans is defined by the occurrence of a blood group A epitope substituted by an (α1-3)-linked GlcNAc [48]. Recently, egg jelly from *Xenopus borealis* was analyzed in order to explain the different fertilization behavior of *X. laevis* and *X. borealis*. Mainly, the observed structures are mucin-type core 1 or 2 structures but previously unobserved structures with hexose residues at the reducing end were found as well. In contrast to the findings in *X. laevis* (mostly sulfate-groups) only neutral oligosaccharides were detected and surprisingly not even one O-glycan structure has been found in both species [49].

1.8 Glycosylation in gastropods and parasites

Even though the research on glycans from snail origin has been quite limited in the past, gastropods have already shown a tremendous capability of producing a broad variety of different glycan structures. They are capable of producing glycan structures such as sialyation or (α1,6)-linked fucose which are known, for example, from mammals, (β1,2)-linked xylose which occurs, for example, in plants and trematodes, (α1,3)-linked fucose which has been found, for example, in insects and methylation which can be found among other species in nematodes.

The neutral N-glycans of *Arion lusitanicus*, *Limax maximus*, *Cepaea hortensis*, *Planorbarius corneus*, *Arianta arbustorum* and *Achatina fulica* have been analyzed previously in this working group [50, 51] and a review on N-glycosylation in gastropods was recently published [52].

Especially, the ability of snails to methylate glycans is interesting. So far, the biosynthetic pathways for this modification are not elucidated and it can only be speculated if the methyl groups are added to the glycan or if methylated sugar nucleotides are used for the synthesis. Apart from that, the exact reasons for the use of methylated sugar constituents remain unclear. However, methylation was first reported in *Helix pomatia* by Hall and Wood in 1977

and later on affirmed in other snails (for example: *Rapana thomasiana*, *Rapana venosa* and *Biomphalaria glabrata*) and mollusks such as the giant clam *Hippopus hippopus* and the fresh water bivalve *Hyriopsis schlegelii* [53-59]. Finally, 3-*O*-methyl-mannose as well as 3- and 4-*O*-methyl-galactose have been confirmed to be frequent snail glycan constituents in N- and O-glycans [60].

As well interesting are investigations that deal with the glycosylation pattern of snails infected by the blood fluke *Schistosoma mansoni* [61, 62]. By shuttling between invertebrate vectors and vertebrate hosts, they continually have to adapt to various extremely harsh environments. Host-cell invasion and the protracted deception of the host's immune system are obviously the most striking features. Studies on various different parasites have shown the strong participation of glycoconjugates in these processes to circumvent host defence mechanisms.

Trematodes of the genus *Schistosoma* are responsible for a chronic human disease called schistosomiasis which affects 200 million people worldwide [63]. It is one of the most formidable trematode related diseases which results in a wide-ranging pathology and is the second most devastating parasitic disease concerning socioeconomic and public health impact after malaria. Various studies have shown that the major humoral immune response to schistosomiasis is to carbohydrate-based antigens. The three major species *Schistosoma japonicum*, *S. mansoni* and *S. haematobium* synthesize a rich array of N- and O-glycans on glycoproteins, glycosphingolipids, polysaccharides and GPI-anchors [64]. Many structural elements resemble those found in mammalian sources. High mannose-type N-glycans with up to nine mannose residues are found as well as complex di-, tri- and tetraantennary structures. Terminal GalNAc(β1-4)GlcNAc (LacdiNAc), GalNAc(β1-4)[Fuc(α1-3)]GlcNAc (fucosylated LacdiNAc) and Gal(β1-4)[Fuc(α1-3)]GlcNAc (Lewis X) are frequently found [65-67]. Especially fucosylation seems to be a common theme for schistosome glycoconjugates. Also remarkable is the absence of sialic acids and sialyltransferase activity [1]. O-glycans are typically simple structures including GalNAc(α1)-Ser/Thr, Gal(β1-3)GalNAc(α1)-Ser/Thr or GlcNAc(β1)-Ser/Thr [39, 68].

Recent studies have identified the N-glycan structures of *Biomphalaria glabrata* and *Megathura crenulata* that show cross-reactivity with *Schistosoma mansoni* glycoconjugates [56, 69]. There is evidence that the susceptibility of the snail to an infection is dependent on the occurrence of specific carbohydrate determinants in the snail and the trematode [70].

Since antibody responses to hemocyanin of *Megathura crenulata* can be used for the serological distinction between acute and chronic infection with *Schistosoma mansoni* in humans [71, 72], there might also be other medical applications for snail derived glycans.

Apart from snail transmitted diseases further including fascioliasis, clonorchiasis and paragonimiasis, damages to crops and vegetables are reasons to spend more effort on the pest control of snails. The inhibition of snail specific glycosylation enzymes or targeting of snail specific glycan structures could be possible ways to suppress snail reproduction or to hinder snail survival.

1.9 Glycosylation analysis

The analysis of the complete glycosylation pattern of an organism (glycome) or the elucidation of the various glycoforms of an isolated, single protein poses numerous different obstacles to the scientists. The vast structural heterogeneity of glycan structures usually demands some kind of separation before the analysis and the often small sample amounts limit the possibilities of detection. Many handling steps and transfer procedures can lead to a significant loss of sample, for example through adsorption on laboratory glassware, and also contamination, for example with reagent impurities. Finally the structural investigation arises as third big challenge. This explains why until now no universal method for the analysis of protein glycosylation has been found. Depending on the specific research goals a combination of various procedures and analyses has to be applied [73].

Mainly, glycan analysis is possible on three levels: intact protein, glycopetide or isolated glycan. All three have their advantages and drawbacks which will be discussed below and are reviewed nicely in [74, 75]. An overview on methods especially suited for O-glycome analysis is given in [76].

1.9.1 Analysis of intact glycoproteins

One of the most commonly used protein separation techniques is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) where proteins are separated according to their molecular weight. Although this is a very stable and reliable method, it has to be kept in mind that glycoproteins can migrate slightly differently than non-glycosylated proteins. While heavy glycosylation leads to a lower SDS binding and therefore lower mobility in the gel which results in an overestimation of the molecular weight, certain carbohydrate moieties can cause the opposite. Naturally occurring negative charges, for example from sialic acids, can drive a faster passage through the gel und therefore an underestimation of the protein weight. As extension to this method, 2-dimensional separation can be performed where proteins are not only separated according to their molecular weight but also according to their isoelectric points. Typically, the analysis of glycoproteins shows characteristic clusters of protein spots due to the slightly changed molecular weight or isoelectric points of various

different glycoforms of the same protein. However, especially heavily glycosylated proteins are often underrepresented on 2-D gels after application of common staining techniques because the high carbohydrate content interferes with the dyeing process.

However, this can be compensated by the use of carbohydrate-specific lectins which are applied to the electroblotted proteins. By using various different lectins at least some preliminary data on the carbohydrate epitopes of the separated proteins can be generated [77].

Apart from detection of blotted glycoproteins, lectins can also be used for the fractionation of complex protein matrices. Lectin affinity chromatography is a very valuable tool to separate glycoforms with a distinct glycan structure from a total protein pool [78, 79]. By using various lectins with different selectivity in one single column it is even possible to capture nearly all glycoproteins of a protein sample and to elute them stepwise with specific displacers [80].

Apart from protein separation via gel electrophoresis or lectin affinity chromatography, capillary electrophoresis (CE) has been used successfully to resolve different glycoforms of, for example, therapeutically used proteins like recombinant human erythropoietin or alpha1-acid glycoprotein [81-83]. Although it has to be noticed that CE alone cannot elucidate the glycan structures attached to the proteins and additional analysis such as mass spectrometry is necessary.

Even though mass spectrometry is capable of analyzing glycoproteins, some limitations have to be considered. First, the large heterogeneity of glycans attached to the proteins makes the analysis of one single glycoforms quite difficult. While relatively small glycoproteins like erythropoietin with a mass below approx. 40 kDa can be analyzed by matrix assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF/MS) [84], larger molecules tend to show unresolved peaks. Since mass accuracy and low resolution pose a large problem at the analysis of glycoproteins using this ionization technique, various glycoforms tend to form unresolved, broad peaks and cannot be discriminated [85]. The second, commonly used MS technique is electrospray (ESI) MS. Although developments in this analysis technique enabled successful characterization of some highly glycosylated glycoproteins such as recombinant antibodies [86], analysis of highly glycosylated proteins is by far more difficult than the analysis of proteins without carbohydrates attached. The desolvation of the sample during the spraying process is hindered due to the hydrophilic properties of carbohydrates and salts which bind to the carbohydrate moieties form additional adducts and therefore raise the complexity of the chromatogram.

1.9.2 Analysis of glycopeptides

To gain a more detailed insight in the function and location of various glycan moieties within the protein, the analysis of glycopeptides is usually used. Therefore, proteins are digested with endoproteinases to reduce the molecular weight and allow a more detailed investigation. Most commonly endoproteinases like pepsin, trysin or chemotrypsin are used that have defined cleavage sites and produce peptides that typically carry just one glycosylation site [87, 88]. Digestion steps with non-specific endoproteases like pronase tend to result in glycans attached to very small peptides or just one single amino acid [89].

The next step of analyzing glycopeptides usually is an enrichment or separation step to remove non-glycosylated peptides. These peptides not only often are the majority of peptides but they also have higher signal intensities and can suppress glycopeptide signals [90]. For this separation multiple options such as size exclusion [91] or hydrophilic interaction chromatography are available. The latter separation methods works through the fact that the peptides are rather hydrophobic while glycopeptides carry a hydrophilic moiety [92, 93]. Alternatively, lectins can be applied to extract glycopeptides from the total peptide mixture. Before using this approach, the used lectins have to be carefully selected. While lectins with a narrow selectivity can act as “lectin affinity selectors” [94], lectins with a broad selectivity like Con A are used to extract most glycopeptides in one single step [95, 96]. A combination of multiple lectins sequentially or together in one single column have also successfully been used [97, 98]. A more detailed insight into the possibilities of lectin affinity chromatography will be given in 1.9.4.2.

Purified glycopeptides can further be analyzed on reversed phase (RP)- or normal phase (NP)-HPLC [99, 100] with or without enzymatic digestion steps to allow structural elucidation. However, nowadays coupling with tandem mass spectrometry is more convenient and delivers more data on the investigated structures. Specific markers ions such as low-molecular weight oxonium ions help at identifying peptides from glycopeptides [101]. By using versatile ion trap spectrometers it is possible to set up the instrument setting to either elucidate the glycan structure or the peptide sequence and the glycan attachment site. Low energy collsion-induced dissociation (CID) fragmentation experiments can be used to fragment the glycosidic bonds in the first step, while the peptide stays intact. For N-glycopeptides the first GlcNAc residue often stays attached to the peptide backbone. This allows further fragmentation of the resulting peptide in a second step. Here, electron-transfer dissociation (ETD) fragmentation is used to cleave the peptide into amino acids. With the attached GlcNAc residue the exact position of the N-glycan can be determined [102]. MALDI-

TOF-TOF/MS is also capable of analyzing the carbohydrate and peptide part of glycopeptides [103].

1.9.3 Analysis of released glycans

A variety of different methods are being used for the analysis of glycans. Unfortunately none of them can be considered as method of choice under all possible circumstances. While NMR, for example, fully elucidates the structure, does not need any preceding labeling step and besides does not destruct the sample, the sample demands in terms of purity and amount can often not be achieved. So, usually a combination of different methods is used to generate enough data to be able to ascertain a final structure.

Of course, first of all glycans have to be released from the peptide backbone. This can be done either enzymatically or chemically. While on the one hand side chemical methods usually ensure a complete release but are on the other hand rather harsh and can alter the glycan, substrate specificities are crucial for a complete release when enzymes are used. The enzyme most commonly used for the analysis of N-glycans is PNGase F (peptide-n-glycosidase F) of *Flavobacterium meningosepticum*. While it is capable of releasing N-glycans from undigested proteins, it will not cleave carbohydrate chains from a single asparagine residue or from an asparagine that is at the very end of a polypeptide chain. Additionally, the action of PNGase F is hindered when the terminal GlcNAc residues is substituted by an α(1-3)-linked fucose [104]. However, the sugar hindrance can be avoided by using PNGase A which can be applied to glycopeptides only. In both cases, the asparagine residues are converted into aspartic acid during the release process. This is especially interesting if the glycosylation attachment sites are evaluated by mass spectrometry.

Another class of enzymes used for partially releasing N-glycans are endo-β-N-acetylglucosaminidases which split the chitobiose unit between the two GlcNAc residues. Again, substrate specificities are crucial for the success of the release. While Endo H and Endo F1 accept oligomannosidic- and most hybrid-type N-glycans, Endo F2 and F3 can be used on certain complex-type glycans only [105].

Unfortunately, the enzymatic arsenal for the release of O-glycans is quite limited. Since O-glycans are much more diverse in terms of amino acid attachment sites and also have no common core structure of the sugars, the lack of a universal O-glycan releasing enzyme is to bewail. Unlike the name would suggest, commercially available O-glycanase from *Diplococcus pneumoniae* or *Alcaligenes sp.* is limited to the recognition of mucin-type core 1 O-glycans only.

So, especially for the liberation of O-glycans chemical methods are applied. The two methods that are usually used are hydrazinolysis and β-elimination. Release of carbohydrates via hydrazinolysis has been first reported more than 45 years ago [106] but still today after quite some improvements and refinements on the original protocol [107-109], the work is quite troublesome. In theory, six hours of hydrazinolysis at 60 °C should be sufficient to cleave all O-glycans while leaving N-glycans still attached to the peptide backbone. Incubation at 95 °C instead was reported to totally cleave N- and O-glycans [110]. The release of intact and non-reduced glycans [111] should easily allow subsequent derivatization with 2-aminobenzamide [112] or 2-aminobenzoic acid (anthranilic acid) [110]. Nevertheless, the use of highly toxic hydrazine and undesired chemical side reactions such as the loss of N-acetyl groups are disadvantages and a comparison of the number of publications during the last decade on hydrazinolysis and β-elimination clearly shows that most researchers favor the latter.

Therefore β-elimination under alkaline conditions is nowadays commonly used for the release of O-glycans. Apart from the original protocol [113, 114], modifications using milder conditions at a lower reaction temperature [115] were found to release almost exclusively O-glycans while N-glycans remain attached to the peptide backbone and do not interfere with O-glycan analysis [116]. However, a reducing agent is added to the reaction mixture in order to prevent “peeling” reactions that would occur under the alkaline conditions. This converts the first sugar of the carbohydrate chain into an alditol which is not susceptible to peeling reactions but unfortunately cannot be derivatized with any kind of label. This not only hampers, for example, subsequent HPLC analysis but also makes MS analysis more difficult because alditols have been found to have less intense MS peaks compared to their corresponding derivatives labeled with a chromophore or fluorophore [117].

Alternatively, non-reductive β-elimination can be applied. The use of aqueous ethylamine instead of the typical sodium borohydride/sodium hydroxide solution was suggested [118] and later the application of a borane-ammonia complex in aqueous ammonia was published to non-reductively release glycans [119]. Very recently, two working groups published methods which combine the release of O-glycans directly with their labeling using 1-phenyl-3-methyl-5-pyrazolone. As releasing agent, an ammonia aqueous solution [120] or dimethylamine [121] is suggested.

As mentioned before, labeling of glycans enables HPLC or CE analysis and improves MS analysis results. Reductive amination is used to attach a chromogenic or fluorescent group to the aldehyde group of the reducing end. Usually aromatic amines such as 2-aminopyridine (PA), 2-aminobenzamide (AB) or anthranilic acid (AA) are used for chromatographic profiling

[122, 123], while 2-aminonaphtalene-1,3,6- trisulfonic acid (ANTS) or 8-aminopyrene-1,3,6-trisulfonate (APTS) are widely used in CE analysis [124, 125].

1.9.4 General methods for separation of glycostructures and glycosylation analysis

1.9.4.1 High performance liquid chromatography

Various high performance liquid chromatography (HPLC) approaches are useful for the separation of complex glycan mixtures or a preliminary profiling. It is especially useful for the fractionation of isobaric compounds which are hardly discriminated via MS, and furthermore allows a more reliable quantification than those based on MS signal intensities.

1.9.4.1.1 Normal-phase chromatography

Normal-phase (NP) chromatography as well as its variant hydrophilic interaction chromatography (HILIC) [126] are based on the combination of a polar stationary phase with a non-polar, non-aqueous mobile phase. Column packings usually consist of silica bonded with amide, cyano or other strongly polar groups. For the use at highly acidic or basic conditions, polymeric packings have been developed. Elution times generally increase with size and by comparison of retention times of labeled glycans (for example with PA) with glucose oligomers, predictions on structures can be made [127].

1.9.4.1.2 Reversed-phase chromatography

As complementary technique to NP-HPLC, analytes are separated according to their hydrophobicity on non-polar stationary phases during reversed-phase (RP) chromatography. The most commonly used material is silica with bonded octadecyl groups. Polar solvents which are mixed with an increasing amount of organic solvent are used for the extraction. It can be noticed that analytes roughly elute with increasing molecular mass [128], but branched oligosaccharides show shorter retention times than their linear counterparts [129].

In conjunction with NP-HPLC, both techniques can be used for the 2-dimensional separation of labeled glycans which cannot be resolved with one separation step alone [130].

1.9.4.1.3 Porous graphitized carbon chromatography

Compared to other HPLC media, porous graphitized carbon (PGC) is a relatively new and still today not completely understood technique. It is clear so far that hydrophobic and polar interactions [131] as well as ionic interactions are responsible for the retention of

oligosaccharides [132, 133]. Elution times increase with growing molecular weight but linear molecules show shorter retention times that branched ones. But not only sequence and branching influence the adsorption, linkage and the anomeric conformation contribute to the retention time. As a result, PGC can be used for the separation of isobaric compounds that are difficult to discriminate using MS [134]. It was demonstrated that PGC is capable of resolving numerous isomers, for example, five different fractions of high-mannose type N-glycans carrying eleven mannose residues from a recombinant invertase [135].

Since PGC material strongly retains labeled as well as unlabeled glycans which are hardly retained on RP material, solid phase extraction (SPE) using PGC is nowadays frequently used for the desalting of glycan samples [133]. Therefore oligosaccharides in aqueous solution are applied to PGC SPE cartridges. While glycans bind strongly to the column, salts can be removed by extensively washing the column with water. Later on glycans are released using acetonitrile with trifluoroacetic acid or formate buffer [136].

Nevertheless, PGC-HPLC is still a rather seldom exploited technique which is probably due to the lack of databases containing data on glycan retention times on such columns [137].

1.9.4.1.4 Anion-exchange chromatography

Anion-exchange chromatography is a way to selectively remove negatively charged glycans from a total glycan pool. This is especially useful to reduce the heterogeneity in a complex mixture but is also a prerequisite for some analysis steps. Negatively charged glycans carrying, for example, sialic acids or sulfate groups demand special protocols in order to enable subsequent MS analysis. The separation of uncharged from negatively charged glycans allows the use of different analytical methods which fit every type best.

Another method that has been used for many years in carbohydrate analysis is high performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD). In a very alkaline environment (pH > 12), carbohydrates form negatively oxyanions which interact with anion-exchange media. While this is a fast and reliable method that does not require any kind of sample derivatization and allows great separation of various structural isomers, it also has its drawbacks. Epimerization of GlcNAc to ManNAc and a possible degradation of the reducing end as well difficult quantification and high salt concentrations which poses a problem for subsequent MS analysis. Although online desalting devices are available [138], high salt concentrations demand desalting via gel filtration or dialysis.

1.9.4.2 Lectin affinity chromatography

A valuable tool for the separation of glycoproteins or -peptides from a mixture of glycosylated and non-glycosylated proteins and peptides or released glycans from a total pool of glycans, in order to reduce the heterogeneity of the available glycans and to facilitate the analysis, is lectin affinity chromatography. A number of different lectins, which are proteins that bind specific carbohydrate determinants, have been successfully used for this approach [79]. Just to mention one example, concanavalin A (Con A) is frequently used for the enrichment of N-glycans. This lectin binds oligomannosidic as well as hybrid and di-antennary complex type N-glycans. Even though this lectin is suitable for the binding of many N-glycans, it has to be noted that tri- and tetraantennary complex type structures are not bound and certain types of fucosylation can also interfere with the binding interaction [139]. It also has to be mentioned that binding affinities vary significantly from structure to structure. Other well known lectins are *Sambuccus nigra* agglutinin (SNA), *Maackia amurensis* leukoagglutinin (MAL), *Ulex europaeus* agglutinin I (UEA-I) or *Lens culinaris* agglutinin (LCA). There are even snail derived lectins used: *Helix pomatia* agglutinin (HPA) and *Limax flavus* agglutinin (LFA). To employ these glycan binding proteins in a chromatography step, they are covalently bound to a carrier such as agarose or sepharose. By using several lectin affinity columns in succession, it is not only possible to capture a large number of different glycans but some structural predictions can be made as well. Of course these have to be confirmed by subsequent MS analysis steps to allow a complete structural assignment. To speed up this purifications procedure, different lectins may be combined in one single column [140, 141], and direct coupling with ESI/MS to screen large numbers of samples is also possible [100].

1.9.4.3 Enzymatic sequencing

Some information on the nature of specific glycans might also be extracted with the help of enzymatic digestions. By applying various exoglycosidases to the sample, the terminal sugars and with subsequent digestions steps also the following sugars can be investigated. Depending on the enzymes used, data on the type of sugar, its linkage and anomericity can be generated. Usually multiple aliquots of the sample are incubated with a number of enzymes and the results are assayed using HPLC or MALDI-TOF/MS. An elegant technique requiring only picomolar amounts is to perform the digestion right on the MALDI target [142]. After analyzing the impact of the first exoglycosidase, it is possible to apply the next enzyme for the digestion of the new terminal sugar.

Other methods, such as the reagent-array analysis method, for the complete sequencing of an oligosaccharide using enzymes only, have also been published [143].

However, it has to be noted that exoglycosidases have to be tested thoroughly for their activity and specificity and that for some kind of sugar or linkage no enzyme might be available. Other carbohydrate modifications such as methylation are also impeding this approach.

1.9.4.4 Fluorophore assisted carbohydrate electrophoresis

Based on PAGE, an alternative protocol for the separation of labeled mono- and oligosaccharides was introduced by Jackson [144, 145]. Therefore released glycans are labeled with fluorophores such as ANTS, APTS or 2-aminoacridone (AMAC) [146], separated on an acrylamide gel and then subjected to UV light in order to detect the fluorescence signal. While ANTS and APTS confer charges to the oligosaccharide and can be used on neutral glycans as well, AMAC can be used to selectively detect sialyated and therefore naturally charged glycans. Only by using a different buffer system (borate buffer instead of the usually used Tris-glycine buffer) which leads to the formation of charged complexes, neutral AMAC labeled glycans can be separated. The detection of labeled bands is performed best by using a UV light box which is commonly used for taking pictures of, for example DNA agarose gels.

While this is a convenient method to check changes in the glycosylation pattern of an isolated glycoprotein, analysis of complex mixtures and structural elucidation is difficult. As described in more detail in 1.9.4.3, various exoglycosidases may be used to digest the sample and to check the mobility shifts [147], or gel bands may be excised and be subjected to MALDI-TOF/MS analysis after extraction of the glycan. However, very heterogeneous samples demand complementary techniques such as NP-HPLC in order to allow sufficient resolution of all structures.

1.9.4.5 Capillary electrophoresis

Capillary electrophoresis (CE) is applicable on glycoproteins as well as released glycans. The migration of charged molecules in an electric field is exploited by this technique. While CE is applicable to small sample amounts usually available in the basic research of single glycan structures, also large sample amounts can be separated which is often necessary for the purification of therapeutic glycoproteins in the pharmaceutical industry. To mention only a few examples of medically important glycoproteins which have successfully been separated concerning their glycoforms on CE, human recombinant coagulation factor VIIa (rFVIIa) [148], human recombinant bone morphogenetic protein 2 (rhBMP-2) [149] or natural and recombinant interleukin-2 [150] should be cited. Apart from capillary zone electrophoresis

(CZE) which is usually used for glycoprotein separation, other variations of CE such as capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE) or micellar electrokinetic chromatography (MEKC) are available. An overview on different glycoproteins separated by CE can be found in the review by Mechref and Novotny [75].

Since the separation is driven by the application of an electric field, uncharged molecules cannot be analyzed which makes a significant difference to HPLC [151]. This also explains why derivatization with ANTS or APTS (1.9.3) is a prerequisite for glycans which do not naturally carry charged groups such as sialic acids, phosphate or sulfate groups. The labeling with fluorescent markers also adds the possibility to use a more sensitive detection method than UV absorbance. Alternatively, the complex formation with metal cations or highly alkaline conditions can be used to analyze uncharged glycans in their native state. While CE is capable of separating complex glycan mixtures fast and at high resolution, again coupling with mass spectrometry is necessary to allow detailed profiling of glycans [152]. Even though there are approaches to define the structure only by comparison of retention times that are available in a carbohydrate-mapping database, their use is limited [153]. Since O-glycans which were released using reductive β-elimination cannot be labeled, the possibilities of CE analysis are limited.

1.9.4.6 Mass spectrometry

During the last decades the importance of mass spectrometry (MS) techniques has increased constantly within many fields of biochemical research. Mainly two techniques are used today for the analysis of glycoproteins, -peptides or released glycans: MALDI-TOF/MS and ESI/MS. Both techniques are highly sensitive and allow analysis of samples in the pico- to femtomolar range. Both instruments can be coupled to liquid chromatography or CE and therefore allow a fast and automated screening of large numbers of samples. Since the ionization is rather soft, almost no fragmentation occurs and quasi-molecular ions are produced which allows mass profiling and the assignment of monosaccharide compositions [74].

Unfortunately, stereoisomers such as GalNAc, GlcNAc and ManNAc cannot be distinguished because the mass of all three is similar. Therefore structural assignment of glycans is dependent on the knowledge of the biosynthetic pathways or other methods such as enzymatic digestion or linkage analysis to discriminate the various sugars with the various classes of monosaccharides (for example hexoses, deoxyhexoses or N-acetylhexoses).

1.9.4.6.1 MALDI-TOF/MS

Considering all kinds of mass spectrometry, MALDI-TOF/MS is one of the most commonly used ones. This is mainly caused by the simplicity and speed of the analysis. Singly charged molecules facilitate the analysis of complex mixtures and allow a rapid assignment of known structures.

As indicated by the name, MALDI-TOF/MS requires the addition of a matrix to the sample which supports the desorption/ionization process. 2,5-dihydroxybenzoic acid (DHB) was among the first matrices developed for MALDI-TOF/MS and still remains the first choice for carbohydrate analysis [154]. Many modifications such as the addition of 2-hydroxy-5-methoxybenzoic acid (super-DHB) [155] or α-L-fucose [156] have been found to increase the sensitivity or reproducibility of the analysis. Apart from that, until today many different matrices such as 3-aminoquinoline (3-AQ), 2,5-dihydroxyacetophenone (DHA) or 6-aza-2-thiothymine (ATT) have been successfully used on various kinds of sample and the matrix of choice should be selected depending on sample preparation, type of experiment and instrument available. Especially analyses of permethylated samples or experiments in the negative ion mode might give better results using a different matrix than DHB.

Application of sample and matrix to the target plate is also a crucial step since it influences the crystallization of the sample spot. While some protocols suggest the sequential application, others recommend to mix matrix and sample thoroughly. Application of two layers of matrix has been shown to increase sample intensities and was recommended for low concentrated samples [157]. While some labs seem to prefer a fast drying process in a vacuum chamber, others suggest a slow drying process to generate a heterogeneous spot.

One parameter which clearly negatively affects the ion yield and crystal formation of every matrix is the presence of salts or certain buffers. While this is true for all MALDI-TOF/MS experiments, carbohydrates have been found to be especially susceptible to this obstacle [158]. While drop dialysis as well as short ion exchange or RP columns have been used for that in the past, PGC SPE nowadays seems to be the most practicable way.

As said before, compositional data based on the total molecular mass can easily be calculated according to the mass increments of the various possible constituents. However, analysis of unknown structures generally necessitates the fragmentation of the structure using MALDI-TOF-TOF instruments. Depending on the energy and time used for fragmentation, two types of cleavages may occur: glycosidic cleavages and cross-ring cleavages. While cleavages of the glycosidic bonds between the monosaccharides give some information on the sequence and branching of the glycan, no information on the linkage type is gained. To obtain data of linkage positions, cross-ring fragments have to be

generated and analyzed. The question if glycosidic or cross-ring cleavages will occur, can be answered by checking the method which is used to fragment the analyzed ions. While postsource decay (PSD) and insource decay (ISD) generally lack cross-ring fragments, collision-induced dissociation (CID) is capable of generating cross-ring fragments. This is mainly due to the higher energy that is transferred in the collision cell that is responsible for the CID.

1.9.4.6.2 ESI/MS

The ESI technique acts as link between liquid samples and MS analysis. Unlike MALDI-TOF/MS where samples have to be dried and crystallized, ESI/MS is capable of analyzing liquid samples that are injected directly (offline) or via liquid chromatography (online). Especially online LC-ESI/MS allows separation and analysis of complex glycan mixtures in one step. Most commonly NP, RP or PGC columns are used for the LC separation since it has to be made sure that the LC solvents are compatible with the ionization process [159, 160]. Due to the special ionization process, ESI/MS often generates multiply charged ions which adds to the complexity of the data.

While ESI-quadrupole-time-of-flight (ESI-Q-TOF) instruments are capable of tandem mass spectrometry and allow the fragmentation of analytes, ESI-ion trap (ESI-IT) instruments even allow capturing of relevant ions which then can be repeatedly fragmented.

1.9.4.7 Gas chromatography

Gas chromatography can be used for monosaccharide analysis of glycans as well as linkage analysis. Oligosaccharides (more than 3-4 monosaccharides) are difficult to analyze since they are usually not volatile. While monosaccharide analysis using GC/MS is rarely used today, linkage analysis is still a very valuable tool for the discrimination of glycan structures. However, both techniques are based on the hydrolysis of the carbohydrate chain and subsequent formation of volatile molecules which can be applied to gas chromatography. The retention times compared to known reference substances as well as the electron impact (EI) MS spectra are then used to identify the exact monosaccharide.

Monosaccharide analysis starts with the acid hydrolysis of all glycosidic linkages. The monosaccharides are then reduced and acetylated to result in peracetylated altitol acetates which are ready to be analyzed. Linkage analysis is quite similar but requires one additional step at the beginning. First, the intact glycans are permethylated in order to add an ether-linked methyl group to every free hydroxyl group of the molecule. This increases the hydrophobicity on the one hand side, and also the volatility on the other hand side. These

modifications are not cleaved by the following hydrolysis step and so methylated monosaccharides are generated. Then the ring of the carbohydrate is opened via reduction and an alcohol is formed at C1 position. Then monosaccharides are peracetylated and every hydroxyl group that has been formed during or after hydrolysis gets modified. The finally resulting partially methylated alditol acetates (PMAA) are then subjected to GC/EI-MS. By comparison of the retention times and determining the positions of methyl and acetyl groups in every sugar via EI-MS, it can be elucidated if the analyzed sugar was terminal or if another sugar was linked to it. In the case of a hexose, a methyl group at position C2, C3, C4 and C6 would clearly indicate that the concerned sugar was in a terminal position. Position C1 (linkage to the glycan chain cleaved at the hydrolysis step) and position C5 (ring cleavage at the reduction step) would then be the only acetylated carbon atoms. If, for example, C4 would also be acetylated then this would indicate a building block of a linear glycan region, an acetylated C4 and C6 would correspond to a sugar which acts as branching point [161, 162].

While linkage analysis is capable of elucidating the position (terminal or within the chain) and linkage of a sugar including its ring size (pyranose or furanose), it cannot unambiguously explain the complete structure of large molecules or give information on the anomericity (α or β) of a linkage. Another drawback of this method is a quite high sample demand (low µg-range) and high labor intensity which prevents high throughput of large sample amounts.

1.9.4.8 Nuclear magnetic resonance

When large amounts of one single, purified glycan structure are available, nuclear magnetic resonance (NMR) obviously is the method of choice. It can give extensive information of the monosaccharide constituents, their exact positions and linkages including anomericity. Additionally, data on non-carbohydrate constituents can be collected. NMR is non destructive which means that samples can be further analyzed by destructive methods such as MS after the NMR experiments have been finished. However, there are also disadvantages: NMR spectrometers are expensive and the generation and interpretation of high quality spectra is time consuming and requires a high level of expertise. Additionally, the sample demands often exceed the physiologically available amounts by far and so other techniques are predominantly used.

NMR analysis of glycans is very much dependent on the availability of reference spectra. If such reference spectra are available, the primary structure of a glycan can be deduced from one ^{1}H-NMR spectrum. But on the other hand side, if a completely new structure has to be elucidated, various different experiments have to be performed. Correlation spectroscopy

(COSY) and total correlation spectroscopy (TOCSY) are two dimensional techniques that help to assign the ^{1}H-NMR spectra; heteronuclear single-quantum coherence (HSQC) experiments and two-dimensional heteronuclear multiple-bond correlation (HMBC) are essential for the assignment of the ^{13}C-NMR spectra [163].

2 Aims

The aim of this study was to elucidate the O-glycan patterns of various snail species. After the previously done analysis of N-glycan structures, we wanted to gain some insight into the biosynthetic capacity of gastropods concerning their O-glycosylation potential. The monosaccharide analysis as well as structural determination of O-glycans in land-living and aquatic snails with or without shell is described in this work.

The small amounts of sample as well as the viscid mucous and the tough snail meat demanded the specific adaption of purification as well as analysis methods. Various different clean-up procedures were tested in order to optimize the analysis in terms of sample demand, time consumption and purity of the final analytes. Analysis methods were downscaled to meet the specific needs of gastropod glycan analysis.

3 Materials and Methods

3.1 Snail clean-up, protein and glycan purification

3.1.1 Glycan purification overviews

3.1.1.1 Oligosaccharide preparation from fibrin

Depicted below (Table 1), you find a schematic overview of a glycan purification protocol for preparation of GnGn-oligosaccharides from fibrin, which can be useful for example as acceptor for glycosyl-transferase assays. All purification and separation steps are monitored by measuring the optical density at 280 nm and by checking the carbohydrate content using the orcinol method (3.1.16). All methods are described in detail later.

Glycoprotein
• Fibrin
Generation of glycopeptides
• Pepsin digestion (3.1.7.1)
Separation of peptides from glycopeptides
• Gel filtration - G25 coarse (3.1.8) • Gel filtration - G50 superfine (3.1.8)
Glycan release
• PNGase A digestion (3.2.1.1)
Separation of glycans from peptides
• Ion exchange chromatography - AG 50W-X2 (3.1.9.2) • Reversed phase chromatography - LiChroprep RP-18 (3.1.10)
Glycan modification
• Desialylation (3.2.4) • β-galactosidase digestion (3.2.5.7)
Glycan purification
• Gel filtration - G25 fine (3.1.8)
Analysis
• MALDI-TOF/MS (3.5.2)

Table 1 - Oligosaccharide preparation protocol

3.1.1.2 Original snail glycan purification protocol

At the beginning of our work we purified snail glycans according to the depicted procedure (Table 2). Briefly, snails were dissected and after homogenization in Tris/HCl buffer subjected to ammonium sulfate precipitation. To reduce heterogeneity, desalted samples were divided into two fractions (flow-through and sodium chloride eluted fraction) in an anion exchange chromatography step. Samples were further digested with pepsin to generate

glycopeptides as substrates for PNGase A digestion. Cation exchange chromatography was used to separate split-off N-glycans from O-glycopeptides and non-glycosylated peptides. Finally non-reductive β-elimination was used to cleave O-glycans from the peptides while leaving the reducing end intact for further derivatization steps such as pyridyl-amination.

Snail
• Dissection and removal of gut (3.1.2)
Protein purification
• Homogenization in Tris/HCl buffer (3.1.3)   • Ammonium sulfate precipitation • Dialysis (3.1.5) • Ion exchange chromatography - DEAE (3.1.9.1) • Gel filtration - G25 coarse (3.1.8)
Preliminary analysis
• SDS-PAGE (3.3.1) • SDS-PAGE (3.3.1) after enzymatic/chemical degradation (3.2.5)
Generation of glycopeptides and N-glycan release
• Pepsin digestion (3.1.7.1) • Gel filtration - G50 superfine (3.1.8) • PNGase A digestion (3.2.1.1) • Gel filtration - G25 fine (3.1.8)
Separation of N-glycans from O-glycopeptides and O-glycan release
• Ion exchange chromatography - AG 50W-X2 (3.1.9.2) • non-reductive β-elimination (3.2.2.2)
Analysis
• HPLC (3.4) • MALDI-TOF/MS (3.5.2) • GC/MS (3.5.1)

Table 2 - Original snail glycan purification protocol

3.1.1.3 Final snail glycan purification protocol

In a number of optimization steps we developed the original protocol during this work. The insight we gained through experiments at the beginning, allowed us to reduce the overall number of steps to allow a faster clean-up but also forced us to introduce additional steps to facilitate analysis (Table 3).

Especially the introduction of precipitation steps instead of gel filtration is a time saving factor, and also the abandonment of pepsin and PNGase A digestion were important milestones. On the other hand, the occurrence of storage carbohydrates required additional purification steps to get rid of non-protein bound carbohydrates.

Snail
• Dissection and removal of gut (3.1.2)
Protein purification

• Homogenization in CHAPS-based lysis buffer (3.1.4)
  • Reduction
  • Alkylation
• Acetone precipitation (3.1.13)

Removal of storage carbohydrates
• α-Glucosidase digestion (3.2.5.1) • Ion exchange chromatography - AG 50W-X2 (3.1.9.2) • Acetone precipitation (3.1.13) • Methanol precipitation (3.1.14)
Glycan release
• β-elimination (3.2.2.1)
Desalting and separation of glycans from peptides
• PGC chromatography (3.1.12) • Reversed phase chromatography (3.1.10)
Analysis
• HPLC (3.4) • MALDI-TOF/MS (3.5.2) • GC/MS (3.5.1) • LC-ESI/MS (3.5.3)

Table 3 - Final snail glycan purification protocol

3.1.2 Snail preparation and dissection

During this work, several different snail species were subject of investigation. Most of them were collected in areas close to Vienna under the supervision of Dr. Manfred Pintar (Department of Integrative Biology and Biodiversity Research, Institute of Zoology, University of Natural Resources and Life Sciences, Vienna), two of them were bred in our laboratory and one was obtained from a specialists shop for aquaristics. All of them were frozen at -80 °C immediately after collection and thawed right before dissection. While water-living snails could be dissected right away, land-living snails were extensively washed to remove the extraneous mucous components. Shell-carrying snails were pulled carefully out of their shell, which exposes the gut for removal. Slugs were opened with a surgical blade with great care not to lacerate the intestinal tract.

In special investigations, viscera and skin were separated before analysis. Since the viscera is usually isolated from the outside environment, it is free of surface contaminants, bacterial or fungal contamination.

Species	Origin	Land-/ Water-living	Shell-carrying
Achatina fulica	Bred in laboratory	Land	Yes
Arianta arbustorum	Collected	Land	Yes
Arion lusitanicus	Collected	Land	No
Biomphalaria glabrata	Bred in laboratory	Water	Yes
Cepaea hortensis	Collected	Land	Yes
Clea helena	Specialist shop	Water	Yes
Helix pomatia	Collected	Land	Yes
Limax maximus	Collected	Land	No
Lymnaea stagnalis	Collected	Water	Yes
Planorbarius corneus	Collected	Water	Yes

Table 4 - Overview of used snails during this work.

3.1.3 Homogenization in Tris/HCl buffer and ammonium sulfate precipitation

As first step of protein purification after dissection, an Ultra Turrax T25 dispersing instrument (IKA Labortechnik, Janke und Kunkel GmbH, Staufen, Germany) was used at 15000 rpm for 2 × 20 s to homogenize sample tissue in 0.05 M Tris/HCl buffer, pH 7.5 containing 3 mM dithiothreitol. Afterwards 176 g/l of ammonium sulfate were added to raise the salt concentration up to 30%. After 30 minutes samples were centrifuged at 15000 × g at 4 °C for 30 minutes and the precipitate was discarded. Salt concentration was further increased up to 80% (356 g/l) to precipitate relevant proteins. Samples were stirred for 60 minutes at room temperature and afterwards centrifuged at 25000 × g at 4 °C for 30 minutes. The supernatant was discarded while the precipitate was resolved in water and subjected to dialysis.

3.1.4 Homogenization in CHAPS-based lysis buffer

As alternative to the method described above, a CHAPS-based lysis buffer was used in the first purification step followed by protein reduction and alkylation [164] and omitted ammonium sulfate precipitation. Approximately 1 g of snail tissue was homogenized in 10 ml of CHAPS-based lysis buffer (0.5 % (w/v) CHAPS, 150 mM sodium chloride, 20 mM Tris/HCl, 2.5 mM sodium pyrophosphate, 1 mM ethylene-glycol-bis(2-aminoethylether)-

N,N,N´,N´-tetraacetic acid and 1 mM EDTA, pH 7.5) as described above (3.1.3) and incubated at 4 °C for 72 hours or at room temperature for 1 hour in an ultrasonic bath.

Following the original protocol [164], samples are now centrifuged at 100000 × g for 90 minutes to separate cytosolic (supernatant) from membrane (pellet) proteins. Membrane proteins are then resolved in CHAPS-based lysis buffer by a sonication step of 30 minutes. Since re-solubilization of membrane proteins was always a big struggle, this step was eliminated in later purifications.

Snail proteins are then reduced by addition of 500 µl of 10 mM dithiothreitol and incubation at 56 °C for 45 minutes. Finally 500 µl of 55 mM iodoacetamide are added and samples are incubated for 30 minutes in the dark to alkylate reduced proteins. To remove all salts, samples were then dialyzed against water overnight.

3.1.5 Dialysis

To remove small contaminants as well as salts, we usually performed dialysis overnight against water. Dialysis membranes from regenerated cellulose with a molecular weight cut off (MWCO) of 12000-14000 Da and a pore diameter of 25 Å were used in various sizes (Servapor; SERVA, Prod. No. 44139 [6 mm diameter], 44145 [16 mm diameter], 44144 [21 mm diameter], 44146 [29 mm diameter] and 44148 [50 mm diameter]).

3.1.6 Ultrafiltration

Ultrafiltration was used mainly for sample volume reduction of delicate samples when usage of a rotary evaporator was not applicable or desalting/buffer exchange was desired. Stirred ultrafiltration cells with 50 and 400 ml maximal process volume (Amicon Bioseparations 8050 and 8400; Millipore, Prod. No. 5122 and 5124) with regenerated cellulose filters and a nominal molecular weight limit of 30000 Da (Ultracel PL-30; Millipore, Prod. No. PLTK04310 [44.5 mm diameter] and PLTK07610 [76 mm diameter]) were used.

3.1.7 Protein digestion

3.1.7.1 Digestion with pepsin from porcine gastric mucosa

- Dissolve glycoprotein in 5% formic acid (20 mg/ml).
- Denature protein at 95 °C in a waterbath for 20 minutes.
- Cool down sample to room temperature.
- Add pepsin (Sigma-Aldrich, Prod. No. P6887) in a ratio of 1:50.

- Incubate at 37 °C for 16 hours.

3.1.7.2 Digestion with pronase from *Streptomyces griseus*

- Dissolve 1 mg of pronase (ROCHE, Prod. No. 165921) in 1 ml of pronase buffer (0.15 M Tris/HCl, pH 7.8, + 1 mM calcium chloride).
- Dissolve sample in pronase buffer.
- Add enzyme solution in a ratio of 1:100.
- Incubate overnight at 37 °C.

3.1.8 Gel filtration

All gel-filtration steps during this work were performed on Sephadex media manufactured by GE Healthcare listed in Table 5. G15 media was used for desalting of samples, G25 and G50 media were used for sample fractionation in the specified areas as well. 1% acetic acid was used as mobile phase.

	Fractionation Range (Mr): dextrans	Fractionation Range (Mr): globular proteins	Prod. No.
G15	$< 1.5 \times 10^3$	$< 1.5 \times 10^3$	17-0010-01
G25 coarse	1×10^2–5×10^3	1×10^3–5×10^3	17-0034-01
G25 fine	1×10^2–5×10^3	1×10^3–5×10^3	17-0032-01
G50 superfine	5×10^2–1×10^4	1.5×10^3–3×10^4	17-0041-01

Table 5 - Sephadex gel filtration media.

3.1.9 Ion exchange chromatography

3.1.9.1 Anion exchange chromatography

For separation of negatively charged proteins, diethylaminoethyl (DEAE) cellulose media (DE52; Whatman, Prod. No. 4057-050) was used in a batch procedure. Samples were dissolved in 50 mM Tris/HCl buffer, pH 8.0 and incubated with DE52 media for 45 minutes at room temperature. Binding proteins were eluted with 50 mM Tris/HCl buffer, pH 8.0 containing 1 M sodium chloride.

3.1.9.2 Cation exchange chromatography

To separate uncharged, released glycans from peptides or digested storage carbohydrates from proteins, cation exchange column chromatography was usually used. A strong cation

exchange media composed of sulfonic acid functional groups attached to a styrene divinylbenzene matrix (AG 50W-X2; Biorad, Prod. No. 142-1251) was chosen. 2% acetic acid was used as starting buffer. Elution was achieved by raising the ionic strength and pH value with 0.4 M ammonium acetate buffer, pH 6.0, for the elution of peptides and 1.0 M ammonium acetate buffer, pH 9.0, for the elution of proteins.

3.1.10 Reversed phase chromatography

As an alternative to ion exchange chromatography (3.1.9), reversed phase media were used to remove peptides. Octadecyl carbon chain bonded silica media (LiChroprep RP-18; Merck, Prod. No. 1130901) was used with 5% acetic acid as aqueous phase and 25% isopropanol in 5% acetic acid for elution.

Prepacked single-use columns (Strata, C18-E, 55 µm, 70 Å, 50 mg bed volume, 1 ml column volume; Phenomenex, Prod. No. 8B-5001-DAK) were used as well. They were conditioned with 1 column volume of 50% acetonitrile in water and washed before use with 1 column volume of water. While non-binding glycans were washed through with 1 column volume of water, peptides were eluted with 1 column volume of 50% acetonitrile in water.

3.1.11 Hydrophobic interaction chromatography

To remove strongly hydrophobic substances such as lipids, hydrophobic interaction chromatography (HIC) using octyl sepharose CL-4B (GE Healthcare, Prod. No. 17-0790-01) was applied as described in [165]. Briefly, the precipitate of the 80% ammonium sulfate precipitation step (3.1.3) was resolved in 10 mM phosphate buffer, pH 7.0, containing 1.2 M ammonium phosphate and applied to a HIC column equilibrated in the same buffer. After one column volume of starting buffer, a linear gradient with three column volumes of 10 mM phosphate buffer, pH 7.0, containing 1.2 M ammonium phosphate and 10 mM phosphate buffer, pH 7.0 was applied.

3.1.12 Porous graphitized carbon (PGC) chromatography

As method of choice for desalting of released oligosaccharides, solid phase extraction (SPE) cartridges containing porous graphitized carbon were used. This method has been shown to be superior to other desalting methods for oligosaccharides and glycopeptides such as dialysis, salt precipitation, mixed-bed ion exchange or size exclusion gel chromatography [133]. While salts and other small contaminants (e.g. sodium borohydride after β-elimination

or uncharged monosaccharides) on the one hand side do not bind to PGC and proteins on the other hand side bind irreversibly, our analytes can conveniently be eluted with an acetonitrile containing formate buffer [166].

PGC SPE columns

Supelclean Envi-Carb; Sigma-Aldrich

- 100 mg bed weight, 1 ml column volume (Prod. No. 57109-U)
- 500 mg bed weight, 6 ml column volume (Prod. No. 57094)

Preconditioning of PGC SPE columns

- Wash column with 1 column volume of 60% acetonitrile in formate buffer (0.3% formic acid adjusted to pH 3.1 with ammonia).
- Equilibrate column by washing with 1 ml of water.

Sample application

- Apply sample (resolved in water or aqueous buffer).
- Discard flow-through.
- Wash column with 1 column volume of water.
- Elute released neutral glycans with ⅓ column volume of 60% acetonitrile in formate buffer (0.3% formic acid adjusted to pH 3.1 with ammonia).

In case of charged oligosaccharides or glycopeptides, use formate buffer adjusted to pH 9.0 for the elution instead.

- Dry sample and resolve in water.

3.1.13 Acetone precipitation

Aqueous samples were mixed with the 4-fold amount of pre-chilled acetone (-20 °C) and incubated at -20 °C for at least 4 hours. Samples were then centrifuged at 4 °C and 32000 × g for 60 minutes. The supernatant was discarded and the pellet was again washed with chilled acetone to ensure a complete removal.

3.1.14 Methanol precipitation

Aqueous samples were mixed with the 3-fold amount of pre-cooled methanol (4 °C) and incubated at -80 °C for at least 1 hour. Samples were then centrifuged at 4 °C and 32000 × g

for 30 minutes. The supernatant was discarded and the pellet was again washed with cold methanol to ensure a complete removal.

3.1.15 Measurement of protein concentration

To check the complete removal of protein and peptide contaminations in glycan preparations we used the Pierce Micro BCA Protein Assay Kit (Thermo Scientific, Prod. No. 23235) according to the manufacturer's guidelines.

3.1.16 Qualitative and quantitative measurement of carbohydrate content

To determine the carbohydrate content of samples and to get reference points for the choice of fractions after chromatographic purification steps, we used the orcinol-sulfuric acid reaction. Furfurals and hydroxymethylfurfurals which are derived from heated carbohydrates in the presence of a strong mineral acid condense with phenolic reagents (orcinol) to give colored products which can easily be detected visually or by measurement of the absorbance at 405 nm.

Fast determination and rough quantification was done on precoated thin layer chromatography aluminum sheets with a 0.2 mm layer of unmodified silica (Alugram SIL G; Macherey-Nagel, Prod. No. 818033). Approximately 20 µl of reagent solution (100 mg orcinol in 50 ml of 20% sulfuric acid) were applied per square centimeter of TLC plate and dried in a stream of warm air. 1 µl of sample was applied and incubated at 100°C for 5-10 minutes. 25 ng of sugar per µl sample solution is the approximate detection limit where a faint brownish-pink staining can be seen.

For exact quantification, 50 µl of sample were mixed with 200 µl reagent solution (80 mg orcinol in 5 ml of water mixed with 37.5 ml of 60% sulfuric acid) in a 96-well plate and incubated at 80 °C for 45 minutes. Then the absorbance was measured at 405 nm.

3.2 Glycan release and modification

3.2.1 Enzymatic release

To release N-glycans from the peptide backbone, usually peptide N-glycosidases (Peptide-N(4)-(N-acetyl-β-glucosaminyl)asparagine amidase or PNGase) are employed. These enzymes cleave the linkage between the asparagine and the inner N-acetyl-glucosamine of the glycan core. In this work we used: PNGase A and PNGase F. While PNGase F (from *Flavobacterium meningosepticum)* is able to work on complete (denatured) proteins, PNGase A (from almonds) needs glycopeptides as substrates and therefore demands a preceding proteolytic digest. Nevertheless the use of PNGase A is inevitable as soon as α-1,3-linked core fucose is expected like in insect or plant tissues, as this constituent inhibits degradation by PNGase F.

3.2.1.1 PNGase A

(EC 3.5.1.52; Roche, Prod. No. 11 642 995 001)

Buffer: 0.05 M citrate phosphate buffer, pH 5.0

Samples were resolved in a sufficient amount of buffer (dependent on the batch size of the preparation), usually 1-2 ml, and 0.1-0.2 U of PNGase A were added and incubated at 37 °C for 16 hours.

3.2.1.2 PNGase F

(EC 3.5.1.52; Roche, Prod. No. 11 365 185 001)

Buffer: 0.05 M sodium phosphate buffer, pH 8.0

0.1% SDS

1% 2-mercaptoethanol

Samples were resolved in a sufficient amount of buffer (dependent on the batch size of the preparation), usually 1-2 ml, and 2-4 U of PNGase F were added and incubated at 37 °C for 16 hours.

3.2.2 β-elimination

3.2.2.1 Reductive β-elimination

During this work, two different protocols for the reductive release of O-glycans were used. The method suggested by Iyer and Carlson [114] is commonly used but previous research [116] has shown that the N-glycan release during β-elimination can be minimized by using the protocol of Spiro and Bhoyroo [115].

3.2.2.1.1 β-elimination according to Iyer and Carlson [114]

- Dissolve dry sample in 0.05 M sodium hydroxide containing 1 M sodium borohydride.
- Incubate sample at 50 °C for 16 hours.
- Neutralize cautiously by drop wise addition of 2 M acetic acid.
- Dry sample in a vacuum centrifuge.
- Wash sample 3 times with 30% methanol.

3.2.2.1.2 β-elimination according to Spiro and Bhoyroo [115]

- Dissolve dry sample in 0.1 M sodium hydroxide containing 0.8 M sodium borohydride.
- Incubate sample at 37 °C for 68 hours.
- Neutralize cautiously by drop wise addition of 2 M acetic acid.
- Dry sample in a vacuum centrifuge.
- Wash sample 3 times with 30% methanol.

3.2.2.2 Non-reductive β-elimination

In 2001, a protocol for the non-reductive β-elimination which should enable release and subsequent derivatization of O-glycans was published [167].

- Resolve dried sample in 1 ml of 25% ammonia saturated with ammonium carbonate.
- Add 100 mg of dry ammonium carbonate.
- Seal vial tightly and incubate at 60 °C for 40 hours.
- Dry sample in a vacuum centrifuge or rotary evaporator.

- Resolve sample in water and dry again repeatedly to remove the ammonium carbonate salt-crust.
- Dry sample completely.
- Add 10 µl of 0.5 M boric acid.
- Incubate at 37 °C for 30 minutes.
- Dry under a stream of nitrogen.
- Wash sample 2 times in 30% methanol.

3.2.3 Hydrazinolysis

Following this protocol by K.R. Anumula, released glycans should resemble glycans released by PNGase-digestion and should be ready for further derivatisation [110].

- Dissolve 10-20 mg of glycoprotein in 1.0 ml of 1% ammonium bicarbonate solution to neutralize residual acids.
- Transfer 5-10 µl of protein solution (approx. 70-100 µg of protein) into 1.6-ml screw cap vials.
- Dry sample in a vacuum centrifuge.
- Dry sample at 80 °C in an oven for additional 10 minutes to ensure complete removal of ammonium bicarbonate.
- Add 20-25 µl of hydrazine under dry argon atmosphere.
- Cap vials tightly.
- Place in a deep well heating block so that vial are completely submerged and cover with an insulator.
    - Incubate at 60°C for 6 hours or at 50 °C for 16 hours to release O-linked glycans only.
    - Incubate at 95°C for 6 hours or at 90 °C for 16 hours to release N-linked glycans (with some O-linked sugar chains).
- Evaporate hydrazine in a chemical hood with a gentle stream of dry argon while the vials are being heated in the heating block at 80°C (<10 minutes).
- Dissolve samples in 50 µl of sodium acetate trihydrate solution (80 mg/ml) by mixing vigorously in a vortex mixer.
- Add 1.5-2.0 µl of acetic anhydride and mix immediately.
- Incubate at room temperature for at least 60 minutes up to16 hours.

3.2.4 Desialylation

- Dried samples are resolved in 50 mM sulfuric acid.
- Incubate samples at 80 °C for 60 minutes.
- Neutralize reaction mixture by addition of 50 mM sodium hydroxide.
- Desalt sample via G15 gel filtration (3.1.8).

3.2.5 Digestion with endo-/exoglycosidases

For HPLC or MALDI-TOF/MS analysis of samples digested with endo- or exoglycosidases, samples were usually resolved in 20 µl of buffer. The amount of added enzyme is given as an approximate value in the following subsections. All digestions were performed at 37 °C and incubated for 16 hours.

3.2.5.1 α-Glucosidase from rice

(EC 3.2.1.20; Sigma-Aldrich, Prod. No. G9259)

Buffer: 0.1 M sodium citrate buffer, pH 4.6

Amount: 0.005 U (in 20 µl)

For the digestion of storage carbohydrates in large scale snail preparations, samples were resolved in approx. 20 ml of buffer and 5 U of α-glucosidase were added.

3.2.5.2 β-Glucosidase from almonds

(EC 3.2.1.21; Sigma-Aldrich, Prod. No. G4511)

Buffer: 0.1 M sodium citrate buffer, pH 4.6

Amount: 0.5 U

3.2.5.3 α-Glucosidase from *Bacillus stearothermophilus*

(EC 3.2.1.20; Sigma-Aldrich, Prod. No. G3651)

Buffer: 0.1 M sodium citrate buffer, pH 4.6

Amount: 0.5 U

3.2.5.4 Endo-β-Galactosidase (recombinant from *Bacteriodes fragilis*)

(EC 3.2.1.103; QA Bio, Prod. No. E-XBG01)

Buffer: 0.25 M sodium phosphate buffer, pH 5.8

Amount: 0.01 U

3.2.5.5 α-Mannosidase from *Canavalia ensiformis* (Jack bean)

(EC 3.2.1.24; Sigma-Aldrich, Prod. No. M5573)

Buffer: 0.05 M sodium citrate buffer, pH 4.6
+ 0.2 mM zinc chloride

Amount: 0.2 U

3.2.5.6 α-L-Fucosidase from bovine kidney

(EC 3.2.1.51; Sigma-Aldrich, Prod. No. F5884)

Buffer: 0.05 M sodium citrate buffer, pH 4.6

Amount: 0.01 U

3.2.5.7 β-Galactosidase from *Aspergillus oryzae*

(EC 3.2.1.23; produced in-house)

Buffer: 0.05 M sodium citrate buffer, pH 4.6

Amount: 0.3 U

3.2.5.8 α-1,2-Fucosidase

(EC 3.2.1.51; Sigma-Aldrich, Prod. No. F9272)

Buffer: 0.05 sodium phosphate buffer, pH 5.0

Amount: 0.01 mU

3.2.5.9 β-N-Acetylglucosaminidase from bovine kidney

(EC 3.2.1.52; Sigma-Aldrich, Prod. No. A2415)

Buffer: 0.05 M sodium citrate buffer, pH 4.6

Amount: 0.05 U

3.2.5.10 Endoglycosidase H from *Streptomyces plicatus* (recombinant from *E. coli*)

(EC 3.2.1.96; Roche, Prod. No. 11 088 726 001)

Buffer: 0.05 M sodium acetate, pH 5.0

Amount: 0.0025 U

3.2.5.11 Neuraminidase from *Clostridium perfrigens* (*C. welchii*)

(EC 3.2.1.18; Sigma-Aldrich, Prod. No. N2133)

Buffer: 0.05 M sodium acetate buffer, pH 5.0

Amount: 0.005 U

3.2.6 Hydrolysis

Hydrolysis of glycans for monosaccharide analysis was done by incubation in 4 M trifluoroacetic acid for 2 hours at 115 °C or 4 hours at 100 °C.

3.3 Electrophoresis

3.3.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Usually, 50-150 µg of protein are subjected to SDS-PAGE. To purify samples in advance, methanol precipitation (3.1.14) is performed before application to the gel. After the residual methanol is completely removed via evaporation, samples are resolved in 7 µl of water. Then 7 µl of 2-fold concentrated sample buffer are added. The samples are then incubated at 95 °C for 5 minutes to denature proteins and applied to the gel.

Electrophoresis was performed at a constant voltage of 200 V for 45-60 minutes.

3.3.1.1 Gel and buffer preparation

	Resolving gel (T12.5, C1.0)	Stacking gel (T5.7, C2.23)
Acrylamide (30% w/v)	3750 µl	760 µl
Bisacrylamide (1% w/v)	1170 µl	520 µl
Resolving gel buffer (pH 8.8)	2250 µl	
Stacking gel buffer (pH 6.8)		1000 µl
Water	1710 µl	1710 µl
SDS (10% w/v)	90 µl	40 µl
APS (10% w/v)	54 µl	32 µl
TEMED	5.4 µl	3.2 µl

Table 6 - SDS-GEL recipe for 2 gels.

Resolving gel buffer: 1.5 M Tris/HCl, pH 8.8

Stacking gel buffer: 0.5 M Tris/HCl, pH 6.8

Sample buffer (2x):
31 mg DTE or DTT
200 mg SDS
5 ml stacking gel buffer
2.8 ml glycerol (87% w/v)
2.7 ml water (distilled)
a few drops of bromphenol blue

Electrode buffer (5x): 15 g/l TRIS

72 g/l glycine

5 g/l SDS

Standard Ladder used throughout this work if not indicated otherwise:

PAGE Ruler (prestained protein ladder), Fermentas, Prod. No. SM0671

3.3.1.2 Coomassie staining

Staining solution

3.5% perchloric acid

0.04% Coomassie Brilliant Blue G250 (0.4 g dissolved in 10 ml methanol)

Fixing solution

500 ml methanol

70 ml acetic acid

430 ml water

- Add 10 ml of fixing solution for 5-10 minutes.
- Wash gel with water.
- Add 10 ml of staining solution for 15-20 minutes.
- Wash gel with water.
- Decolor gel with 10 ml of 5% acetic acid.
- Exchange 5% acetic acid as often as necessary.

If further analysis of Coomassie stained bands is required, the following **destaining protocol** can be applied:

- Cut gel band out of gel.
- Cut gel into small pieces (≤ 1 mm²) and transfer it into a micro centrifugation tube.
- Rinse the pieces with water.
- Discard the water and add 50 µl of 50% acetonitrile.
- Incubate for 15 minutes at room temperature and discard liquid.
- Add 50 µl of 50% acetonitrile.
- Incubate for 15 minutes at room temperature and discard liquid.

- Add 50 µl of 100% acetonitrile, vortex briefly and discard liquid.
- Add 30 µl of 0.1 M NH_4HCO_3 (7.9 mg/ml) and reswell gel for 5 minutes.
- Add 30 µl of 100% acetonitrile.
- Incubate for 15 minutes at room temperature and discard liquid.

Important: If gel is not properly destained at this stage, repeat this protocol once!

- Dry gel in a vacuum centrifuge for approx. 15 minutes without heating.

3.3.1.3 Silver staining

All steps of this protocol [168] are performed at room temperature with shaking and gel is washed with water after every incubation step.

- Incubate gel for 5 minutes in 50% methanol with 12% trichloroacetic acid and 2% copper (II) chloride
- Incubate gel for 5 minutes in 10% methanol with 5% acetic acid.
- Incubate gel for 5 minutes in 0.01% potassium permanganate.
- Incubate gel for 1 minute in 10% methanol with 5% acetic acid.
- Incubate gel for 5 minutes in 10% ethanol.
- Incubate gel for 5 minutes in water.
- Stain gel for 5 minutes in 0.1% silver nitrate.
- Incubate gel for 0.5 minutes in water.
- Develop staining for 3-5 minutes in 1% potassium carbonate with 0.01% formaldehyde.
- Stop the reaction with 5% acetic acid.

3.3.2 Fluorophore assisted carbohydrate electrophoresis (FACE)

According to previous publications, this method can be used for the high resolution separation of reducing saccharides [144-146]. Gels are run with 15 mA during the first hour and 30 mA during the next two hours and analyzed by UV illumination.

3.3.2.1 Gel and buffer preparation

	Resolving gel	Stacking gel
Acrylamide/Bisacrylamide-Stock	5000 µl	315 µl
Stock gel buffer (pH 8.5)	2500 µl	1250 µl
Water	2500 µl	3380 µl
APS (10% w/v)	50 µl	50 µl
TEMED	10 µl	5.0 µl

Table 7 - FACE-gel recipe for AMAC and ANTS derivatization

Acrylamide/Bisacrylamide-Stock: 60% (w/v) acrylamide
1.6% (w/v) N,N'-methylene-bis(acrylamide)

Stock gel buffer (4x): 1.5 M Tris/HCl, pH 8.5

Stock electrode buffer (10x): 1.92 M glycine
0.25 M Tris, pH 8.5

ANTS sample buffer: glycerol:water = 1:4 (v/v)

AMAC sample buffer: dimethyl sulfoxide:glycerol:water = 2:1:7 (v/v/v)

Since AMAC is an uncharged molecule, only negatively charged samples may be analyzed using the buffer and gel recipes described above. If positively or uncharged samples have to be applied, the stock gel and stock electrode buffer have to be exchanged as follows:

Stock gel and electrode buffer (10x) 1 M Tris/boric acid, pH 8.3

	Resolving gel	Stacking gel
Acrylamide/Bisacrylamide-Stock	2500 µl	315 µl
Stock gel buffer (pH 8.3)	500 µl	500 µl
Water	2000 µl	4130 µl
APS (10% w/v)	25 µl	50 µl
TEMED	5 µl	5.0 µl

Table 8 - FACE-gel recipe for AMAC derivatization of uncharged/positively charged samples

3.3.2.2 Derivatization with ANTS (8-Aminonaphthalene-1,3,6-trisulfonic acid)

- Dry sample.
- Add 5 µl of 0.15 M ANTS in 15% acetic acid in water.
- Shake vigorously.
- Add 5 µl of 1 M sodium cyanoborohydride in dimethyl sulfoxide.
- Shake vigorously.
- Incubate at 37 °C for 16 hours.
- Dry sample.
- Resolve dry sample in 20 µl of ANTS sample buffer.

3.3.2.3 Derivatization with AMAC (2-Aminoacridone)

- Dry sample.
- Add 5 µl of 0.1 M AMAC in 15% acetic acid in dimethyl sulfoxide.
- Shake vigorously.
- Add 5 µl of 1 M sodium cyanoborohydride in water.
- Shake vigorously.
- Incubate at 37 °C for 16 hours.
- Dry sample.
- Resolve dry sample in 20 µl of AMAC sample buffer.

3.4 HPLC separation and analysis

3.4.1 Monosaccharide analysis

3.4.1.1 Anthranilic acid (AA) derivatization

Derivatization with anthranilic acid, also known as 2-aminobenzoic acid, as a fluorescent tag can be either used on mono- or on oligosaccharides. We focused on the monosaccharide analysis and adaption of existing protocols to our needs.

In general, the fluorescent tag enabling detection at very high sensitivity is linked to the reducing end of the carbohydrate by reductive amination via Schiff base. Extensive reviews of reaction chemistry have been published by K.R. Anumula [122, 169]. Previously published protocols [110, 170] were downscaled to fit our needs of fast handling of multiple samples with low carbohydrate content.

3.4.1.1.1 Derivatization

Solution (1): 2% (w/v) boric acid in methanol [can be stored at room temperature for several months]

Solution (2): 3 mg of anthranilic acid and 30 mg of sodium cyanoborohydride in 1.0 ml of solution (1) [fresh]

Solution (3): 80 mg/ml sodium acetate trihydrate in water

- Dry sample (working range: 0.35 pmol – 240 nmol of total sugar; for standard preparation use 180 nmol).
- Dissolve monosaccharides in 5 µl of solution (3) by mixing vigorously in a vortex mixer.
- Add 10 µl of solution (2).
- Incubate at 80 °C in an oven or heating block for 60 minutes.
- Store concentrated samples at -20 °C.
- Dilute 1:500 prior to HPLC analysis.
- Use 5 µl of diluted sample for HPLC analysis.

3.4.1.1.2 Analysis

Column: RP C18 (ODS Hypersil, 5 µ, 250 × 4 mm, Thermo Scientific, Part No. 30105 – 254030)

Flow:	1 ml/min		
Solvent	A:	1.0% tetrahydrofuran	
		0.5% phosphoric acid	
		0.2% 1-butylamine	in water (v/v/v/v)
	B:	50% of solvent A	in acetonitrile (v/v)
Detection:	Ex/Em = 360/425 nm		

Timetable:

Time (min)	%B
0	5
17	25
23	100
29	100
30	5
35	5

3.4.1.2 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization

Since AA derivatives allow separation of most sugars commonly occurring in glycans but not of all our reference substances, we used another monosaccharide derivatization method in parallel. Unlike AA, 1-phenyl-3-methyl-5-pyrazolone is an UV active tag which is added to the reaction in vast excess. This allows derivatization of larger amounts of sugar but makes the removal of unused reagent after derivatization necessary in order not to interfere with the HPLC analysis. This is achieved by multiple liquid-liquid extraction with an organic solvent. Reaction chemistry was first described by S. Honda [171]; our protocol was modified from D. Fu [172].

3.4.1.2.1 Derivatization

- Dry sample (working range: 3 pmol – 500 nmol of total sugar; for standard preparation use 180 nmol).
- Dissolve monosaccharides in 25 µl of 0.5 M PMP in methanol by mixing vigorously in a vortex mixer.

- Add 15 µl of 0.5 M sodium hydroxide.
- Add 10 µl of water.
- Incubate at 70 °C in an oven or heating block for 120 minutes.
- Neutralize the reaction solution by addition of 20 µl of 0.5 M hydrochloric acid.
- Perform liquid-liquid extraction with diethyl ether 5 times.
    - Add 500 µl of diethyl ether.
    - Mix extensively in a vortex mixer.
    - Remove organic (upper) layer carefully.
- Dry sample completely in a vacuum centrifuge.
- Dissolve dried samples in 1 ml of water.
- Store standards at -20 °C.
- Use 5 µl of undiluted sample for HPLC analysis.

3.4.1.2.2 Analysis

Column: RP C18 (ODS Hypersil, 5 µ, 250 × 4 mm, Thermo Scientific, Part No. 30105 – 254030)

Flow: 1.5 ml/min

Solvent A: 8.0% acetonitrile in 0.1 M ammonium acetate solution (pH 5.5)

B: 30.0% acetonitrile in 0.1 M ammonium acetate solution (pH 5.5)

Detection: UV absorbance at 245 nm

Timetable:

Time (min)	%B
0	45
10	50
26	85
26.5	85
27	45
32	45

3.4.2 Glycan separation and analysis

3.4.2.1 2-aminopyridine (PA) derivatization

This method was used for derivatization of glycans set free by enzymatic digestion or non-reductive β-elimination. Since the fluorophore is added to the reducing end of the sugar-chain by reductive amination, glycans set free by β-elimination cannot be labeled.

3.4.2.1.1 Derivatization

PA-stock-solution:	1 g of 2-aminopyridine in 0.76 ml of concentrated hydrochloric acid
Solution (1):	Add 2 parts of water to 1 part of PA-stock-solution (pH 6.2) [fresh]
Solution (2):	10 mg of sodium cyanoborohydride in 20 µl of solution (1) and 30 µl of water

- Dry sample (approx. 300 µg of oligosaccharide).
- Add 80 µl of solution (1).
- Incubate in a boiling water bath for 13 minutes.
- Add 4 µl of solution (2).
- Incubate at 90 °C for 16 hours.
- Remove excess PA-reagent via G15 gel-filtration (see section 3.1.8).
- Pool carbohydrate containing fractions and lyophilize sample.
- Resolve samples in 500 µl of water.
- Use 5-25 µl of undiluted sample for HPLC analysis.

3.4.2.1.2 Analysis on reversed phase column

Column:	RP C18 (ODS Hypersil, 5 µ, 250 × 4 mm, Thermo Scientific, Part No. 30105 – 254030)
Flow:	1.5 ml/min
Solvent	A: 0.1 M ammonium acetate in water (pH 4.0)
	B: 30.0% methanol in water (v/v)
Detection:	Ex/Em = 320/400 nm
Standard:	3-11 isomaltose units (PA derivatized)

Timetable:

Time (min)	%B
0	0
30	30
30.5	0
35	0

3.4.2.1.3 Analysis on normal phase column

Column: NH_2-bonded NP (Palpak Type N, Pore size 120 Å, 5 µ, 250 × 4.6 mm, Takara Bio Inc., Part No. CA8100)

Flow: 1.0 ml/min

Solvent A: 3% acetic acid adjusted to pH 7.3 with triethylamine in 50% acetonitrile (v/v)

B: 100% acetonitrile

Detection: Ex/Em = 310/380 nm

Standard: 3-11 isomaltose units (PA derivatized)

Timetable:

Time (min)	%B
0	45
5	45
45	0
46	0
47	45
53	45

3.4.2.2 Separation of unlabeled glycans on porous graphitized carbon (PGC)

To reduce glycan heterogeneity and facilitate further analysis steps such as ESI/MS or GC/MS, reduced glycan samples obtained from β-elimination, which cannot be labeled, can be fractionated on PGC columns.

Column: PGC (Hypersil-Keystone, Hypercarb, 5 µ, 150 × 3 mm, Thermo Scientific, Part No. 35005-153030)

Flow: 0.6 ml/min

Solvents: A: 0.3% formic acid adjusted to pH 3.1 with ammonia
B: 95% acetonitrile in solvent A

Detection: UV absorbance at 212 nm

Fractionation: 1 fraction per minute

Timetable:

Time (min)	% B
0	0
2	0
30	25
31	60
35	60
36	0
40	0

3.5 MS analysis

3.5.1 GC/MS analysis

All methods concerning gas chromatography described hereafter were published in [161].

3.5.1.1 Monosaccharide analysis

3.5.1.1.1 Generation of alditol acetates

- Start with approx. 2-3 µg of dried sugar.

Hydrolysis

- Add 500 µl of 4 M trifluoroactic acid.
- Incubate 4 hours at 100 °C.
- Dry sample (vacuum centrifuge or stream of nitrogen).

Reduction

- Add 600 µl 1% sodium borohydride (or sodium borodeuteride).
- Incubate overnight at room temperature.
- Stop reaction by addition of 200 µl of 2 M acetic acid.
- Dry sample (vacuum centrifuge or stream of nitrogen).
- Wash 3-4 times with 2.5 ml of 1% acetic acid in methanol.
- Dry sample (vacuum centrifuge or stream of nitrogen).

Acetylation

- Add 100 µl of pyridine.
- Add 400 µl of acetic anhydride.
- Incubate overnight at room temperature.
- Dry sample in a stream of nitrogen.
- Add 4 ml of dichloromethane.
- Add 4 ml of water for liquid-liquid extraction and remove aqueous layer again 3 times.
- Dry sample in a stream of nitrogen.
- Dissolve in acetone for GC analysis.

3.5.1.1.2 GC/MS analysis of alditol acetates

Column:	VF 5 ms capillary column (60 m, 0.25 mm inner diameter, 0.1 mm film thickness; Varian, Darmstadt, Germany)
Carrier gas:	helium (2.5 ml/min)
Timetable:	
	Start at 40 °C for 1.5 min.
	Raise to 130 °C with 40 °C/min.
	Raise to 290 °C with 8 °C/min.
	Maintain at 290 °C for 5 min.

Electron impact (EI) mass spectrometry was performed in the positive ion mode using a Polaris Q instrument (ThermoQuest Analytical Systems).

3.5.1.2 Glycan linkage-analysis

3.5.1.2.1 Generation of partially methylated alditol acetates

- Start with approx. 2-3 µg of dried sugar.

Permethylation [173]

- Add 50 µl of waterfree dimethyl sulfoxide.
- Sonicate 90 minutes at room temperature.
- Add 50 µl of lithium methylsulfinyl carbanion.
- Sonicate 90 minutes at room temperature.
- Add 50 µl of iodomethane (or deuterated iodomethane)
- Sonicate 90 minutes at room temperature.
- Stop reaction by addition of 150 µl of water.
- Purification via solid phase extraction (BOND Elut LRC-C18, 100 mg; VARIAN, Prod. No. 12113001) conditioned with
    - 5 ml water
    - 5 ml acetonitrile
    - 5 ml methanol
    - 10 ml water.

- Apply sample and discard flow-through.
- Rinse with 5 ml of water and discard flow-through.
- Elute sample with 4 ml of acetonitrile (100%).
- Dry sample in a stream of nitrogen.

Hydrolysis

- Add 500 µl of 4 M trifluoroactic acid.
- Incubate 4 hours at 100 °C.
- Dry sample (vacuum centrifuge or stream of nitrogen).

Reduction

- Add 600 µl 1% sodium borohydride.
- Incubate overnight at room temperature.
- Stop reaction by addition of 200 µl of 2 M acetic acid.
- Dry sample (vacuum centrifuge or stream of nitrogen).
- Wash 3-4 times with 2.5 ml of 1% acetic acid in methanol.
- Dry sample (vacuum centrifuge or stream of nitrogen).

Acetylation

- Add 100 µl of pyridine.
- Add 400 µl of acetic anhydride.
- Incubate overnight at room temperature.
- Dry sample in a stream of nitrogen.
- Add 4 ml of dichloromethane.
- Add 4 ml of water for liquid-liquid extraction and remove aqueous layer again 3 times.
- Dry sample in a stream of nitrogen.
- Dissolve in acetone for GC analysis.

3.5.1.2.2 GC/MS analysis of partially methylated alditol acetates

Column:	VF 5 ms capillary column (60 m, 0.25 mm inner diameter, 0.1 mm film thickness; Varian, Darmstadt, Germany)
Carrier gas:	helium (1.2 ml/min)

Timetable:

Start at 40 °C for 2.4 min.

Raise to 130 °C with 40 °C/min.

Raise to 200 °C with 2 °C/min.

Raise to 270 °C with 10 °C/min.

Maintain at 270 °C for 2 min.

Electron impact (EI) mass spectrometry was performed in the positive ion mode using a Polaris Q instrument (ThermoQuest Analytical Systems).

3.5.2 MALDI-TOF/MS analysis

MALDI/MS was performed on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with a LIFT-MS/MS facility either in the laboratory of Prof. Rudolf Geyer (Institute of Biochemistry, Faculty of Medicine, Justus-Liebig-University Gießen, Germany) or the laboratory of Prof. Ebrahim Razzazi-Fazeli (Institute for Animal Nutrition, Vetcore, University of Veterinary Medicine, Vienna, Austria).

Typically 1 µl of glycan sample was mixed with 0.5 µl of matrix on an 800 µm hydrophilic anchor of an AnchorChip MALDI sample plate (Bruker) and dried in a stream of cold air.

3.5.2.1 Matrices

ATT 5 mg/ml of 6-aza-2-thiothymine in 50% acetonitrile in 0.1% trifluoroacetic acid

DHB 5 mg/ml of 2,5-dihydroxybenzoic acid in 50% acetonitrile in 0.1% trifluoroacetic acid

3.5.2.2 Permethylation

It is very important to use completely dry samples for this procedure only! Since methyl-groups are added to all free hydroxyl-groups including those of remaining water molecules, moist samples will most likely result in under-methylated samples.

- Dry sample thoroughly in a vacuum centrifuge.
- Add 300 µl of dimethyl sulfoxide.
- Add the tip of a spatula of grinded, dry sodium hydroxide.
- Shake vigorously for 5 minutes.
- Centrifuge samples for 1 minute at 2500 × g.

- Add 50 µl of iodomethane (or deuterated iodomethane).
- Shake vigorously for 5 minutes.
- Centrifuge samples for 1 minute at 2500 × g.
- Incubate at room temperature for 40 minutes on a shaking platform.
- Chill sample in an ice bath.
- Add 1 ml of 10% acetic acid.
- Add 1 ml of dichloromethane.
- Shake vigorously for 5 minutes.
- Centrifuge samples for 1 minute at 2500 × g.
- Discard upper (aqueous) layer.

- Again add 1 ml of 10% acetic acid.
- Shake vigorously for 5 minutes.
- Centrifuge samples for 1 minute at 2500 × g.
- Discard upper (aqueous) layer and repeat last four steps three times.
- Finally freeze the organic phase at -20 °C.
- Take the unfrozen organic phase and therefore remove frozen water residues.
- Dry sample in a vacuum centrifuge.
- Resolve sample in 50% methanol for MALDI-TOF analysis.

3.5.2.3 Solid-state permethylation

This protocol was modified slightly from Y. Mechref [164]. The difference between the common protocol described in 3.5.2.2 and this one is that sodium hydroxide beads are used instead of grinded sodium hydroxide. This facilitates the removal of sodium hydroxide after the methylation step.

- Sodium hydroxide beads (20-40 mesh) are suspended in acetonitrile and filled in micro centrifuge columns.
- Wash the column several times by applying water free dimethyl sulfoxide.
- Resolve dried samples in 50 µl of dimethyl sulfoxide and add 22 µl of iodomethane and 0.3 µl of water,
- Apply sample mixture to sodium hydroxide micro-reactor.

- Re-apply sample for four times.
- Wash reactor twice with 100 µl of dimethyl sulfoxide.
- Add 200 µl of chloroform.
- Add 200 µl of water.
- Mix vigorously.
- Again add 200 µl of water.
- Discard upper (aqueous) layer.
- Mix vigorously.
- Discard upper (aqueous) layer and repeat the last three steps three times.
- Dry sample in a vacuum centrifuge.
- Resolve sample in 50% methanol for MALDI-TOF analysis.

3.5.3 ESI/MS analysis following in-gel release method

For analysis of small sample amounts with ESI/MS a special in-gel release method [174, 175] was established for the analysis of glycans bound to Coomassie-stained proteins isolated by SDS-PAGE. This protocol was further modified for the analysis of low-milligram amounts [176].

3.5.3.1 Snail dissection and protein purification

- Dissect snails after extensive washing.
- Discard gut and cut snails into small pieces (approx. 0.1-1.0 mg).
- Homogenize whole snail tissue without gut (except indicated differently in Table 9) in CHAPS-based lysis buffer (100 mg of snail tissue/ml).
- Sonicate sample for 30 minutes at room temperature.
- Incubate overnight at 4 °C.
- Dialyze samples against water overnight (Servapor, diameter: 16 mm, MWCO: 12000-14000 Da, pore diameter: 25 Å; Serva, Prod.No. 44145).
- Centrifuge sample for 15 minutes at room temperature at 12000 × g.
- Discard insoluble compounds and lyophilize supernatant overnight.
    - Typical yield: 20-100 mg.

Species	Amount	Part of snail
Achatina fulica	<1	foot only
Arianta arbustorum	2	
Arion lusitanicus	<1	skin only
Biomphalaria glabrata	9	
Cepaea hortensis	4	
Clea helena	5	
Helix pomatia	<1	foot only
Limax maximus	<1	skin only
Lymnaea stagnalis	3	
Planorbarius corneus	3	

Table 9 - Snail samples for ESI analysis.

3.5.3.2 SDS-PAGE

(all buffers as described in 3.3.1, except indicated otherwise)

- Dissolve 4.5 mg of dry sample in 200 µl of SDS sample buffer (2x)
- Vortex vigorously
- Centrifuge sample for 3 minutes at room temperature at 16000 × g.
- Take 50 µl of supernatant (2 gel lanes à 25 µl) and boil for 5 minutes at 95 °C.
- Add 6 µl (3 µl per lane) of prestained protein ladder to the sample vial (PAGE Ruler, Fermentas, Prod. No. SM0671).
- Cast SDS-PAGE gel for “in-gel release method” (8 × 8 cm gel with 5 pockets) following Table 10.

	Resolving gel (T17.0, C1.0)	Stacking gel (T3.2, C4.4)
Acrylamide (30% w/v)	3368 µl	285 µl
Bisacrylamide (1% w/v)	1051 µl	390 µl
Resolving gel buffer (pH 8.8)	1500 µl	
Stacking gel buffer (pH 6.8)		750 µl
Water		1280 µl
SDS (10% w/v)	60 µl	30 µl
APS (10% w/v)	36 µl	24 µl
TEMED	3.6 µl	2.4 µl

Table 10 - SDS-GEL recipe for “in-gel release method” (2 gels with elongated stacking gel).

- Apply 28 µl of sample per gel pocket.
- Perform SDS-PAGE for approx. 90 minutes at 80 volts until lowest and highest band of ladder (10-170 kDa) separate 0.5 - 1 cm.
    - Cut gel area (10-170 kDa) for β-elimination directly from the gel and squash gel before transferring it into a micro centrifugation tube.
    - Perform Coomassie staining (as described in 3.3.1.2) with second band to check protein content.

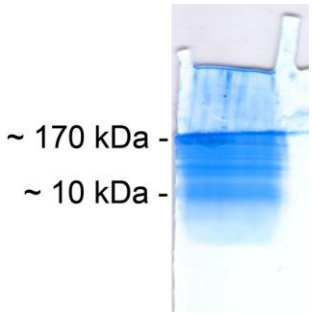


Figure 5 - Typical SDS-PAGE of in-gel release method

A Coomassie staining as shown in Figure 5 relates to a protein content of approx. 200-300 µg within the band from 10-170 kDa and is usually sufficient for ESI/MS analysis.

3.5.3.3 β-elimination

- Wash gel with 500 µl of 0.5 M sodium hydroxide to equilibrate gel.
- Add 300 µl of 1 M sodium borohydride in 0.5 M sodium hydroxide [177].
- Incubate overnight at 50 °C.

3.5.3.4 PGC clean-up

Purification was done according to 3.1.12 on PGC spin columns.

- Wash PGC spin column (Hypersep Hypercarb, 25 mg, Thermo Scientific, Part No. 60302-607) with 1 ml of 60% acetonitrile in formate buffer (0.3% formic acid adjusted to pH 3.1 with ammonia) to precondition column.
- Equilibrate column by washing with 1 ml of water.
- Apply liquid fraction of β-elimination on column.
- Discard flow-through.

- Wash column with 1 ml of water to remove salt from sample.
- Elute released glycans with 0.3 ml of 60% acetonitrile in formate buffer (0.3% formic acid adjusted to pH 3.1 with ammonia).
- Dry sample and resolve in 20 µl of water.
- ESI analysis of 2-3 µl of sample.

3.5.3.5 ESI/MS analysis

ESI/MS analysis was performed as previously described [136, 178] by Dr. Martin Pabst. Selected ion monitoring (SIM) of the *m/z* values was applied to ensure optimal peak intensity.

Column: PGC (Hypercarb, 100 × 0.18 mm, Thermo Scientific)

Flow: 2.0 µl/min without solvent slitting (Ultimate 3000; Dionex)

Solvent: A: 0.3% formic acid adjusted to pH 3.0 with ammonia
B: 100% acetonitrile

Detection: Q-TOF Ultima Golbal (Waters Micromass) in positive-ion mode

Timetable:

Time (min)	%B
0	0
2	0
5	8.8
60	17.2
80	40

4 Results

4.1 Preliminary results derived from snails following the original glycan purification protocol

Starting this project, some batches of snail glycans from *A. fulica* and *A. lusitanicus* were purified following an older protocol which was used previously for N-glycan preparation (3.1.1.2). With some additional purification steps we planned to separate N- from O-glycans to enable an easy comparison of both fractions. To minimize the influence of contamination with foreign glycans, we analyzed the fractions of “skin” and “viscera” separately. After homogenization of the samples in Tris/HCl buffer, ammonium sulfate precipitation followed by dialysis was performed. The separation step of the anion exchange chromatography (DEAE) was meant to reduce the heterogeneity and facilitate further analysis. Both fractions, the non-binding as well as binding fraction that was eluted with sodium chloride, were kept and purified.

4.1.1 SDS-PAGE analysis

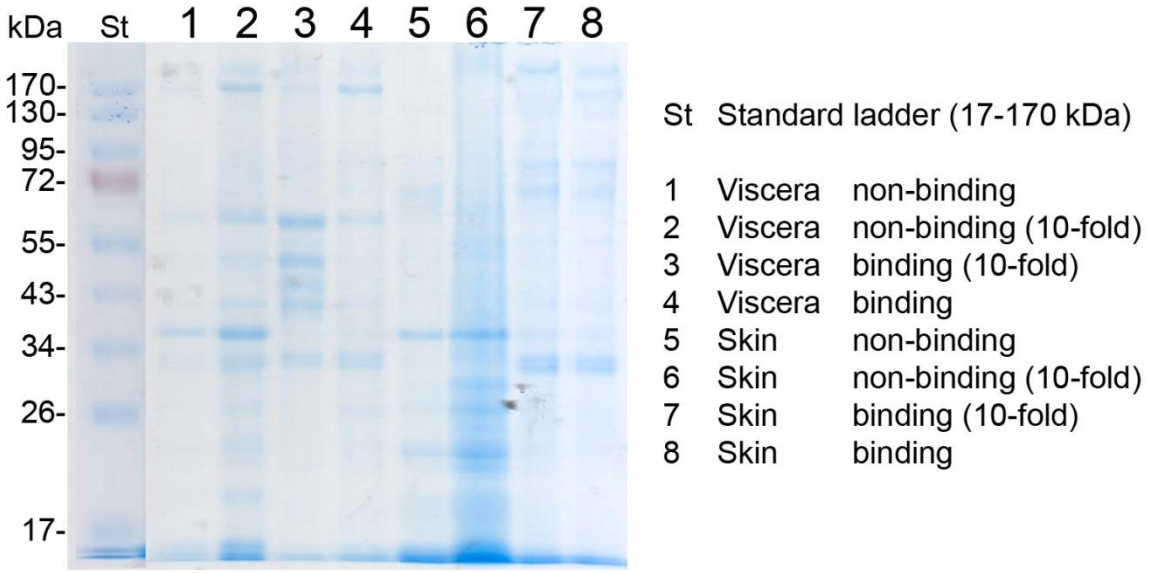


Figure 6 - SDS-PAGE of first *A. fulica* preparation after anion exchange chromatography showing the binding and non-binding fractions of viscera and skin.

Figure 6 shows the first *A. fulica* preparation applied to SDS-PAGE after the anion exchange step. The flow-through (non-binding) as well as the sodium chloride eluted fractions (binding) of the viscera and skin were applied in two different concentrations.

To get a first insight in the glycosylation, a second SDS-PAGE of the same samples after various digestion steps was performed. It should show how the cleavage of N-glycans (digestion with PNGase F), the removal of sialic acids (digestion with neuraminidase) or β-elimination influences the size of the numerous proteins (Figure 7).

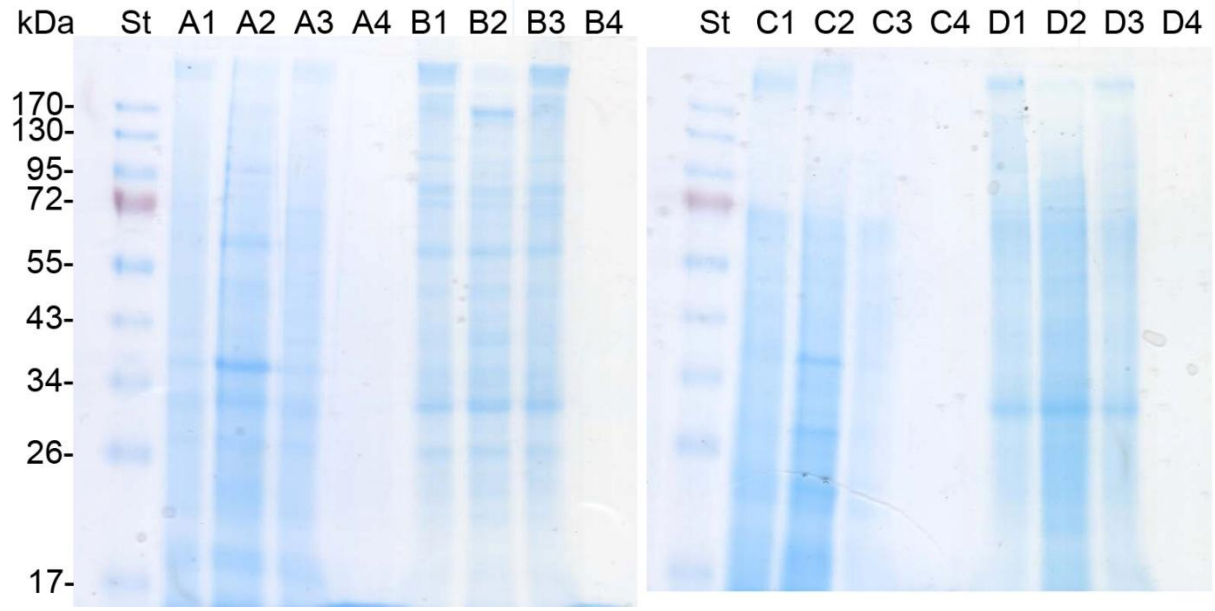


Figure 7 - SDS-Page of non-binding (A) and binding (B) fractions of *A. fulica* viscera and of non-binding (C) and binding (D) fractions of *A. fulica* skin: Standard (St), undigested (1), PNGase F digested (2), neuraminidase digested (3), subjected to β-elimination (4).

Surprisingly, there was no difference found between the control and PNGase F or neuraminidase digested lanes (1-3), while β-elimination led to a degradation of the whole protein. Overall, it has to be noted that all bands showed a rather diffuse and smeared appearance.

4.1.2 RP-HPLC of PA-labeled snail glycans

4.1.2.1 Comparison of N- and O-glycan fraction

Released glycans of *A. fulica* were separated in N- and O-glycan fractions according to the original protocol (3.1.1.2) and were PA-labeled and analyzed using RP-HPLC. Therefore purified snail proteins (Figure 6) were digested with pepsin. PNGase A was used to cleave N-glycans, leaving O-glycopeptides behind. These two fractions were then separated from each other by cation exchange chromatography, and O-glycopeptides were subsequently subjected to non-reductive β-elimination. Finally, 2-aminopyridine was used to label glycans and enable fluorescent detection on RP-HPLC.

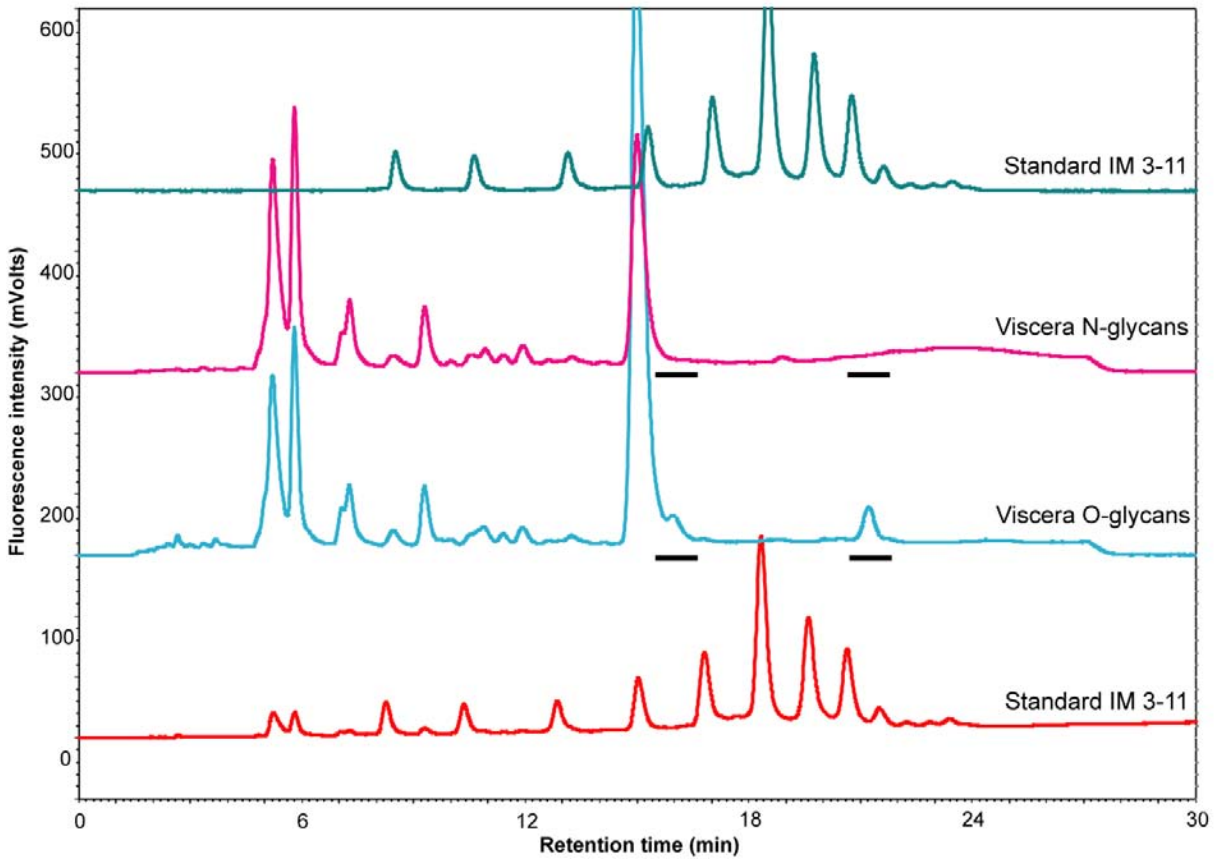


Figure 8 - RP-HPLC of PA-labeled *A. fulica* N- and O-glycans from viscera.

Figure 8 shows a representative result of the viscera flow-through fraction. Apart from two minor peaks (16 and 21 min), there was no significant difference between O- and N-glycan fraction. Certainly this is a clear indication that the purification and separation was not successful. It is surprising as well that N-glycans show quite early eluting peaks (eluting before four standard isomaltose units) and nothing later than the peak at 15.5 min that co-elutes with the standard substance consisting of six sugar residues.

4.1.2.2 Enzymatic digestion of snail glycan samples

Further on it was checked whether some structural elucidation via enzymatic digestions was possible. Therefore the “O-glycan fractions” of viscera and skin (binding fractions of the cation exchange chromatography step) were taken and incubated overnight with several commercially available enzymes which are known to degrade common glycan structures.

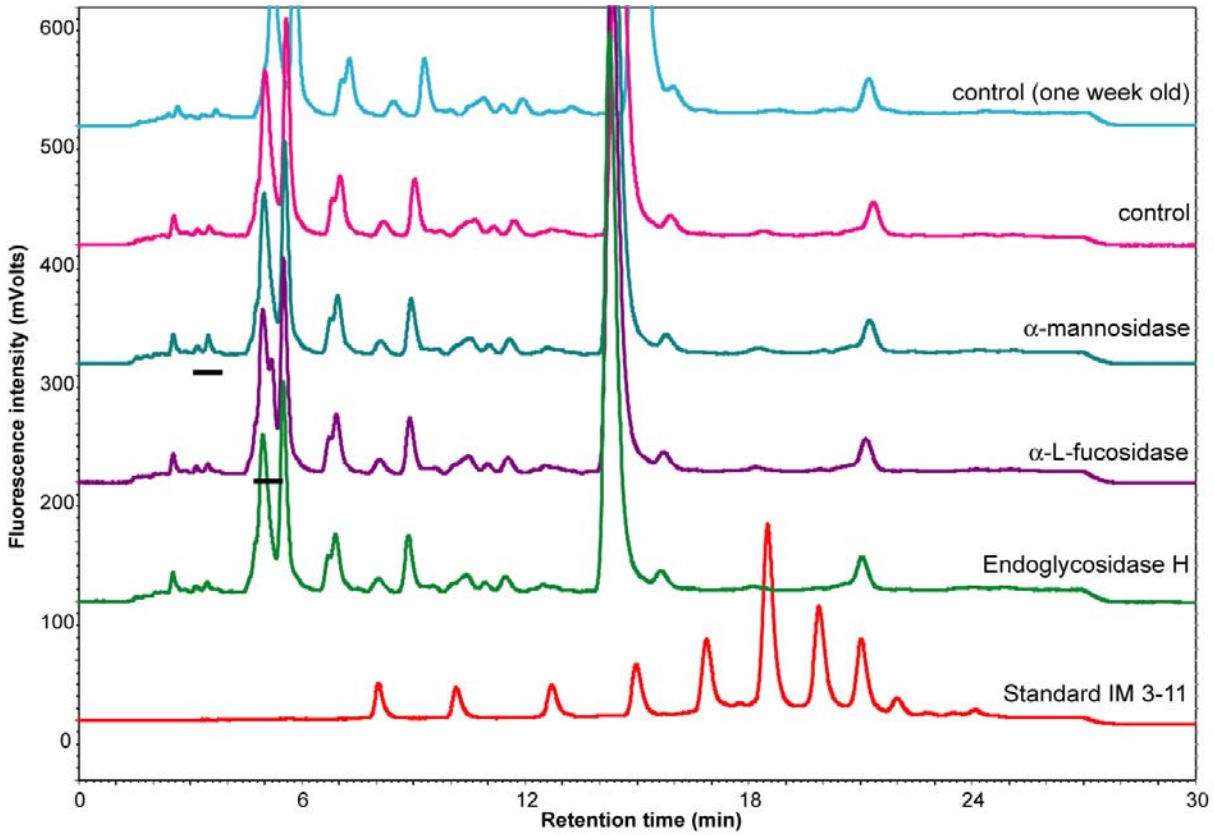


Figure 9 - Enzymatic digestions of putative *A. fulica* viscera O-glycan fraction.

A. fulica viscera ion exchange flow-through O-glycan fraction was digested with α-mannosidase, α-L-fucosidase and Endoglycosidase H. Additionally, a control run which was performed one week earlier is displayed to show the reproducibility (Figure 9). At the first glance no big changes in the spectrum are visible and also after having a closer look only minor peak shifts are obvious. The α-mannosidase digested sample shows a slightly increased peak at 3.5 min, and the α-L-fucosidase treated sample displays an additional peak at 5 min. For the Endoglycosidase H digested sample no differences can be spotted.

Figure 10 illustrates the experiments we performed with *A. fulica* skin ion exchange flow-through O-glycan fraction. Since no big peak shifts occurred in the previous assay (Figure 9), we decided to omit the Endo H digest and to include three additional enzymes: β-N-acetylglucosaminidase, β-galactosidase and α-1,2-fucosidase. While α-1,2-fucosidase did not affect the spectrum and α-L-fucosidase from bovine kidney (5 min) and β-N-acetylglucosaminidase (6 min) only caused one difference compared to the control spectrum, α-mannosidase and β-galactosidase catalyzed some more changes. Apart from some minor additional or lost peaks (3.4; 8.3; 12.2-13.0; 14.7; 15.7; 19.0 and 22.3 min) the additional peak around 18.0 min is the most prominent change in the α-mannosidase pattern. The β-

galactosidase as well displays some minor changes (2.3; 7.8; 8.9; 10.2 and 11.4 min) and two big additional peaks (5.3 and 5.7 min).

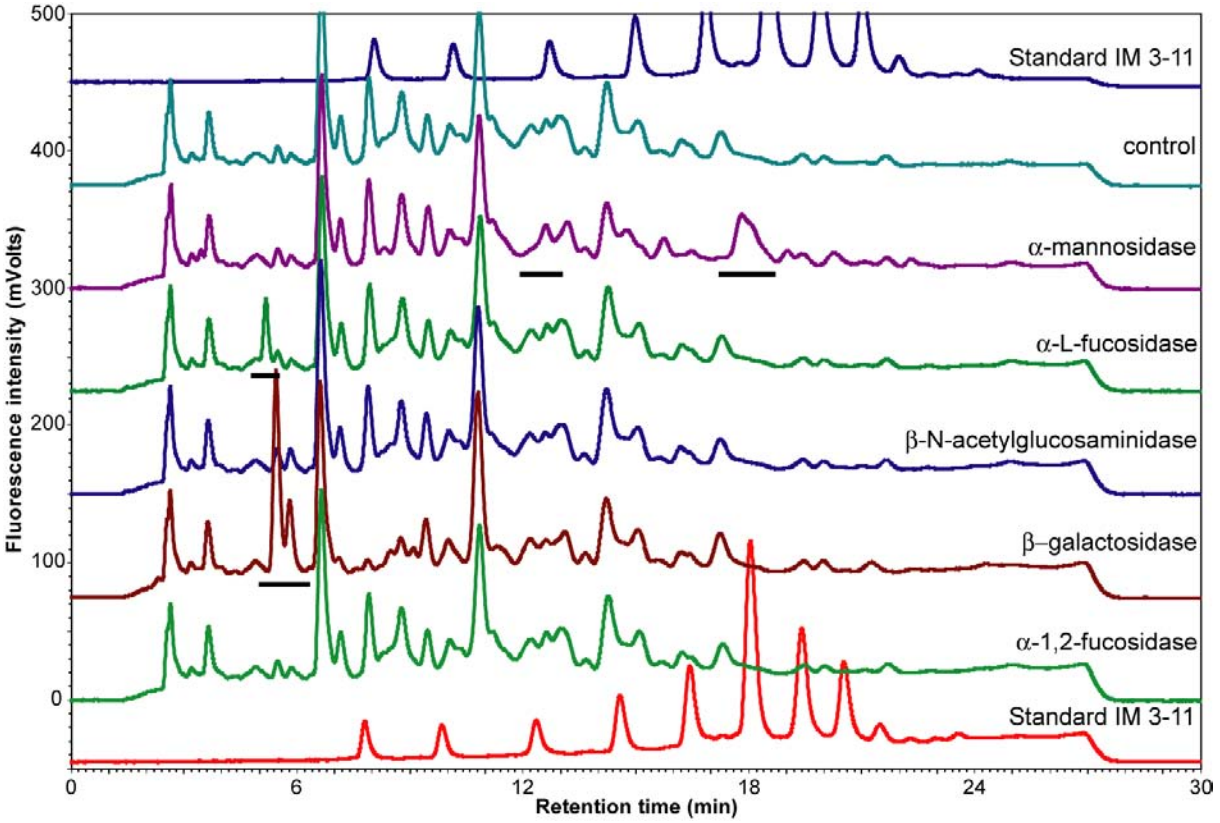


Figure 10 - Enzymatic digestions of putative *A. fulica* skin O-glycan fraction.

4.1.2.3 Fractionation of PA-labeled snail glycans

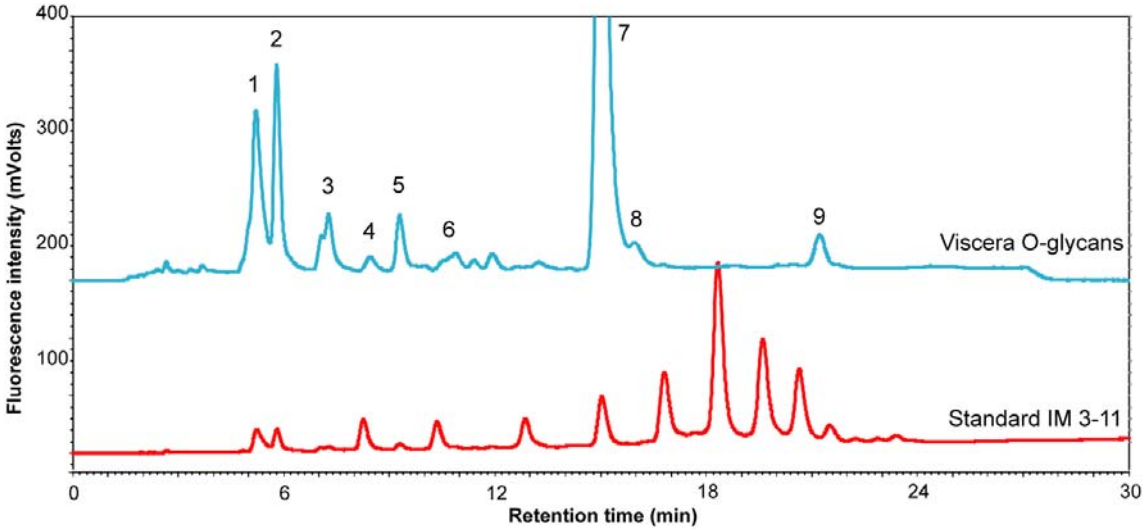


Figure 11 - Pool 1-9 of putative *A. fulica* viscera O-glycan sample.

To reduce the heterogeneity of the sample the putative O-glycans of *A. fulica* were fractionated in 9 pools (indicated in Figure 11) for further analysis.

4.1.3 NP-HPLC of PA-labeled snail glycans

The next step in elucidating the structure of the PA-labeled glycans was to apply fractionated pools (Figure 11) to NP-HPLC in order to get some information on their size.

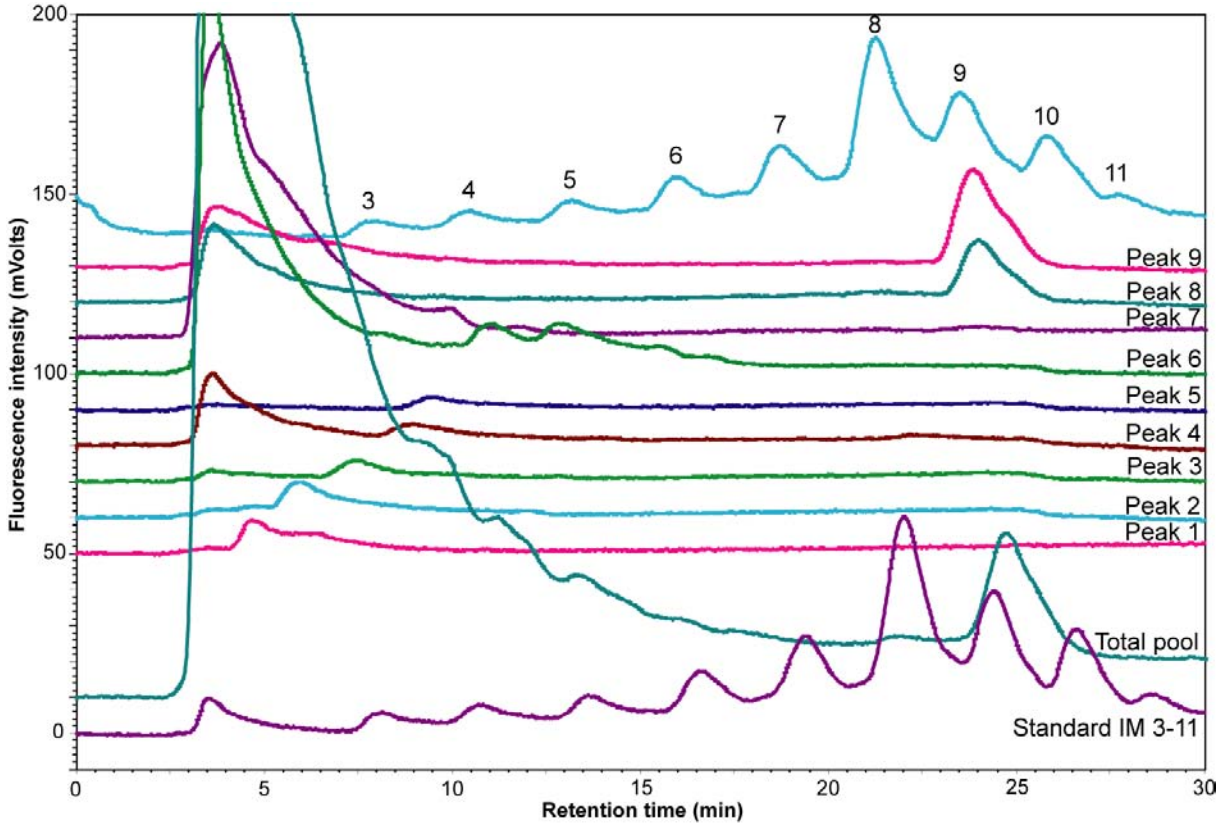


Figure 12 - Size elucidation of fractionated peaks using NP-HPLC.

Figure 12 shows the comparison of the isomaltose standard containing molecules consisting of three to eleven glucose units with the nine fractions derived from 4.1.2.3. Additionally, one unfractionated aliquot (=total pool) was applied as well where it can be clearly seen that the majority of the sample consists of rather small molecules. As expected, peaks 1 to 9 show increasing size. While peaks 1 to 7 are smaller than five glucose units, peak 8 and 9 are significantly larger. Their size can be estimated at about nine glucose units. It has to be noted that peak 7 which was by far the largest peak (Figure 11) only displays a peak right at the beginning of the chromatogram and therefore is a quite small structure. Taking these and former results into account, the suspicion that peak 8 and 9 might be N-glycan impurities arise. Even though the total pool had already been digested with Endo H and α-mannosidase

which should digest some N-glycans at least to some extent, a re-try of these enzymatic digestions on the purified structures was carried out.

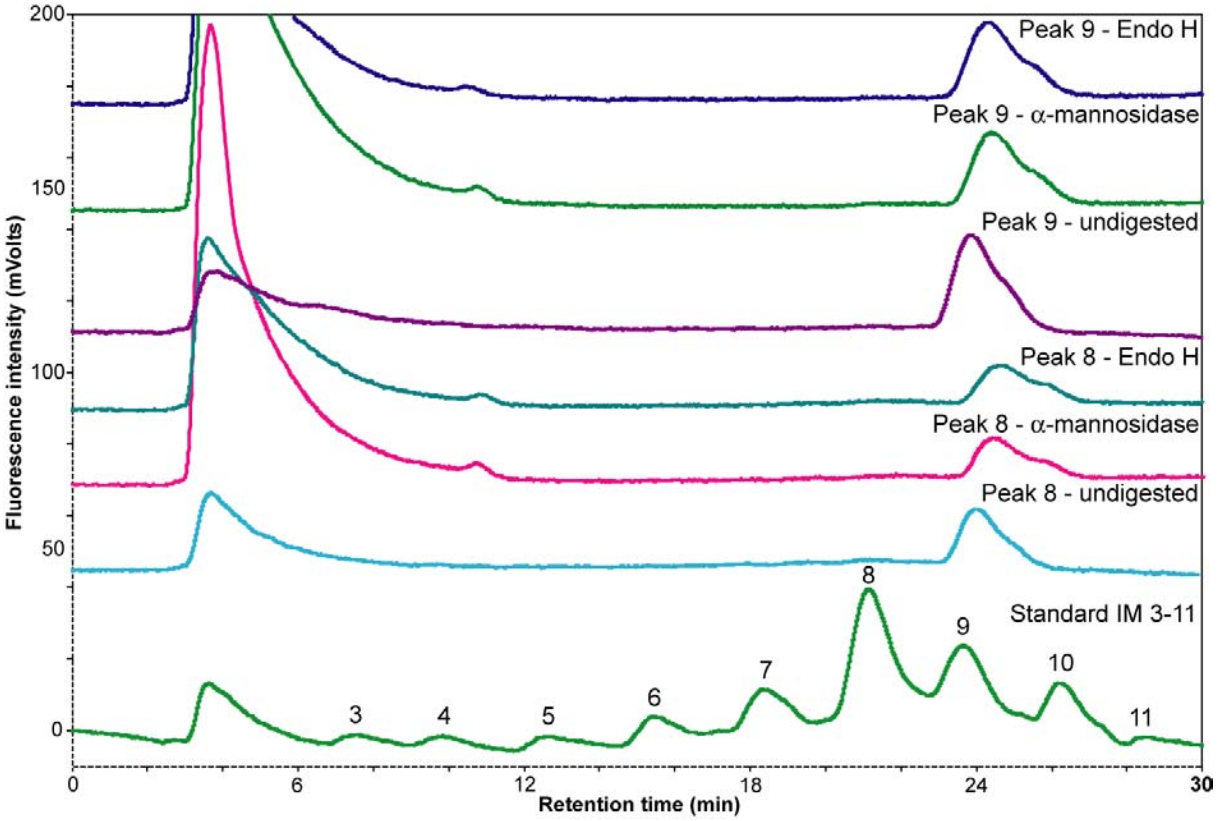


Figure 13 - Endo H and α-mannosidase digestion of fractionated pools 8 and 9 from Figure 11.

Figure 13 displays the undigested assays of peak 8 and 9 as well as both samples digested by Endo H and α-mannosidase. Obviously both peaks are not affected significantly which drastically reduces the possibility of these two peaks being N-glycan impurities.

4.1.4 FACE of purified snail glycans

In theory, FACE should enable the high resolution separation of reducing saccharides. The method can be used for mono- up to oligosaccharides consisting of six or more residues. Due the fluorescent staining, the detection of low amounts of carbohydrates is possible.

Since the snail O-glycans were expected to be about in that range concerning their size, some experiments using this technique were carried out. Unfortunately our equipment (UV transilluminator and camera) is not really capable of taking pictures of a gel containing ANTS-stained bands. The UV filter attached to the camera is made to cut off wavelengths below 580 nm. While this is fine for ethidium bromide stained DNA bands which emit yellow light at about 605 nm, the ANTS stained green bands (515 nm) are not visible on the taken

pictures. On the other hand pictures without the filter attached are overexposed and therefore without value.

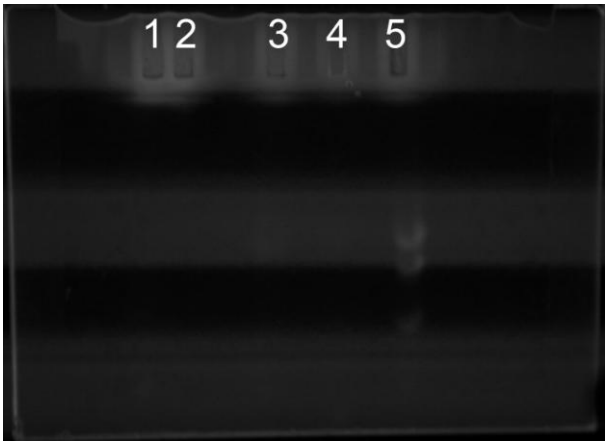


Figure 14 - FACE of AMAC-derivatized oligosaccharides. Dextran-ladder (1,2), putative *A. fulica* O-glycans (3,4) and fetuin (5). [Picture was taken without UV-filter.]

Figure 14 shows the result of an experiment using the Tris/boric acid buffer system to allow the separation of uncharged glycans. Unfortunately, neither the ladder of digested dextran nor the snail sample gave any bands. Just the applied fetuin gave three faint bands. These bands were then cut out and soaked in water overnight to elute the stained glycans for MS analysis. Also this experiment was not successful and therefore it was decided not to go deeper into this separation method. There are no clear technical reasons why this method did not work properly in our hands but obviously much more time would have been necessary to get acquainted to it.

4.1.5 MALDI-TOF/MS analysis of purified O-glycans

The fractions which were pooled after NP-HPLC (Figure 11) were also analyzed by MALDI-TOF/MS. As shown in Figure 15, there is still a rather high heterogeneity within the sample. Apart from many peaks that could not be assigned with a possible monosaccharide composition (mostly below m/z 1000), we found a number of oligosaccharides which are definitely not O-glycans. Chains of hexoses (Hex7-PA up to Hex11-PA) as well as typical N-glycans (HexNAc2Hex6-PA and HexNAc2Hex8-PA) were found.

The found structures show triple peaks; assigned peaks are H^+-adducts with their corresponding sodium (+ 21.99 Da) and potassium adducts (+ 38.10 Da).

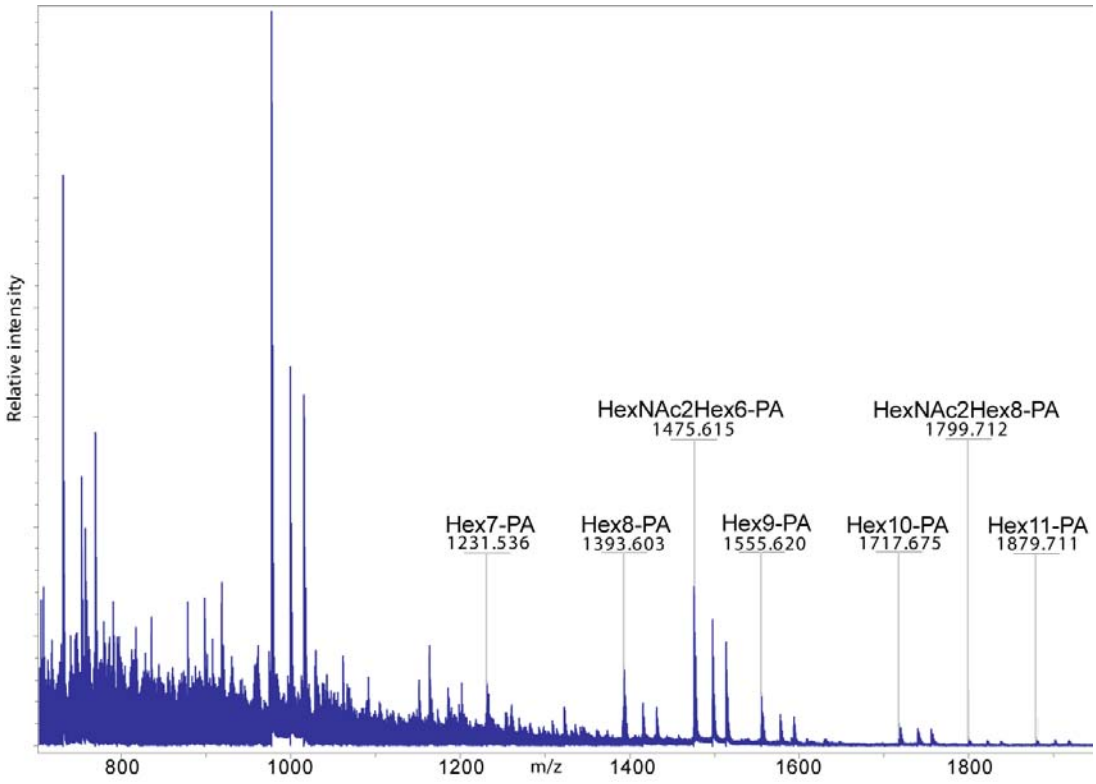


Figure 15 - MALDI-TOF/MS of peak 7 from putative *A. fulica* viscera O-glycan fraction.

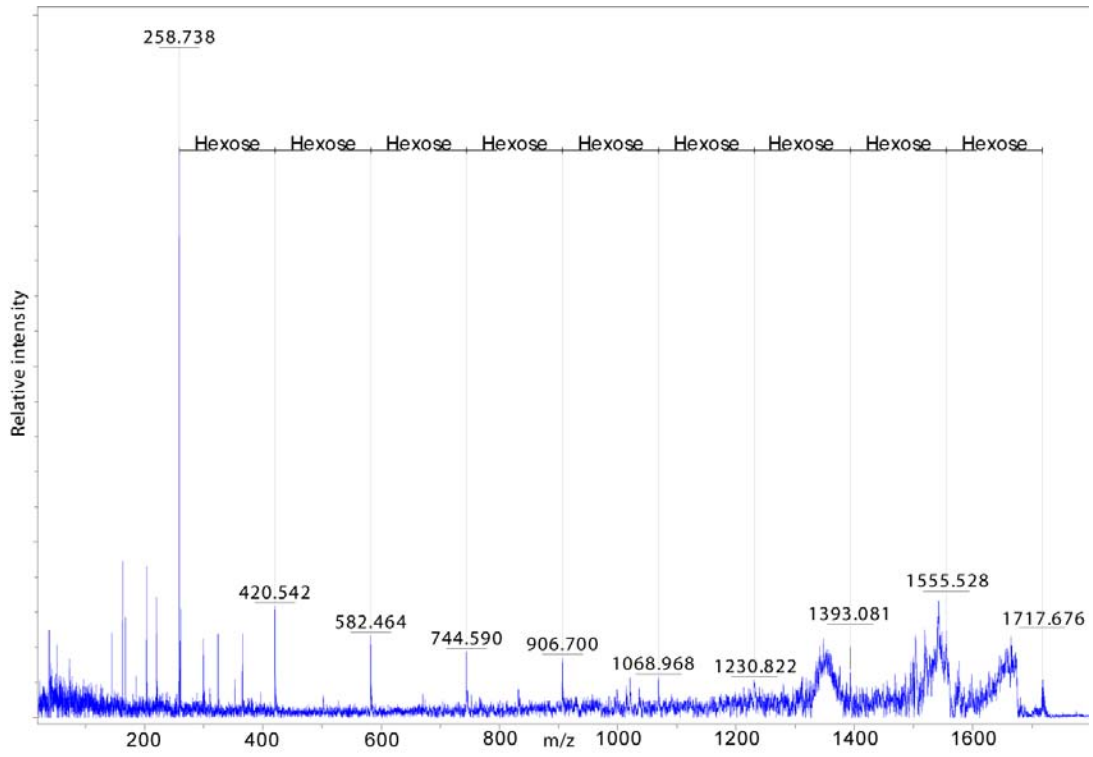


Figure 16 - MALDI-TOF/MS/MS of Hex10-PA (1717.7 Da) structure from Figure 15.

All peaks assigned in Figure 15 were isolated and subjected to MS/MS. The fragmentation pattern of the peak at m/z 1717.7 (Hex10-PA) is shown as example in Figure 16. The fragmentation pattern clearly shows how the parent ion (m/z 1717.7) gets stepwise reduced by one hexose (m/z 162.1) after the other. The inner PA-labeled hexose (m/z 258.7) is the residue after the cleavage of nine hexoses.

In the spectrum shown in Figure 15 derivatives of this structure containing from seven up to eleven hexoses are also prominent. The peak at m/z 1475.6 was elucidated as typical N-glycan structure consisting of two N-acetylhexosamine and six hexose residues, the peak at 1799.7 of two N-acetylhexosamine and eight hexose residues.

4.1.6 Summary of the first results derived from the original snail glycan purification protocol

Even though the glycan purification included various different clean-up steps we were not able to get clean glycan fractions. Especially the O-glycan fractions were spoiled on the one hand side with hexose chains which were suspected to be some kind of storage carbohydrate and on the other hand side with N-glycans. Both contaminations made a reliable monosaccharide analysis impossible and the high heterogeneity of glycans within the sample caused big difficulties during any kind of analysis.

Apart from these obvious impurities the overall condition of our purified glycans was not really inspiring confidence. Samples maintained a sticky and sometimes gel-like appearance. This impression was also confirmed by first GC results which indicated a high amount of water in “dried” samples.

4.2 Intermediate experiments

The next goals were to remove storage carbohydrates, get a clean and dry sample and achieve a reliable separation of N- and O-glycans.

4.2.1 Hydrazinolysis

The first idea was to try the frequently published method of hydrazinolysis. This method should be capable of cleaving N- and O-glycans from the peptide backbone while keeping the reducing end intact for further labeling procedures. By just adjusting the reaction temperature, it should be possible to selectively cleave N- or O-glycans. Later on labeling with AA or PA should give clean fractions of fluorescently labeled glycans.

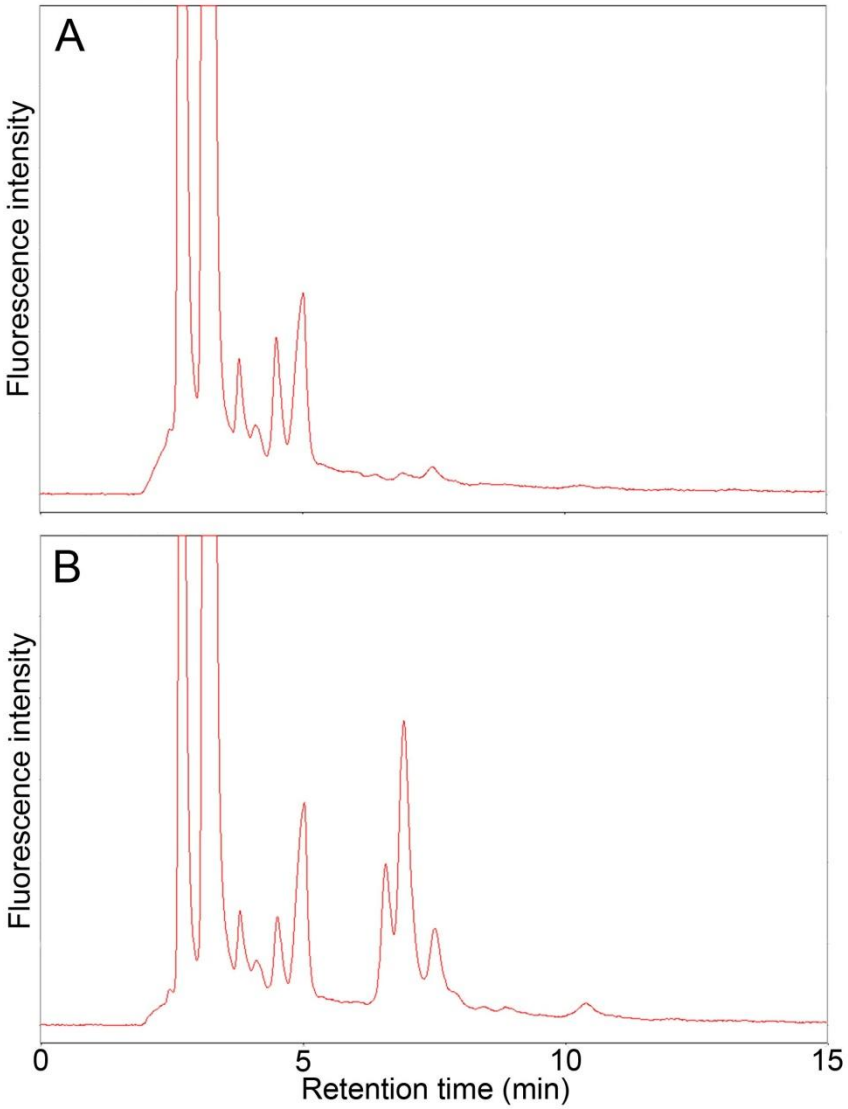


Figure 17 - PA-labeled N-glycans (A) and O-glycans (B) of *A. lusitanicus* after hydrazinolysis on NP-HPLC.

Unfortunately, the results of our experiment were not as positive as publications would suggest. We tried derivatization with AA and PA and separated the samples on RP- as well as NP-HPLC columns.

Figure 17 shows the PA-labeled N- or respectively O-glycans on NP-HPLC. While there are at least some additional peaks for the O-glycan fraction – the peaks at the beginning of the N-glycan pattern are quite similar to those of the O-glycan pattern. Since all these peaks elute very early, just after the dead volume, we do not expect them to be analytically relevant. The first standard peak consisting of three glucose units has a retention time of about eleven minutes which is also an indication that the peaks shown in Figure 17 are not N- or O-glycans. Since the results on RP-HPLC and after AA labeling are very similar, and because the handling of hydrazine is quite challenging, we decided to go back to our original protocol.

4.2.2 Modification of the original protocol

A further purification approach was to add hydrophobic interaction chromatography in order to help to remove the “sticky” components which prevent proper drying of the samples. Additionally various glycosidases were tested on our samples to check whether the storage carbohydrate chains could be digested.

As shown in Figure 18, four different enzymes were tested on a snail sample which displayed a nice set of hexose chains (Hex 3 to 9). In our samples α-glucosidase from rice showed the most convincing results and therefore an incubation step with this enzyme was included in all following clean-up procedures.

While this optimization solved the storage-carbohydrate problem, our other problems were still there. On the one hand side the protocol was getting longer and longer but the purification effect didn’t seem to increase. We were also not able to achieve a clean separation of N- and O-glycans even though we experimented with multiple PNGase A and F digestions followed by various cation exchange steps.

When we finally encountered big problems with permethylating our samples for MALDI/MS analysis, we checked the protein content of our carbohydrate samples and found out that we were obviously struggling with peptide contamination which could not be removed by reversed phase or ion exchange chromatography.

Although monosaccharide analysis on GC and HPLC looked fine, MALDI-TOF analysis was still not possible. The native samples showed a huge amount of peaks which were not fitting to any carbohydrate composition, and the permethylated samples displayed a severe under-methylation (Figure 19).

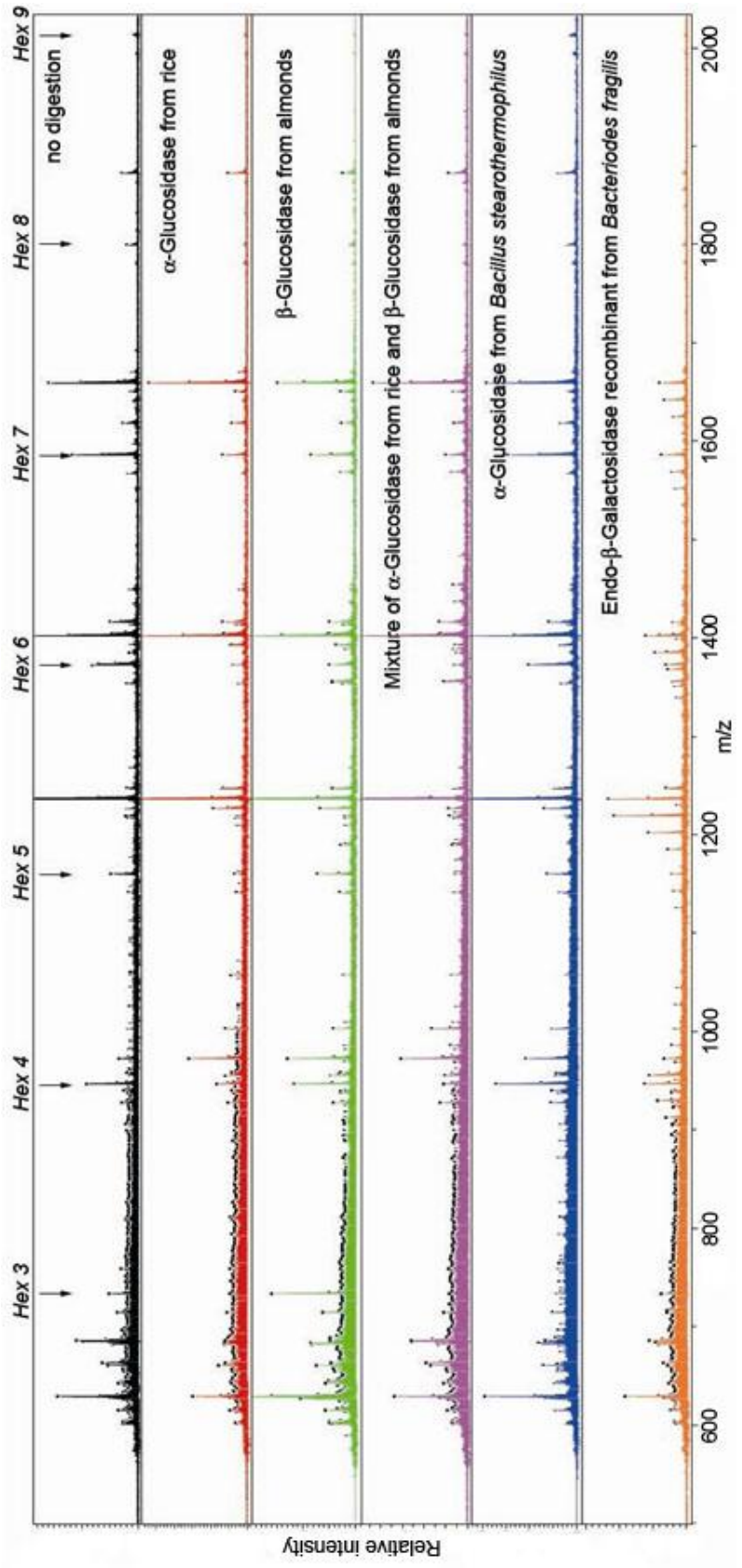


Figure 18 - Testing of various glycosidases.

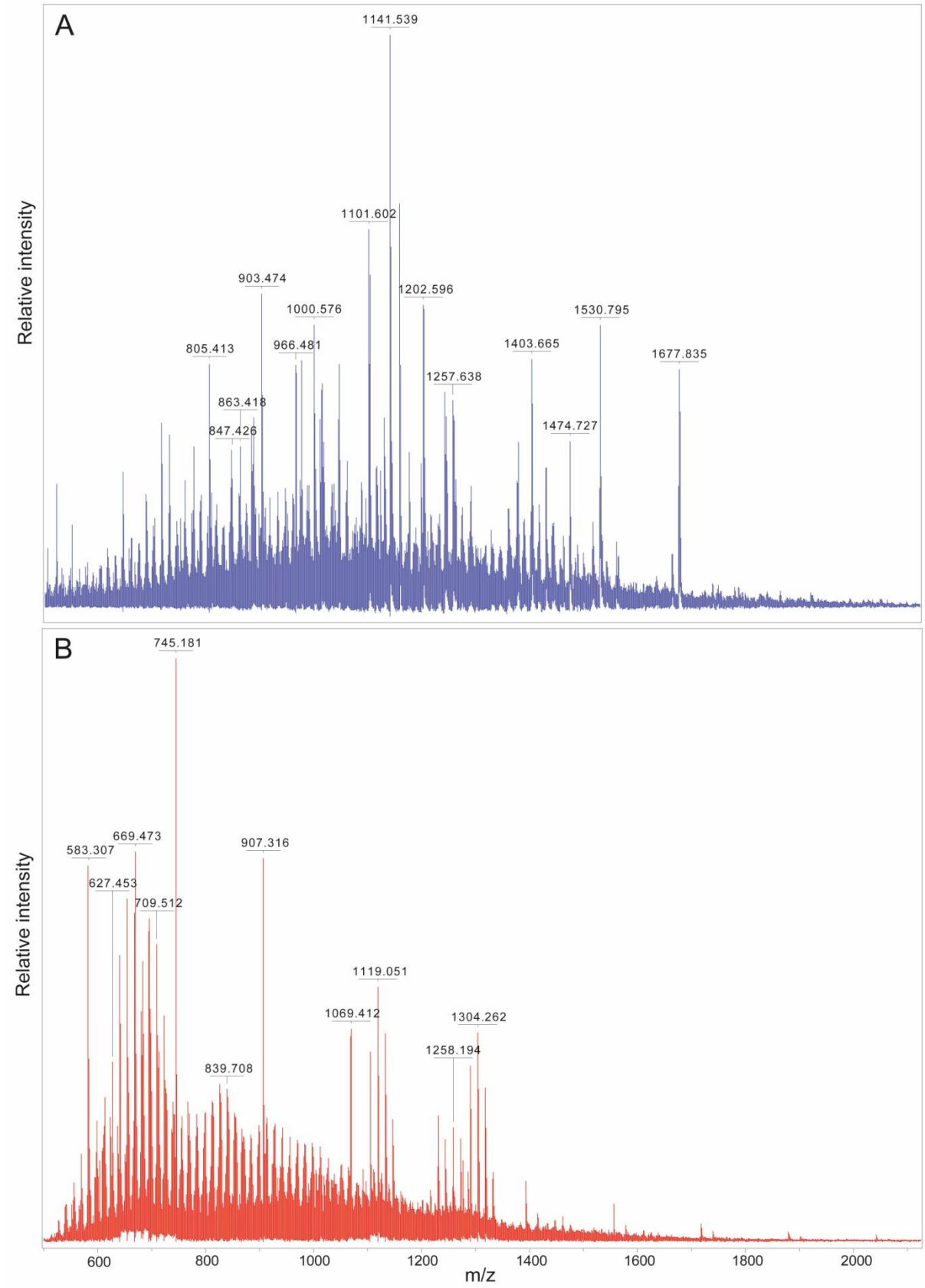


Figure 19 - Final MALDI-TOF experiment (A: native sample, B: permethylated sample).

This was one of the turning points where the standard procedure was drastically changed. As depicted in 3.1.1.3, many gel filtration steps were removed and time saving acetone or methanol precipitation steps were introduced. The PNGase digestion step was omitted as well since all previous results still documented the presence of N-glycans anyway. Instead of a non-reductive β-elimination, commonly used methods were chosen. The method by Iyer and Carlson [114] was tested but replaced by the most commonly used protocol by Spiro and Bhoyroo [115] later on when ESI/MS analysis confirmed that N-glycans were still released. By keeping the clean-up procedure as straight forward as possible sample degradation was also reduced.

4.3 Optimal purification procedure

Finally, after taking many experiments and results into account, one purification procedure was found to be optimal for the extraction of glycans from snail samples (3.1.1.3). Compared to the original protocol (3.1.1.2), this method allows a better extraction of glycans from the crude sample and can be completed in shorter time. Especially, the removal of gel filtration steps which pose a bottleneck when multiple samples are prepared in parallel as well as the removal of most enzymatic digestions (PNGase A and F) which necessitate overnight incubation are a great boost for the convenience of the clean-up procedure.

Although the new protocol is less time consuming, it was possible to raise the quality of the samples significantly. Due to the introduction of three, previously not used, purification approaches it was possible to reduce contaminations of the samples which interfere with the subsequent analyses.

After the dissection of the snail and the removal of the gut, proteins are homogenized in CHAPS-based lysis buffer. They are then reduced and alkylated to disrupt the native protein folding. Further on, they are precipitated in chilled acetone as first purification step. Since large storage polysaccharides which distort monosaccharide content analyses also precipitate in acetone, α-glucosidase is used to digest these molecules. Cation exchange chromatography and two further precipitation steps (acetone and methanol) are then capable of removing most non-protein sample content.

After β-elimination, released glycans (O-glycans with some N-glycans) are subjected to porous graphitized carbon chromatography. By doing this, samples are desalted and purged from peptides conveniently in one step.

Subsequent analyses are then best started with ESI/MS analysis which is capable of discriminating N- and O-glycans or by sample fractionation using PGC-HPLC. Fractionated peaks can be then subjected to GC/MS or MALDI-TOF/MS for a more detailed investigation.

4.4 Optimization of HPLC monosaccharide analysis

One of the first steps of every glycan analysis usually is monosaccharide analysis. This is necessary to get some insights on the glycan constitution, to evaluate possible contaminations with non-glycan carbohydrates or to elucidate untypical monosaccharide constituents such as methylated sugars.

Among various techniques such as capillary electrophoresis or gas chromatography, HPLC analysis using different labeling approaches is one of the most commonly used methods.

4.4.1 Anthranilic acid-labeling

Anthranilic acid derivatization is one of the most commonly used labeling methods for the analysis of monosaccharides on RP-HPLC. After addition of the fluorophore via reductive amination, labeled monosaccharides can be detected with high sensitivity without removal of excess derivatization mixture. Common protocols are tailored for the derivatization of quite large sugar amounts [122, 169]. In a first step the optimal working range for the analysis of one monosaccharide was analyzed.

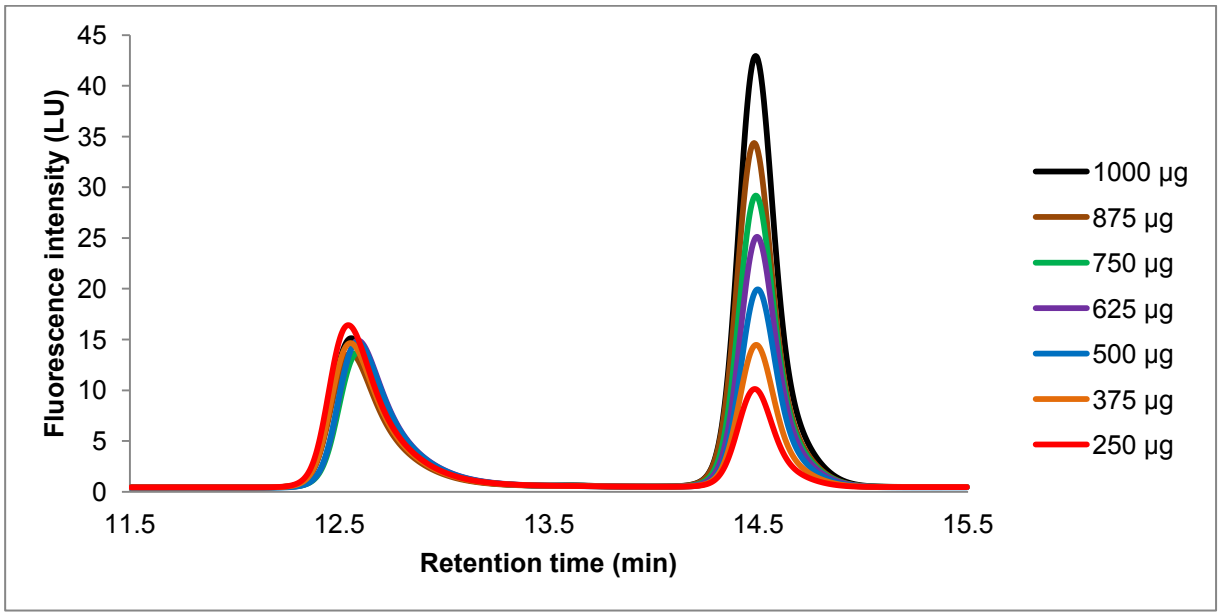


Figure 20 - AA-Derivatization of various amounts of galactose (250-1000 µg) with AA following the old protocol.

As shown in Figure 20, the method published by K.R. Anumula is suitable for sugar amounts of at least 100 µg [122]. Various amounts of galactose were derivatized and the ratios of sugar peak (12.6 min) height to AA peak (14.5 min) height were compared.

Throughout the tested range, the height of the AA peak does not change significantly while the galactose peak is clearly dependent on the derivatized amount of sugar. Derivatization of 1000 µg of galactose gives a sugar to AA peak height ratio of about 3:1, labeling of 375 µg of galactose results in a peak that is already smaller than the AA peak.

Additionally, it can be observed that the size of the large “dirt peak” at the end of the chromatogram (21-33 min) is dependent on the amount of AA (Figure 21). The more AA was needed for labeling, the smaller the peak at the end got. While this “dirt peak” cannot be attributed to a specific substance or reaction by-product, its size obviously increases with the amount of excess reagent.

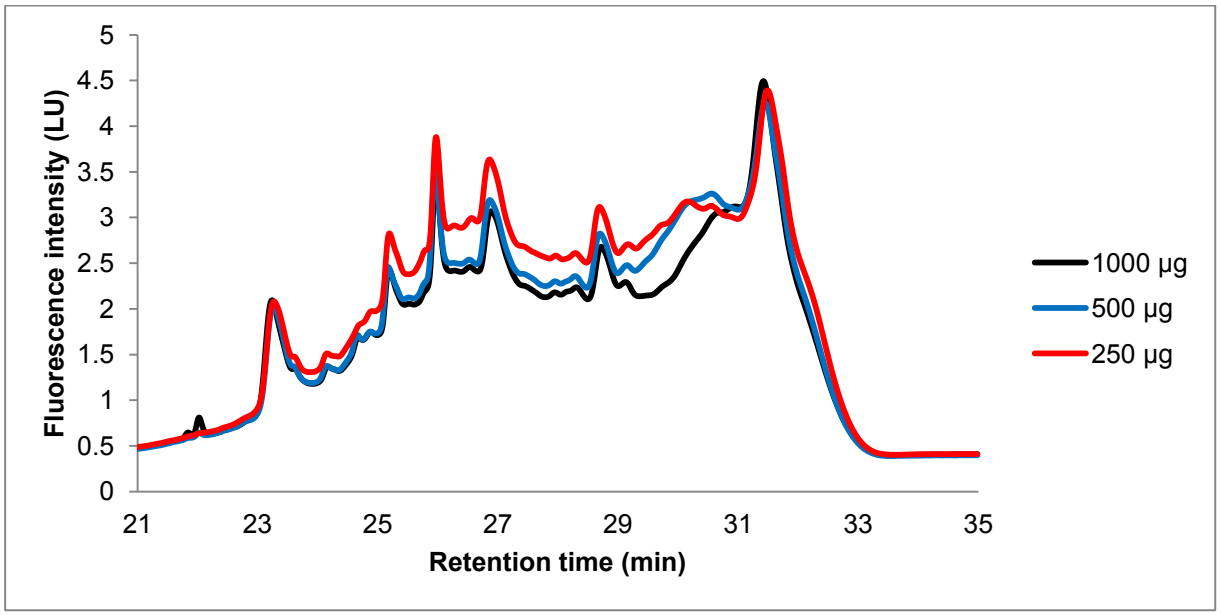


Figure 21 - „Dirt peak“- AA-Derivatization of various amounts of galactose (250-1000 µg) following the old protocol.

According to the original protocol the sample is dissolved in 50 µl of sodium acetate solution and 100 µl of AA reagent solution (30 mg AA and 30 mg sodium cyanoborohydride in 1 ml of 2% boric acid in methanol). The first down-scaling approach was to cut the added AA reagent solution by 90% and thereby reduce the amount of excess AA. Instead of 100 µl of AA reagent solution just 10 µl were added.

The original protocol suggests a total reaction volume of 150 µl. Therefore 140 µl of sodium acetate solution were added to keep the reaction volume constant and to ensure a good dissolving of the sample. However, additional reaction mixtures with reduced reaction volumes containing 5, 50 and 90 µl of sodium acetate solution in addition to the 10 µl of AA reaction solution were tested as well.

	AA peak		Galactose peak	
Sodium acetate solution	Area (LU*s)	Height (LU)	Area (LU*s)	Height (LU)
5 µl	18.9	0.82	48.3	3.6
50 µl	22.5	0.98	39.3	2.9
90 µl	24.9	1.10	18.4	1.4
140 µl	26.8	1.20	7.5	0.6

Table 11 - First down-scaling approach - Derivatization of 100 µg of galactose using 10 µl of AA reagent solution and specified amount of sodium acetate solution (5-140 µl).

As shown in Table 11, the concentrated mixture (5 µl sodium acetate solution and 10 µl AA reagent solution) gives the best galactose peak area and height. The high sample dilution in the mixture consisting of 140 µl of sodium acetate solution and 10 µl AA reagent solution leads to a significant worse galactose to AA peak ratio despite the same amounts of galactose and AA were used (Figure 22).

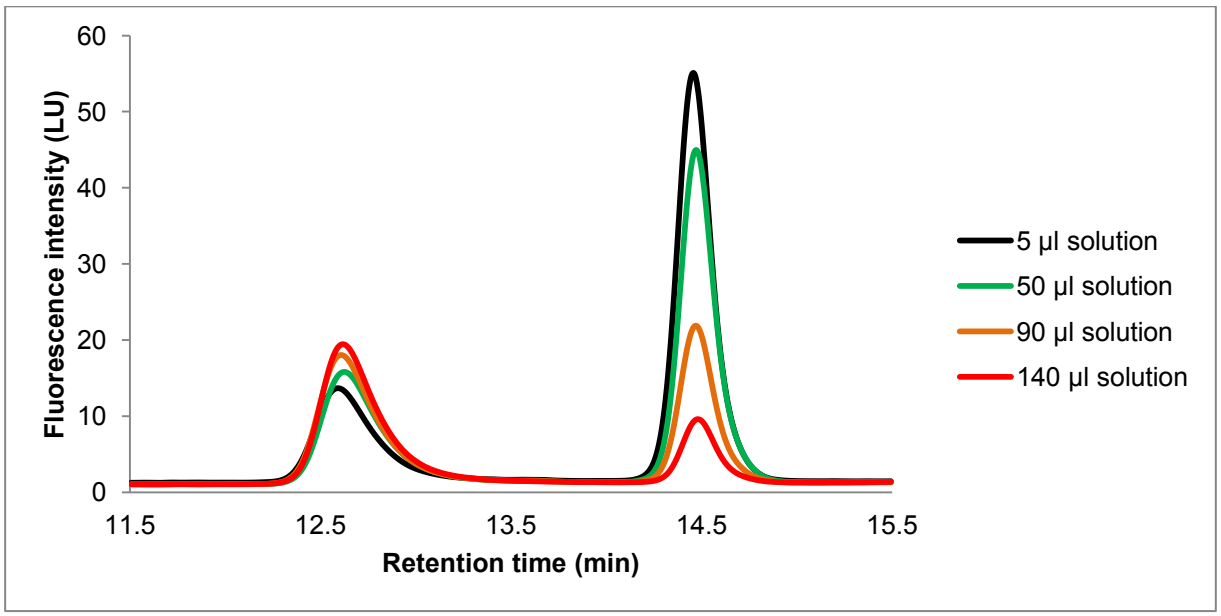


Figure 22 - First down-scaling approach - AA-Derivatization of 100 µg of galactose using 10 µl of AA reagent solution and specified amount of sodium acetate solution (5-140 µl).

To ensure that 5 µl of sodium acetate solution are sufficient to resolve the dried monosaccharide, we tested various standard sample amounts which fit our expected sample amount. As shown in Figure 23, 75 µg of galactose were still giving an appropriate ratio of

sugar to reagent peak. Comparing both peak areas, a sugar to AA peak ratio of 1.7 was found – and since the AA peak is broader than that of the derivatized sugar, the ratio of sugar to peak height was 2.4. By simply down-scaling the standard protocol to one tenth, we therefore achieved a significant optimization compared to Figure 20. The sugar-to-AA peak ratios for the previous derivatization of 750 µg of galactose were 1.5 for the area and 2.2 for the height respectively.

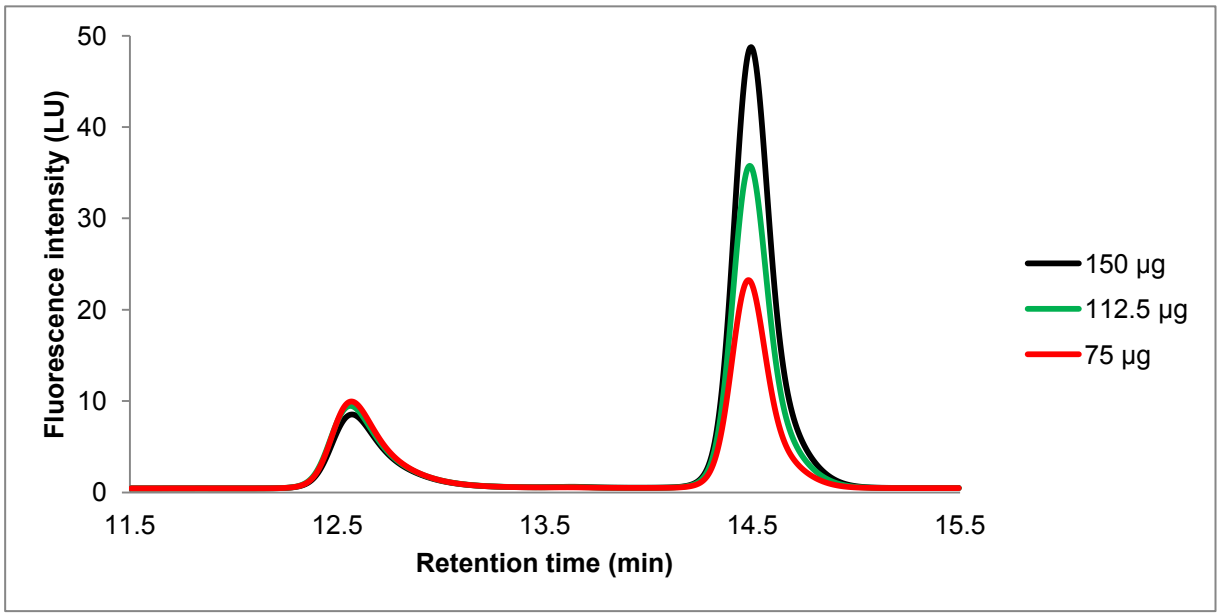


Figure 23 - AA-Derivatization of various amounts of galactose (75-150 µg) after reducing AA reagent and sodium acetate solution by 90%.

Nevertheless, the high sensitivity of fluorescence detection predestines this labeling method for even further down-scaling. To keep handling as easy as possible, a further reduction of the total reaction volume was not considered. While preliminary tests showed that reduction of sodium cyanoborohydride has negative effects on the derivatization yield, we solely decreased the AA amount in the following tests. The original protocol suggests preparation of the AA reagent solution by dissolving 30 mg of cyanoborohydride and 30 mg of AA in 1 ml of 2% boric acid in methanol. Four different reaction mixtures containing 30, 15, 3 and 0.3 mg of AA were tested to derivatize a sugar amount as low as 1 µg.

Figure 24 shows how the AA peak (12.7 min) decreases significantly during our second down-scaling approach. Not surprisingly the reagent peak size decreases with lower amounts of AA added to the reaction.

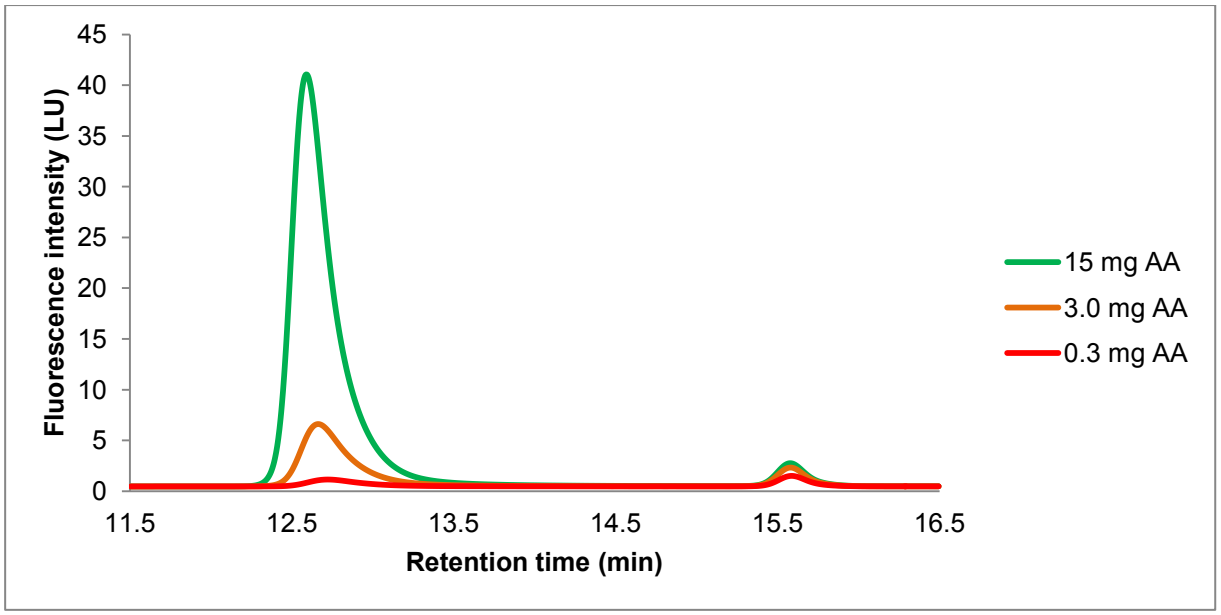


Figure 24 - Second down-scaling approach – AA-Derivatization of 1 µg of glucose using 10 µl of AA reagent solution (specified amount of AA and 30 mg of sodium cyanoborohydride in 1 ml of 2% boric acid in methanol) and 5 µl of sodium acetate solution.

A closer look at the peak height of the glucose peak (15.6 min) revealed how the various amounts of AA affect the derivatization efficiency of the sugar (Figure 25).

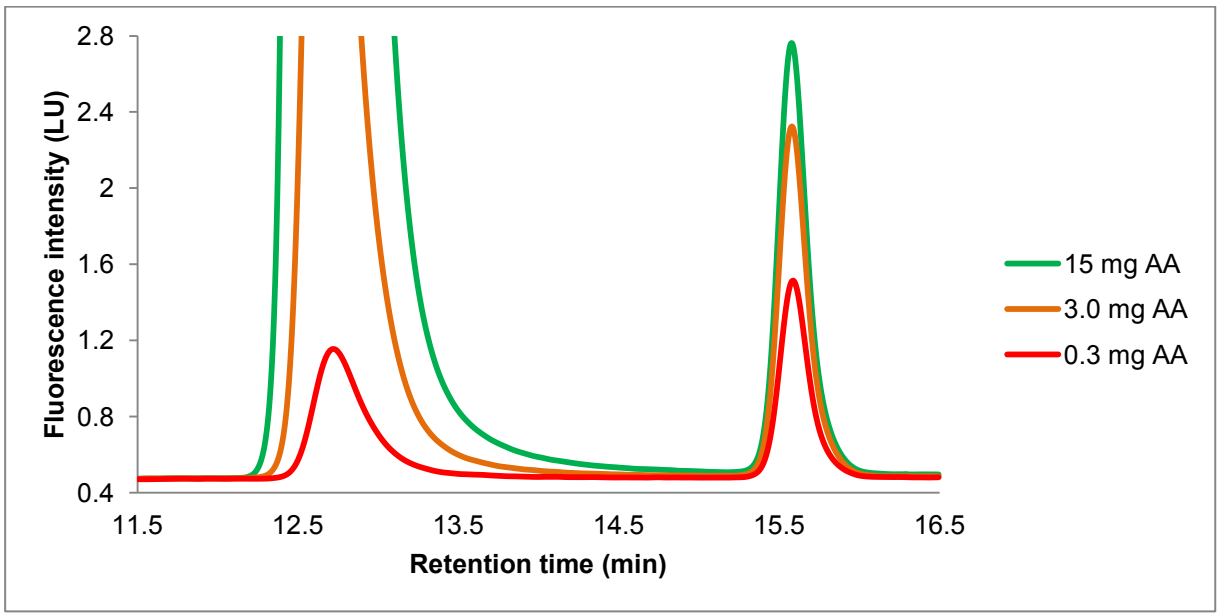


Figure 25 - Second down-scaling approach – AA-Derivatization of 1 µg of glucose using 10 µl of AA reagent solution (specified amount of AA and 30 mg of sodium cyanoborohydride in 1 ml of 2% boric acid in methanol) and 5 µl of sodium acetate solution (detailed view).

Since signal height of glucose didn't increase dramatically in the 15 mg AA test compared to the 3 mg test (area increase = approx. 24%) and addition of just 3 mg of AA led to a vastly smaller AA peak (area reduction= approx. 83%) (Figure 25 and Table 12), 3 mg of AA and 30 mg of sodium cyanoborohydride in 1 ml of 2% boric acid in water were chosen as optimal reaction mixture for the following monosaccharide analyses.

	AA peak		Glucose peak	
	Area (LU*s)	Height (LU)	Area (LU*s)	Height (LU)
15 mg AA	768.3	40.6	29.9	2.3
3.0 mg AA	131.4	6.1	24.2	1.8
0.3 mg AA	16.4	0.7	14.1	1.0

Table 12 - Second down-scaling approach (AA-derivatization of 1 µg of glucose).

As next step, the optimal working range for the new protocol was established. Mannose amounts from 5-300 nmol were derivatized and gave good sugar to AA peak ratios throughout the tested range (Figure 26). While the lower end (approx. 1 µg of sugar) of the test range still gave some room for further investigation on the limit of detection, the upper limit was resolved in this test. Peak areas for 300 nmol of derivatized sugar hardly increased compared to 240 nmol. This can be explained by the fact that the added AA was completely used up, and AA peaks (12.4 min) were almost gone (Figure 27).

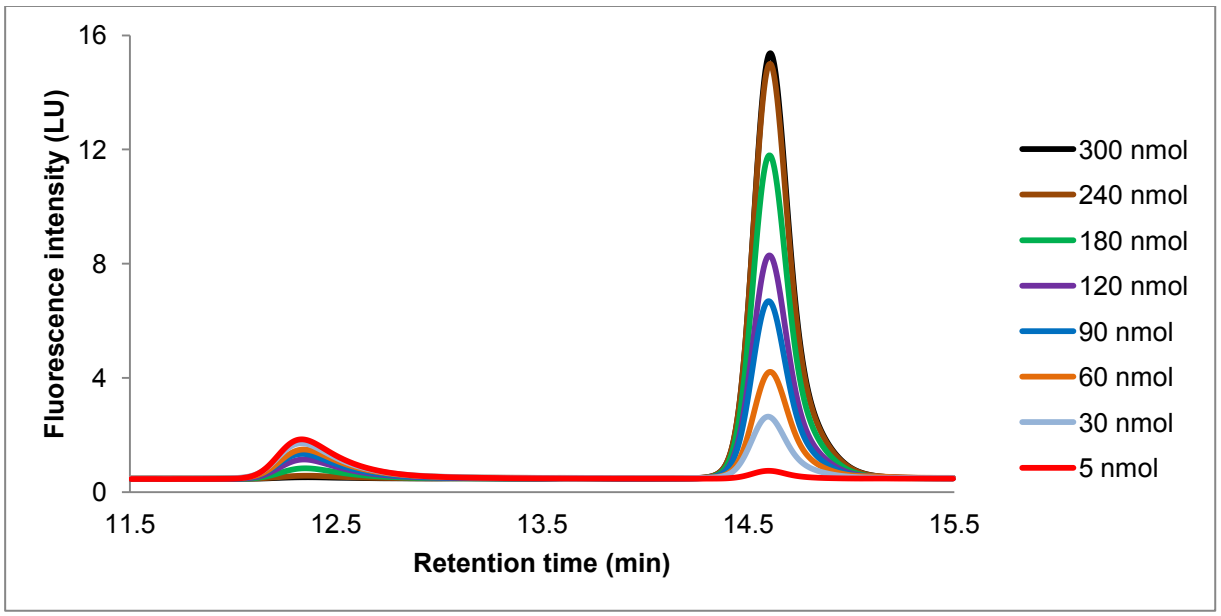


Figure 26 - AA-Derivatization of various amounts of mannose (5-300 nmol) following the new protocol.

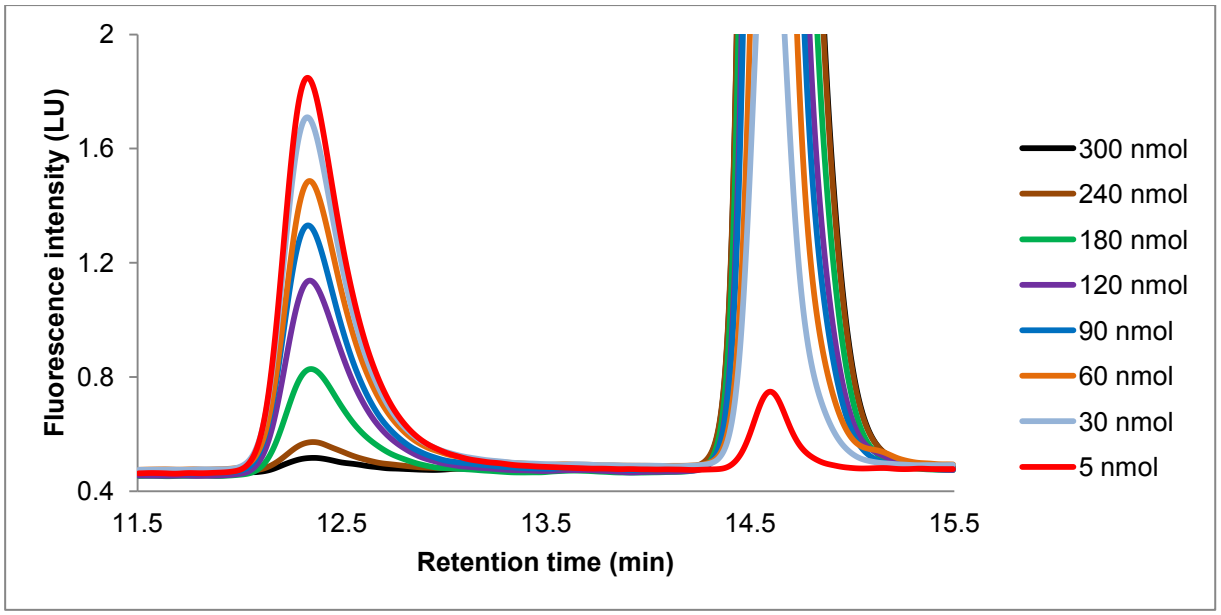


Figure 27 - AA-Derivatization of various amounts of mannose following the new protocol (detailed view).

After establishing the ideal working range, the response values of different standard sugars were checked. Derivatization of 180 nmol of the six standard sugars GlcN, GalN, Gal, Man, Xyl and Fuc clearly showed that there is a difference between amino sugars, hexoses and other sugars like Xyl or Fuc (Figure 28).

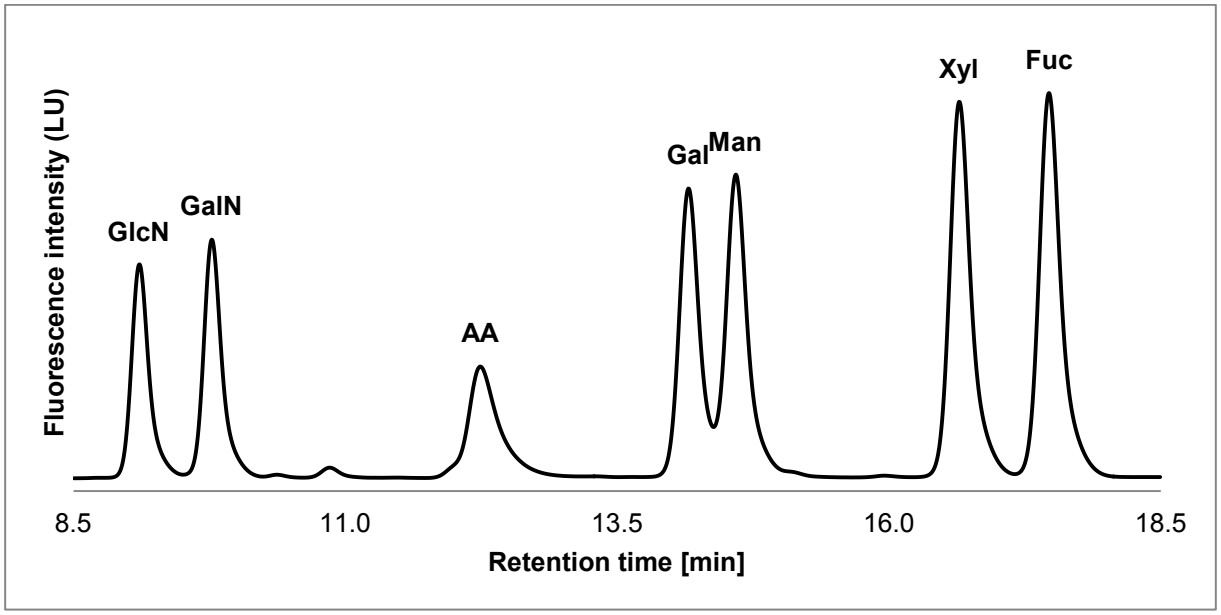


Figure 28 - AA-Derivatization of equimolar amounts of six standard sugars (180 nmol).

Therefore detailed calibration curves for these six sugars were established to enable exact quantification of different monosaccharides Figure 29. The linear range for four of our tested sugars (Man, Gal, Fuc, Xyl) was up to about 240 nmol.

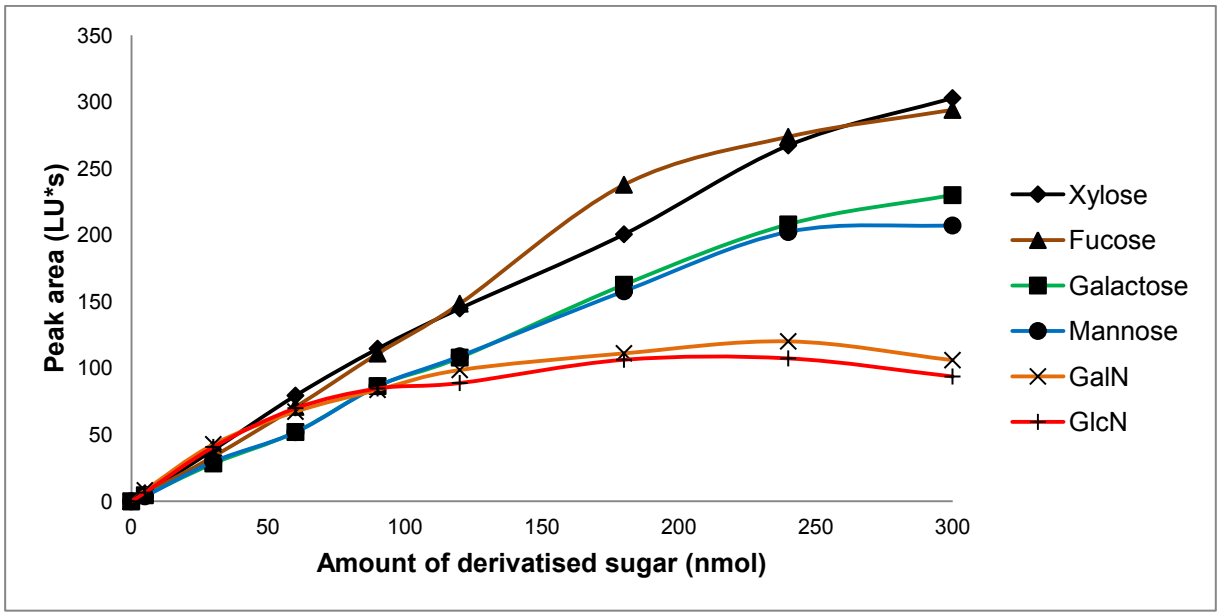


Figure 29 - AA-Calibration curves for six different monosaccharides.

Contrarily, amino sugars get derivatized worse and the linear range is just up to approx. 100 nmol. Probably, the amino group at the second carbon atom of the sugar ring interferes with the labeling process at C1. This theory is also backed up by the bad derivatization efficiency of 2-O-methylated Gal compared to the 4-O-methylated Gal which can be seen later.

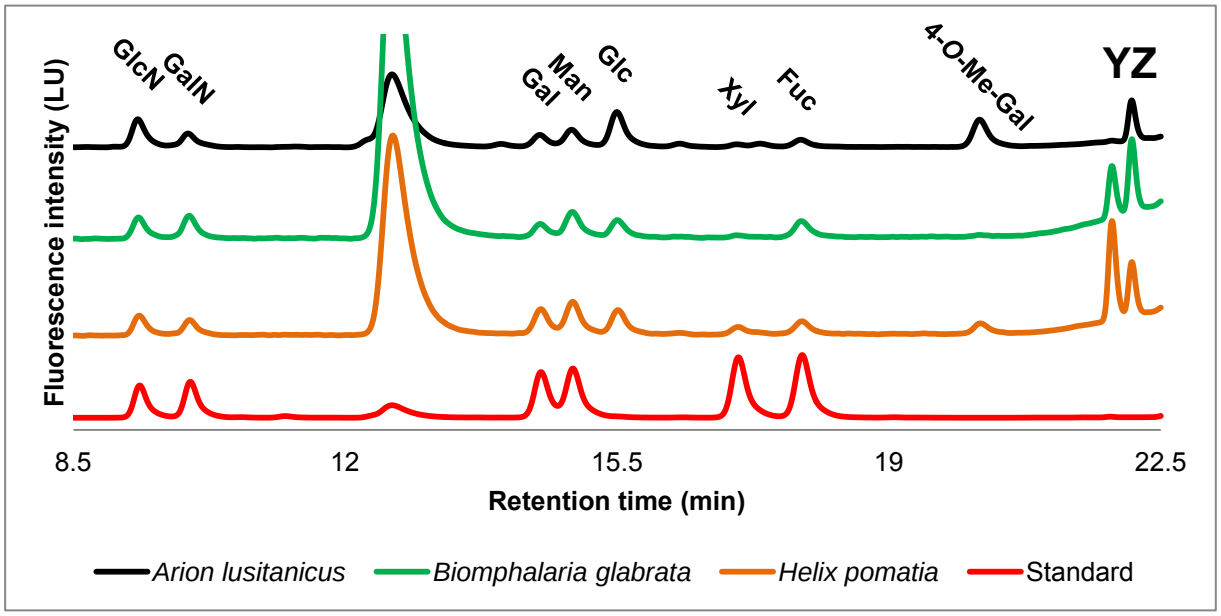


Figure 30 - Elucidation of retention times of methylated sugars.

Retention times (min) of standard AA-labeled sugars on RP-C18 column			
Glucosamine	9.17	Ribose	15.73
Mannosamine	9.65	Arabinose	16.01
Galactosamine	9.84	Altrose	16.03
Galactose	14.23	Xylose	16.73
Talose	14.49	Glucuronic acid	17.03
Allose	14.62	Rhamnose	17.55
Mannose	14.66	Fucose	17.56
Idose	14.92	3-*O*-Me-GlcN	19.06
Glucose	15.20	4-*O*-Me-Gal	19.84
Galacturonic acid	15.33	3-*O*-Me-Glc	19.89
Desoxyribose	15.46	3-*O*-Me-GlcNAc	21.67
Gulose	15.53	2-*O*-Me-Gal	21.70
From snail sample derived retention times (min) of sugars on C18 column			
3-*O*-Me-Gal	21.59	3-*O*-Me-Man	21.85

Table 13 - Retention times of AA-labeled monosaccharides on RP-C18 column.

The range of AA-labeled monosaccharides was extended up to 24 standards including methylated sugars to be able to determine even very special glycan constituents. Through snail analysis and comparison with GC/EI-MS data, the retention times of two methylated sugars (3-*O*-Me-Man and 3-*O*-Me-Gal) where no reference substance was available to us could be added (Figure 30).

Peaks marked with Y and Z could be assigned to 3-*O*-Me-Gal (=Y) and 3-*O*-Me-Man (=Z) because GC/EI-MS analysis found that all investigated snails have 3-O-methylated mannose but there are differences in the occurrence of methylated galactose. While 3- and 4-O-

methylated galactoses both occur in *H. pomatia*, for example, *A. lusitanicus* displays 4-*O*-Me-Gal on its glycans but no 3-*O*-Me-Gal and *B. glabrata* just 3-*O*-Me-Gal but no 4-*O*-Me-Gal.

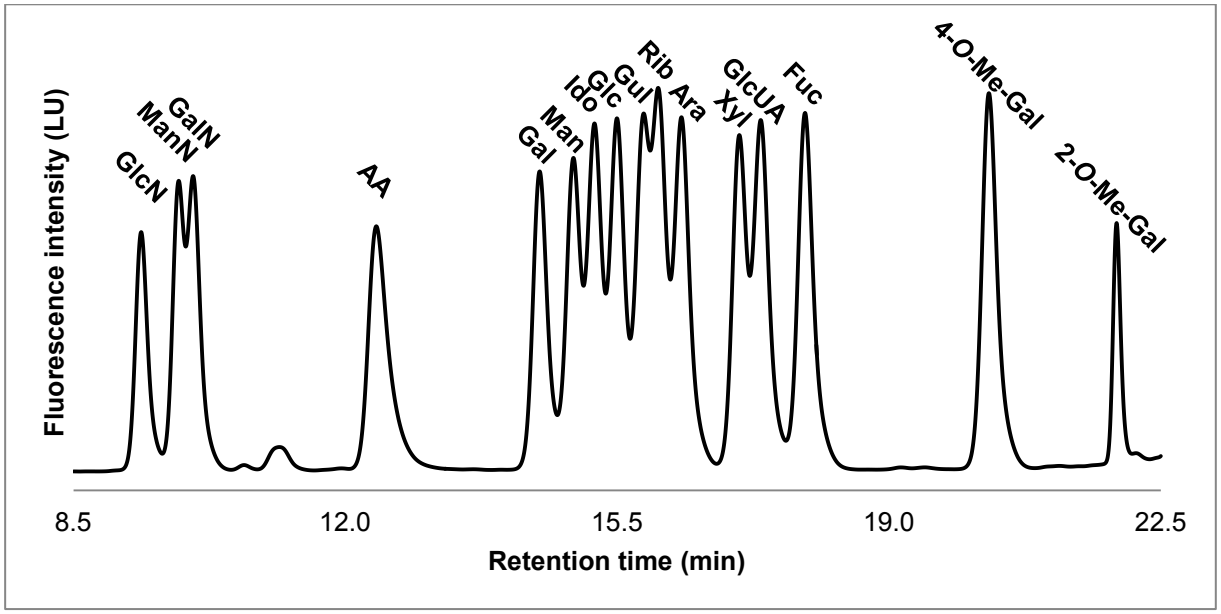


Figure 31 - Resolution of 15 AA-labeled monosaccharides in one single run.

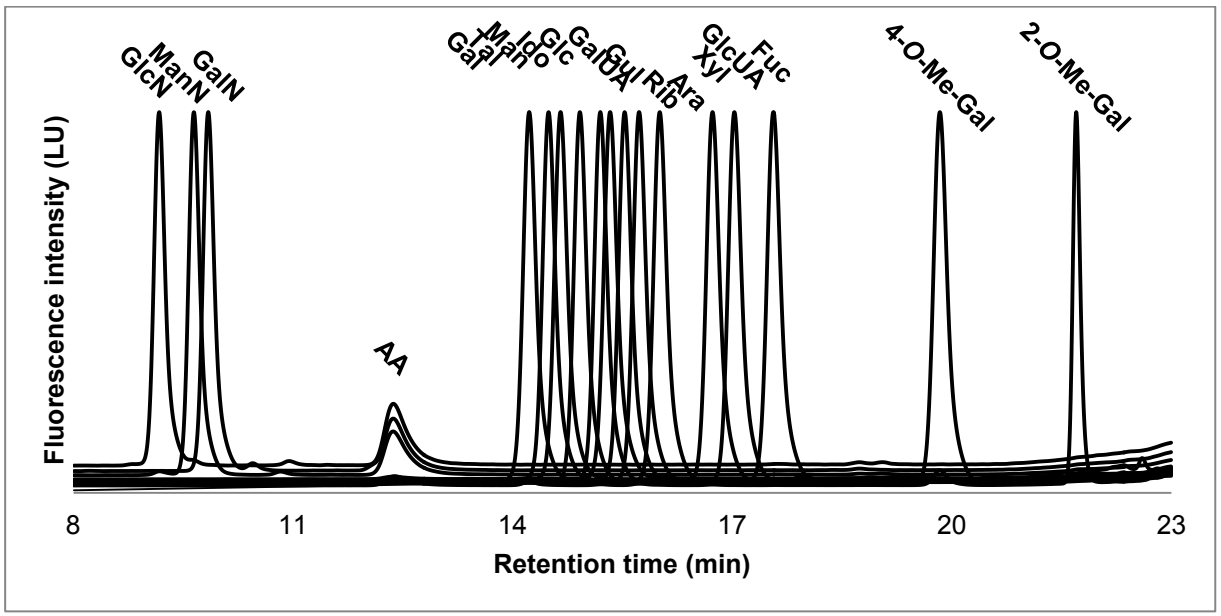


Figure 32 - Resolution of 17 AA-labeled monosaccharides in independent, normalized runs.

Certainly some monosaccharides have retention times close to each other and cannot be resolved under the described conditions. Therefore, different solvent compositions and various gradients were tested but a full separation of all standards could not be achieved and the published gradient seems to be optimal in terms of short run time and high resolution. Analysis temperature was set to 30 °C – while lower temperatures elongated separation time

significantly, higher temperatures up to 60 °C lead to a worse peak separation. Luckily, all sugars which cannot be resolved in the AA labeling system, can be clearly elucidated using the PMP system (see 4.4.2). The resolution power of our system is displayed in Figure 31 where the separation of 15 monosaccharides in one single run is shown. An overlay of 17 independent, normalized runs is depicted in Figure 32.

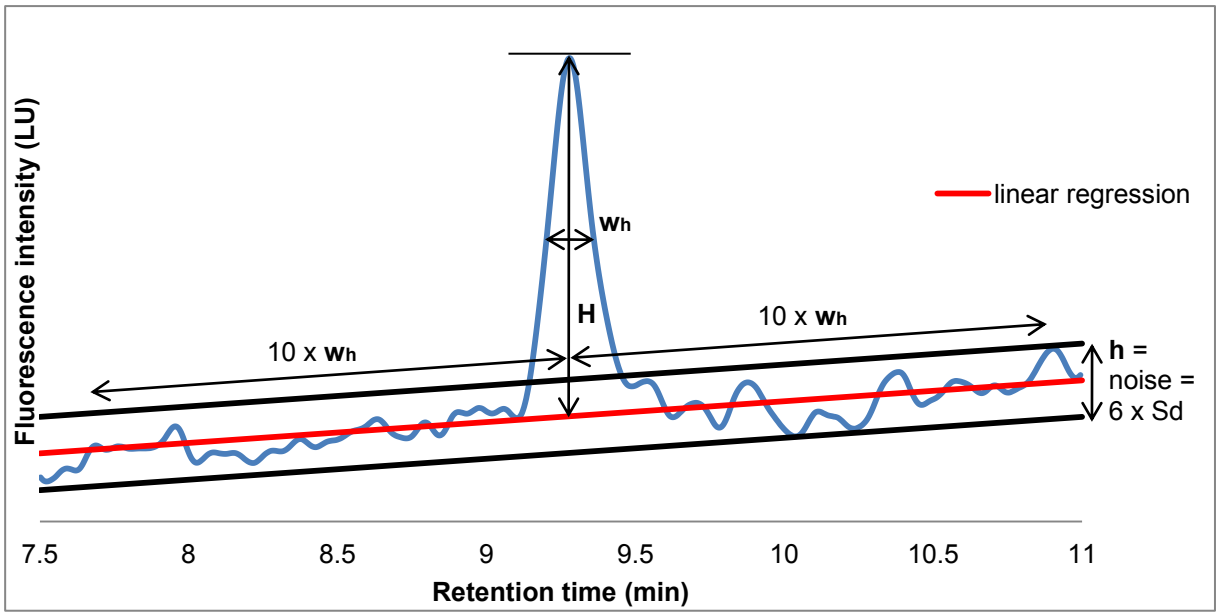


Figure 33 - Calculation of the limit of quantitation (Example: 220 fmol GlcN). H = peak height; w_h = width at half-height; h = background noise range; Sd = standard deviation.

The final step of our system optimization was the evaluation of the detection and quantitation limits. According to Section 2.2.46 (Chromatographic separation techniques) of the European Pharmacopoeia 5.0 (Council of Europe, Strassbourg, France; 2005) the limit of detection of a specified peak is corresponding to a signal-to-noise ratio of 3, while the limit of quantitation is corresponding to a signal-to-noise ratio of 10. The signal-to-noise (S/N) ratio itself is defined through the following formula:

$$S/N = {}^{2\mathrm{H}}/_{\mathrm{h}}$$

H…height of the peak measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to 20 times the width at half-height.

h…range of the background noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to 20 times the width at half-height of the peak (w_h) in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

As illustrated in Figure 33, **h** was calculated as six times the standard deviation (Sd) of the linear regression of the drift by Agilent ChemStation for LC 3D systems software (version B.04.01 SP1). After injection of a blank, noise was calculated in an area equal to 20 times w_h distributed evenly around the respective retention time – 7.8 to 10.8 min for GlcN eluting at 9.3 min. Finally, the limit of quantitation for AA labeled sugars was determined at 220 fmol and the limit of detection at 65 fmol.

This AA optimization has already been published by H. Stepan and E. Staudacher [179] and can be found in 9 Appendix. Final derivatization procedure can be found at 3.4.1.1.

4.4.2 PMP-labeling

According to the optimization of the AA labeling method, the labeling with 1-phenyl-3-methyl-5-pyrazolone (PMP) was optimized as well [171]. Obviously both methods (AA and PMP) differ in their reaction chemistry, derivatization process and detection. While the AA procedure is much faster and allows detection of smaller amounts of sugar, PMP derivatization allows usage of large sample amounts.

Since the PMP derivation procedure requires liquid-liquid extraction to remove the excess PMP reagent [172], there was no need to downscale the addition of labeling agent. While keeping the original reaction mixture untouched, an improvement of the the extraction step where an organic solvent is used to extract the excess PMP from the aqueous sample was made. Three different organic solvents (butyl ether, dichloromethane and diethyl ether) were tested. Finally, diethyl ether gave the best results in terms of sample yield and peak shape.

The liquid-liquid extraction was then further evaluated to check how many extraction steps are necessary to remove most of the residual PMP while avoiding loss of sample. While three extraction steps are sufficient to remove most PMP, we recommend five extraction steps in order to get an appropriate PMP/sample peak ratio even when derivatizing small sample amounts.

One further improvement to increase the reproducibility of quantification experiments was to evaporate the aqueous phase after the liquid-liquid extraction to dryness and resolve it later on in a defined volume of water. Since the organic solvent evaporates during the vacuum centrifugation, it is not necessary anymore to completely remove the organic phase which was a critical point in terms of losing some sample as well.

Gradients, solvents and detection wavelength were found to be optimal in the original protocol. Separation temperature was set to 30 °C.

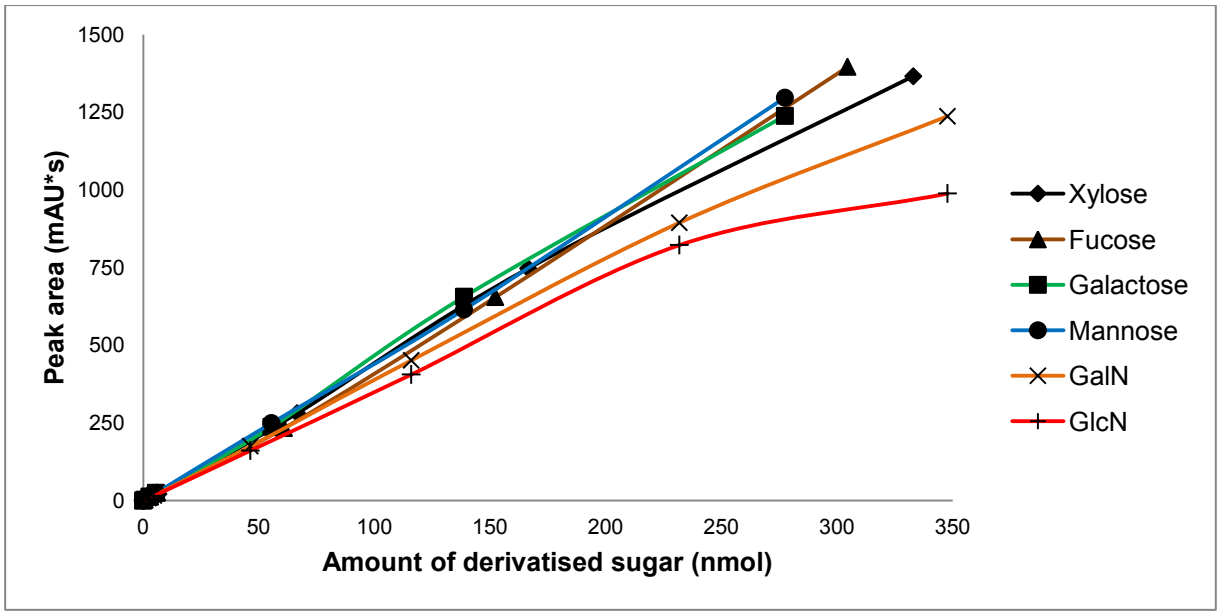


Figure 34 - PMP-Calibration curves for six different monosaccharides.

As for the AA derivatization, the calibration for six different monosaccharides for PMP labeling was checked as well (Figure 34). The calibration curves for Man, Gal, Fuc and Xyl were linear throughout the tested range (0.1-100 µg which equates to approximately 0.5-500 nmol of sugar). GlcN and GalN just showed a linear range up to about 300 nmol, and it has to be noted that, comparable to the AA labeling, derivatization of a certain amount of amino sugar gives a smaller peak area than derivatization of other tested sugars.

Unlike for the separation of AA labeled monosaccharides, amino sugars do not separate as nicely from the other sugars in front of the reagent peak. PMP-labeled amino sugars are scattered through the chromatogram. Further it has to be noted that PMP-labeled monosaccharides do not separate as well as their AA-labeled analogues. While 0.2 min difference in the respective retention time is enough to get peak separation in the AA system with fluorescence detection, this does not apply to the PMP system with UV detection.

The full spectrum of standard sugar retention times and the additional retention times of 3-O-methylated mannose and galactose can be found in Table 14.

The methylated sugars were assigned to their respective peaks as described for the AA spectrum (Figure 35). One single run resolving 15 different monosaccharides is shown in Figure 36. An overlay of 19 independent, normalized runs is depicted in Figure 37.

Retention times (min) of standard PMP-labeled sugars on RP-C18 column			
Mannose	8.25	3-*O*-Me-GlcNAc	16.96
Gulose	8.81	Idose	17.22
Glucosamine	9.90	Glucose	17.57
Mannosamine	10.24	3-*O*-Me-Glc	18.38
3-*O*-Me-GlcN	10.79	Galactose	18.54
Talose	10.92	Altrose	18.84
Ribose	11.05	Arabinose	19.61
Rhamnose	11.12	Xylose	19.69
Allose	11.28	Fucose	21.29
Galactosamine	13.45	4-*O*-Me-Gal	21.84
Glucuronic acid	13.82	2-*O*-Me-Gal	22.75
Galacturonic acid	14.99	Desoxyribose	23.59
From snail sample derived retention times (min) of sugars on C18 column			
3-*O*-Me-Man	14.76	3-*O*-Me-Gal	20.07

Table 14 - Retention times of PMP-labeled monosaccharides on RP-C18 column.

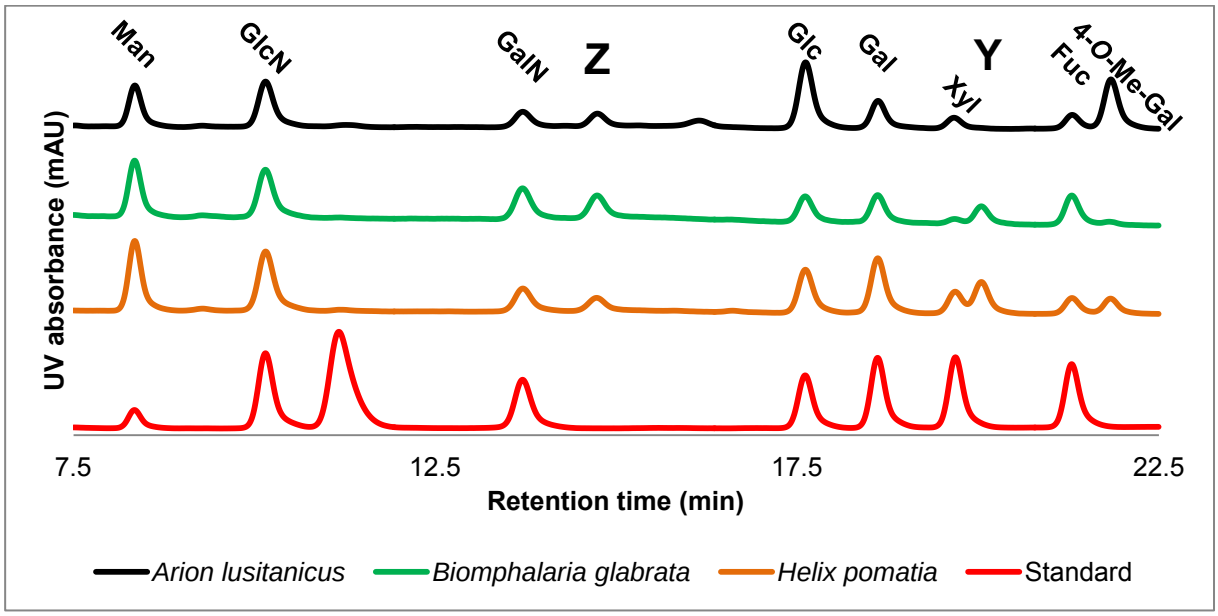


Figure 35 - Elucidation of retention times of methylated sugars.

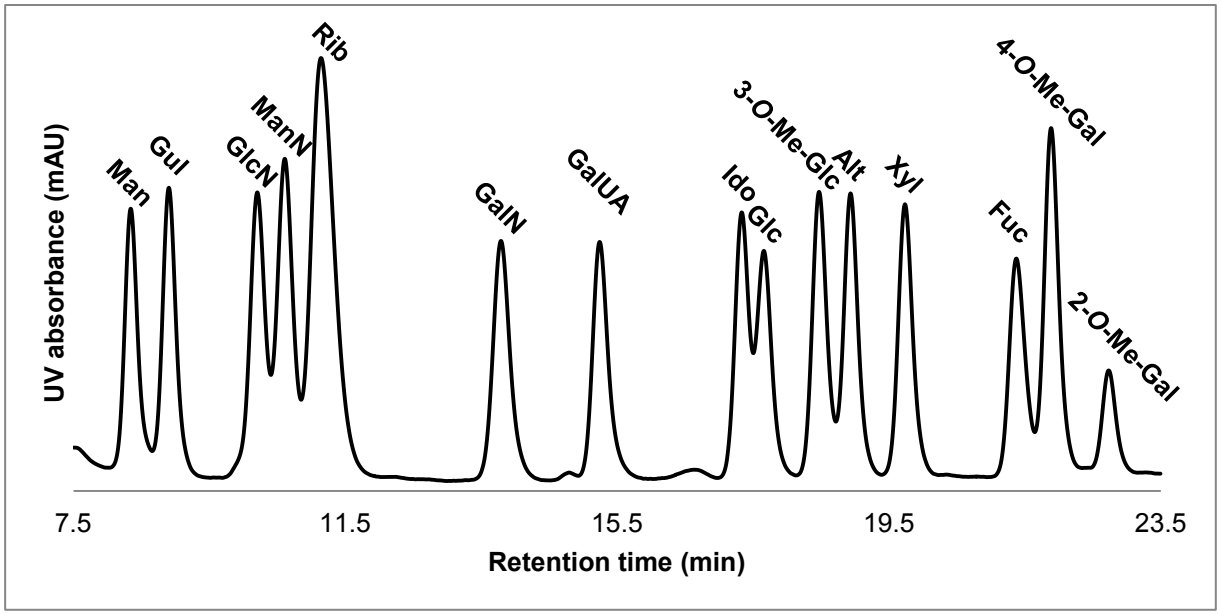


Figure 36 - Resolution of 15 PMP-labeled monosaccharides in one single run.

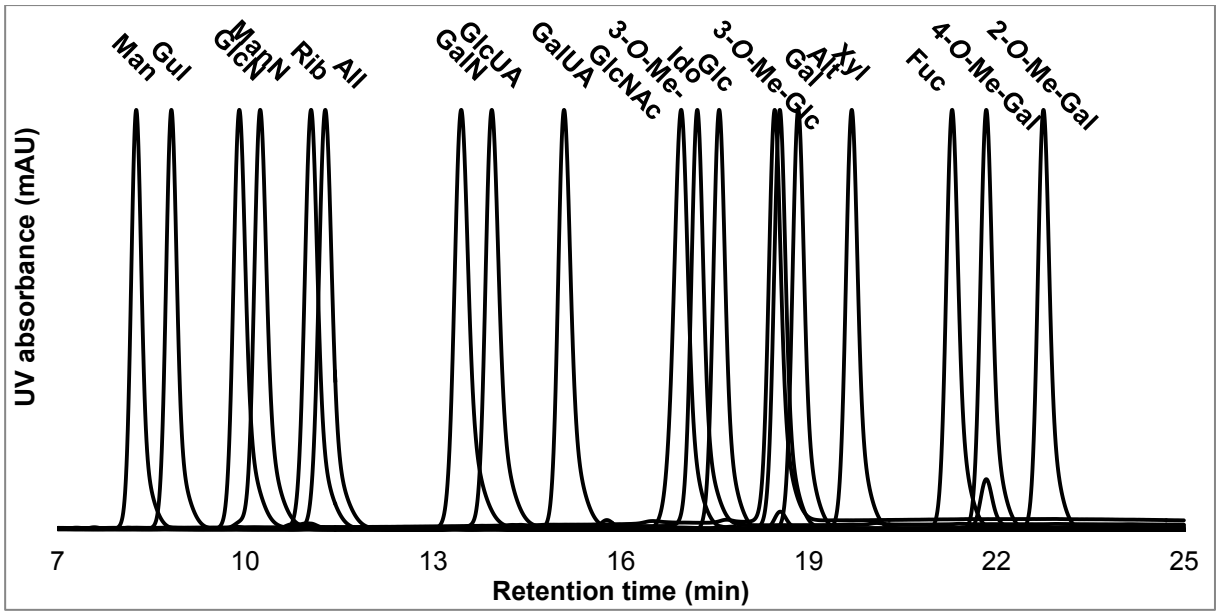


Figure 37 - Resolution of 19 PMP-labeled monosaccharides in independent, normalized runs.

Finally again, the limits of detection and quantitation were checked. As expected the limits are significantly higher than for the AA system but another constraint was encountered as well. While an early eluting sugar such as glucosamine (9.9 min) has a limit of detection of 0.2 pmol and limit of quantitation of 0.7 pmol, later eluting sugars have considerably higher limits. Galactose, for example, eluting at 18.5 min already shows significant peak broadening which does affect value **H** (peak height) in the equation to calculate S/N. Therefore, a limit of detection of 3.2 pmol and limit of quantitation of 10.7 pmol was found.

This PMP optimization has already been published by H. Stepan and E. Staudacher [179] and can be found in 9 Appendix. Final derivatization procedure can be found at 3.4.1.1.

4.5 Application of HPLC monosaccharide analysis on gastropod samples

After optimizing the derivatization protocols for the AA and PMP labeling, monosaccharide analyses of the snail derived samples were generated. The elucidation of the composition of the total glycans as well as of the difference between N- and O-glycans were main objectives.

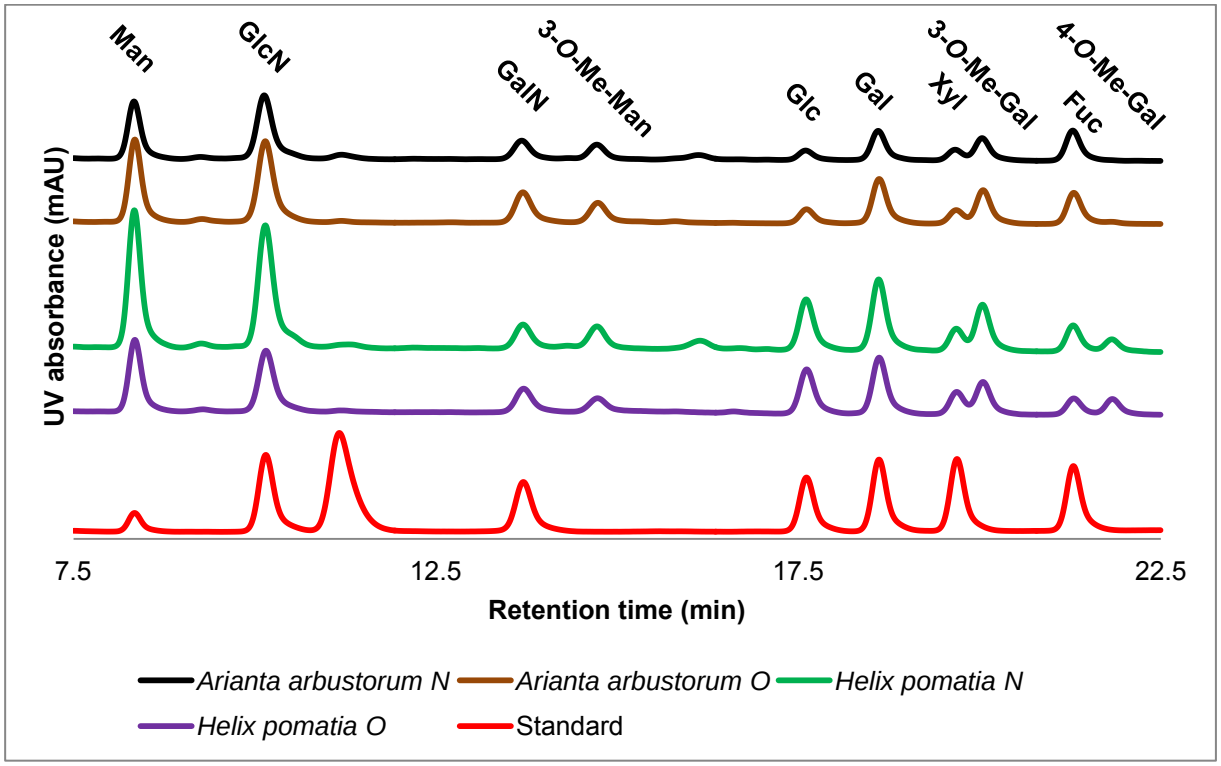


Figure 38 - Comparison of monosaccharide patterns of N- and O-glycan fractions of two selected snails (PMP-labeled).

Figure 38 and Figure 39 show the monosaccharide analysis of two different snails (*A. arbustorum* and *H. pomatia*) after PMP and AA labeling respectively. The N- and O-glycan spectra of *A. arbustorum* (black and brown graphs) and of *H. pomatia* (green and purple graphs) look rather similar, respectively. Obviously, these results indicate that the separation of N- from O-glycans was insufficient. All other tested snails showed similar results.

The complete removal of N-glycans using PNGase A or PNGase F has caused trouble always throughout this work. Even multiple digestions and the use of both enzymes did never result in a pure O-glycan fraction. The large amount of mannose and glucosamine in the O-glycan fraction is an indicator for N-glycans which obviously were not cleaved from the protein backbone completely.

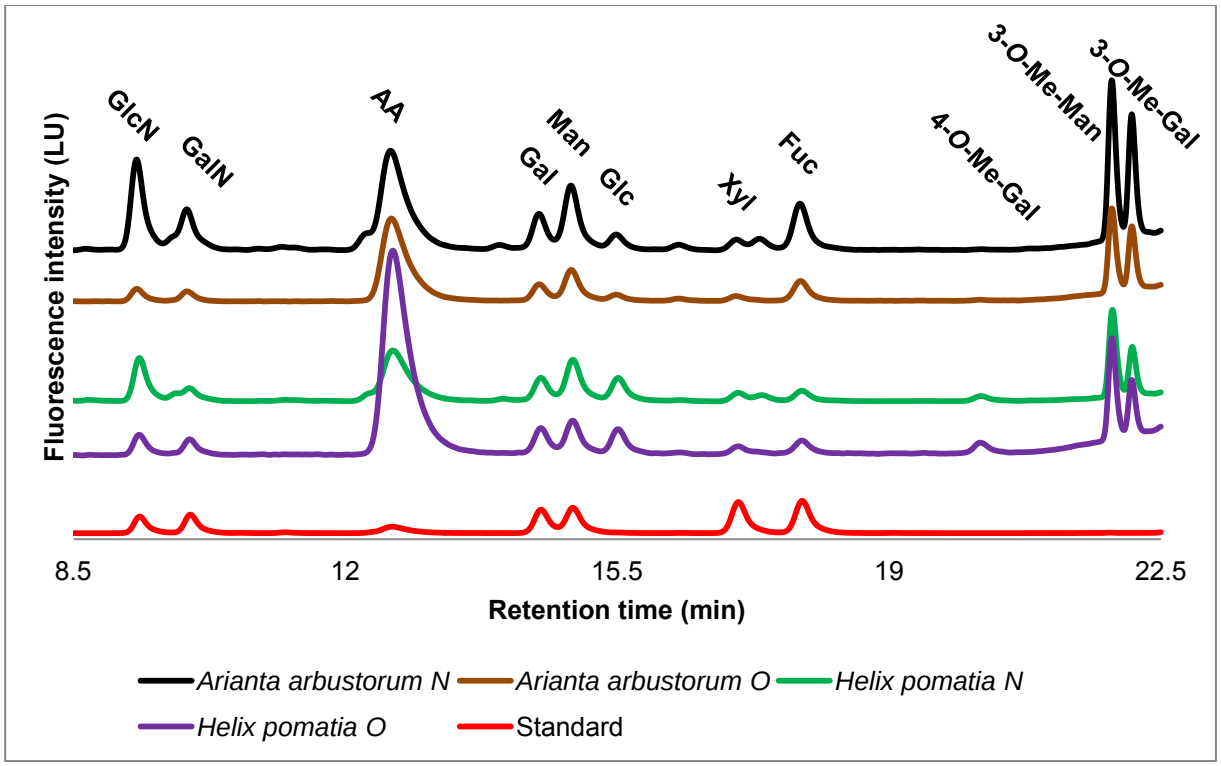


Figure 39 - Comparison of monosaccharide patterns of N- and O-glycan fractions of two selected snails (AA-labeled).

During the later work, we did not try to improve the removal of N- from O-glycans. Figure 40 and Figure 41 show a complete monosaccharide analysis of seven different snails without discrimination between N- and O-glycans. Since we were not able to guarantee clean fractions, this seems to be a more correct depiction.

As expected, the occurrence of GlcN, GalN, Gal, Man, Glc, Fuc and traces of Xyl was verified in all snail samples with the AA and PMP system. Of course, our focus was set on the elucidation of methylated monosaccharides such as 3- and 4-O-methylated mannose and galactose. While 4-O-methylated mannose was not detected, 3-*O*-methyl-mannose is a very frequent monosaccharide in all investigated species.

Methylated galactoses obviously show a larger variability within the tested species. While *C. hortensis*, *H. pomatia* and *P. corneus* decorate their glycans with 3- and 4-O-methylated galactoses, all other investigated snails seem to have a preference for just one type. *A. arbustorum* and *B. glabrata* display 3-*O*-methyl-Gal, *A. fulica* and *A. lusitanicus* mainly 4-*O*-methyl-Gal (Table 15).

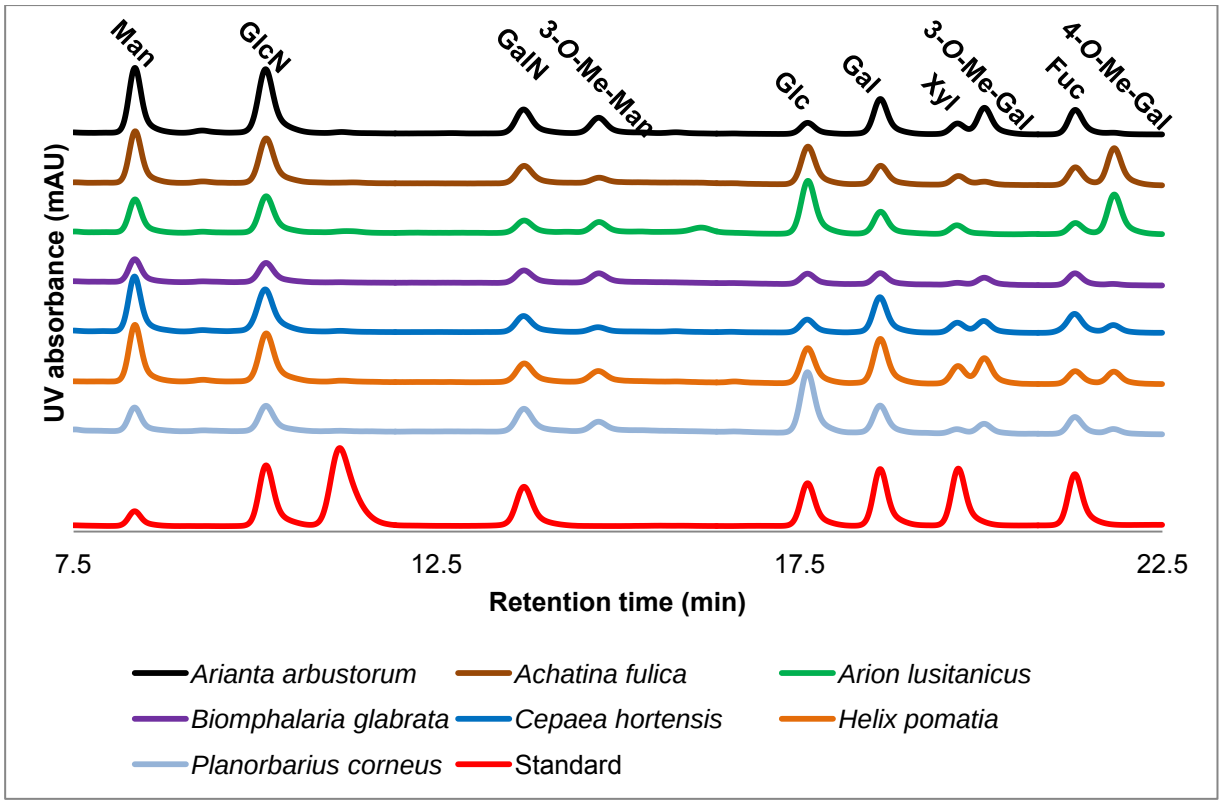


Figure 40 - Snail monosaccharide analysis of total glycans after PMP-derivatization.

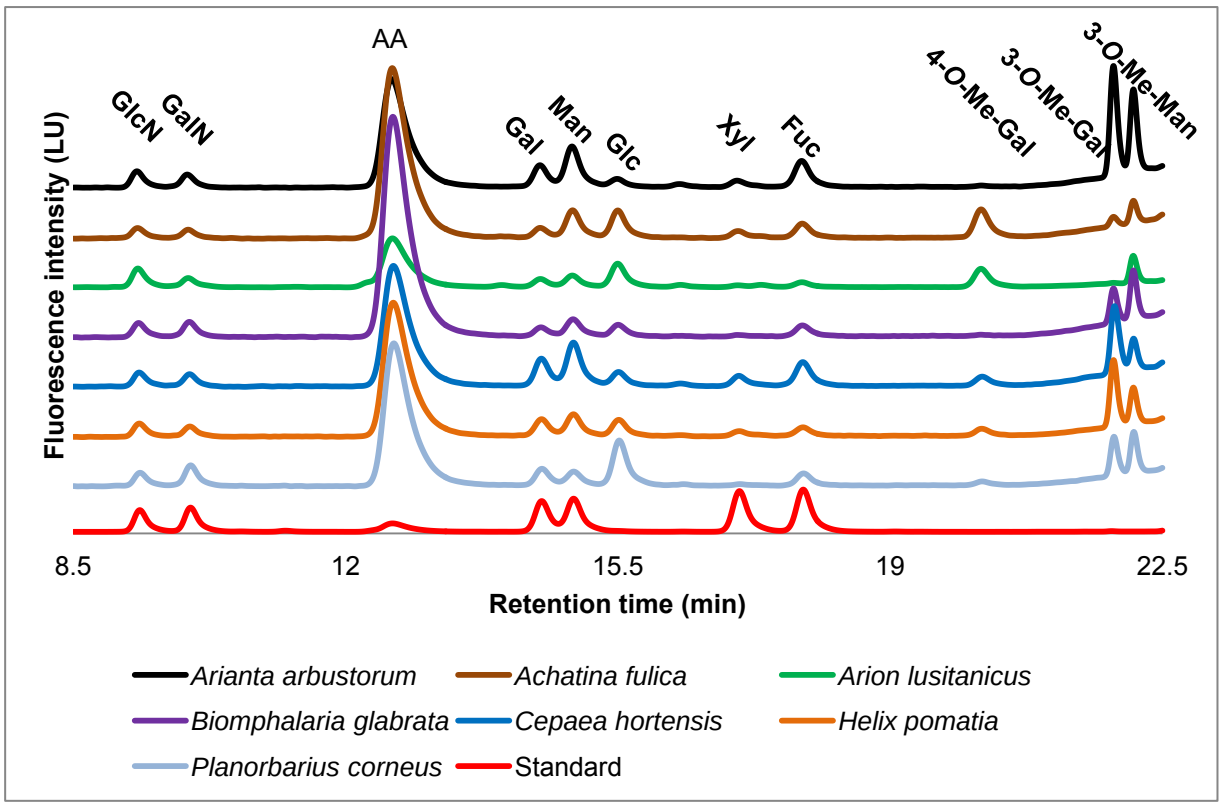


Figure 41 - Snail monosaccharide analysis of total glycans after AA-derivatization.

4.6 GC/MS analysis

HPLC analysis using two different labeling procedures allows a clear determination of monosaccharides which are available as standards. However, for those which are not available GC/MS analysis is the best choice for analysis. To identify those peaks from the HPLC analysis, gas chromatographic separation in combination with electron impact mass spectrometry was performed in the laboratory of Prof. Rudolf Geyer (Institute of Biochemistry, Faculty of Medicine, Justus-Liebig-University Gießen, Germany).

4.6.1 GC/MS snail monosaccharide analysis

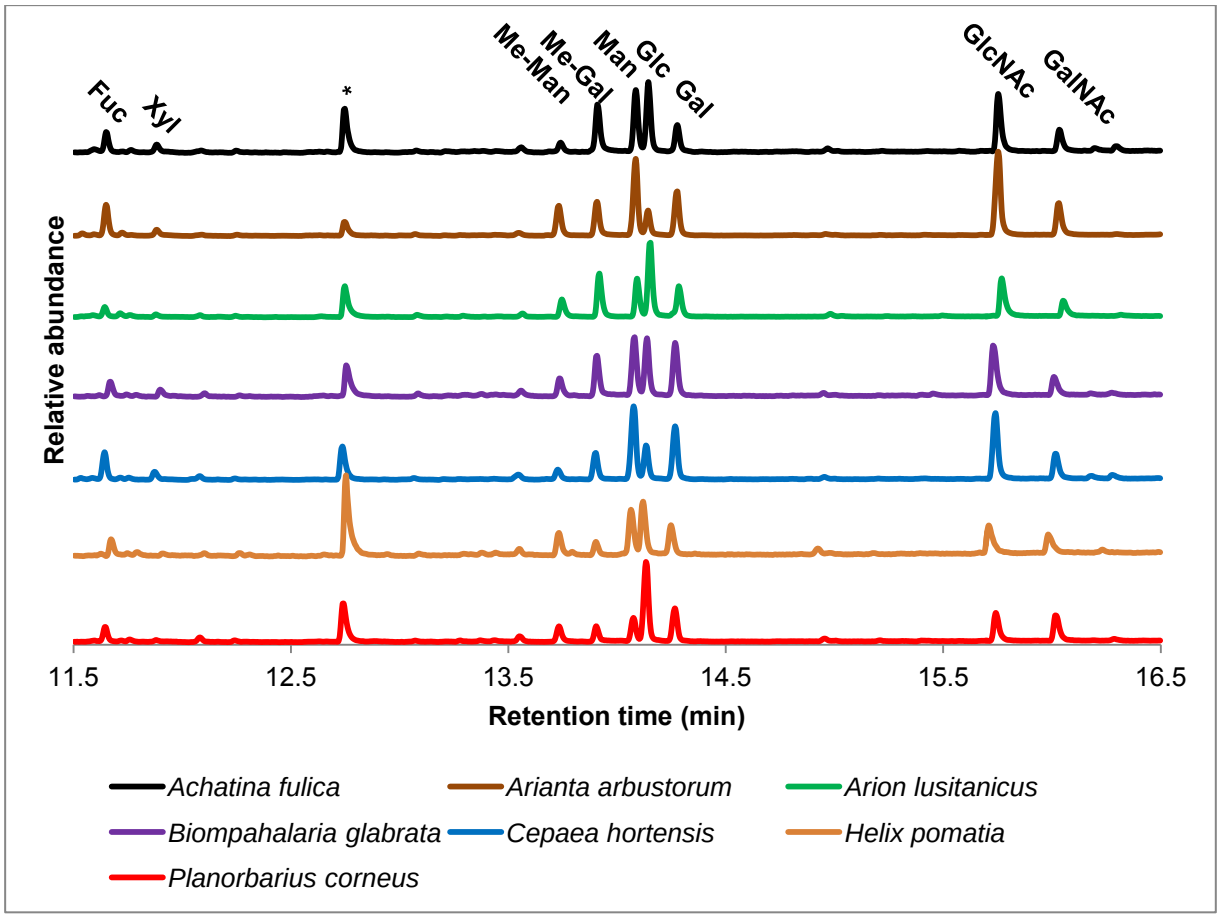


Figure 42 - GC/EI-MS monosaccharide analysis of selected snails (* = peaks of phthalates).

The first step of elucidating the unidentified peaks of the HPLC spectrum was to check the GC spectrum of various snails for unusual monosaccharides apart from Fuc, Xyl, Man, Gal, Glc, GlcNAc and GalNAc. The peak marked with an asterisk (*) was identified as plastic softener (phthalates) but the two peaks at 13.7 and 13.9 min were identified to be sugars: methyl-mannose and methyl-galactose, respectively (Figure 42).

Methylated sugar standards purified by preparative gas chromatography were used to elucidate the type of methylated hexose (mannose or galactose) based on the retention time. However, fragmentation of the molecules using EI-MS was necessary to check where the methyl groups are attached. To clarify the exact position of methylation, the monosaccharides were converted into alditol acetates using sodium borodeuteride as described in 3.5.1.1.1. The incorporation of the deuterium atom at the C1 position allows the localization of the methyl-groups based on the specific fragmentation pattern (Figure 43) during EI-MS analysis. While methylated mannose and galactose have different retention times and can therefore be separated, 3- and 4-O-methylated galactose co-elute under the used conditions.

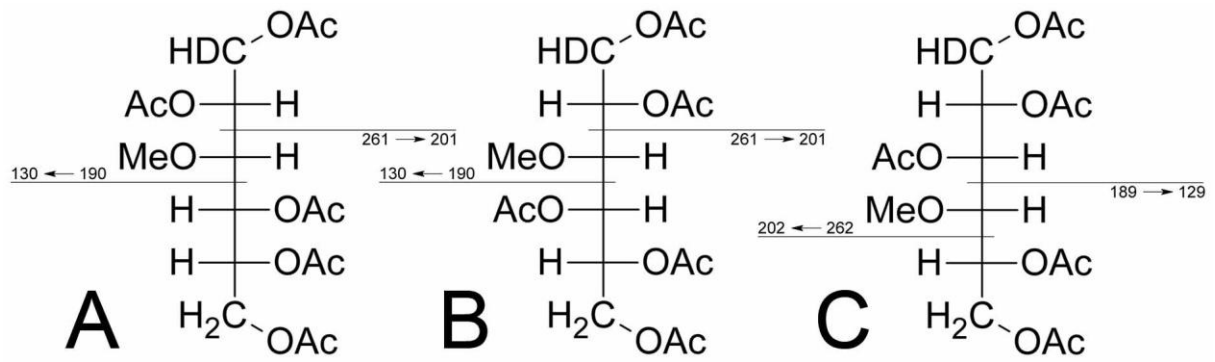


Figure 43 - Indicative fragments of 3-*O*-Me-Man (A), 3-*O*-Me-Gal (B) and 4-*O*-Me-Gal (C).

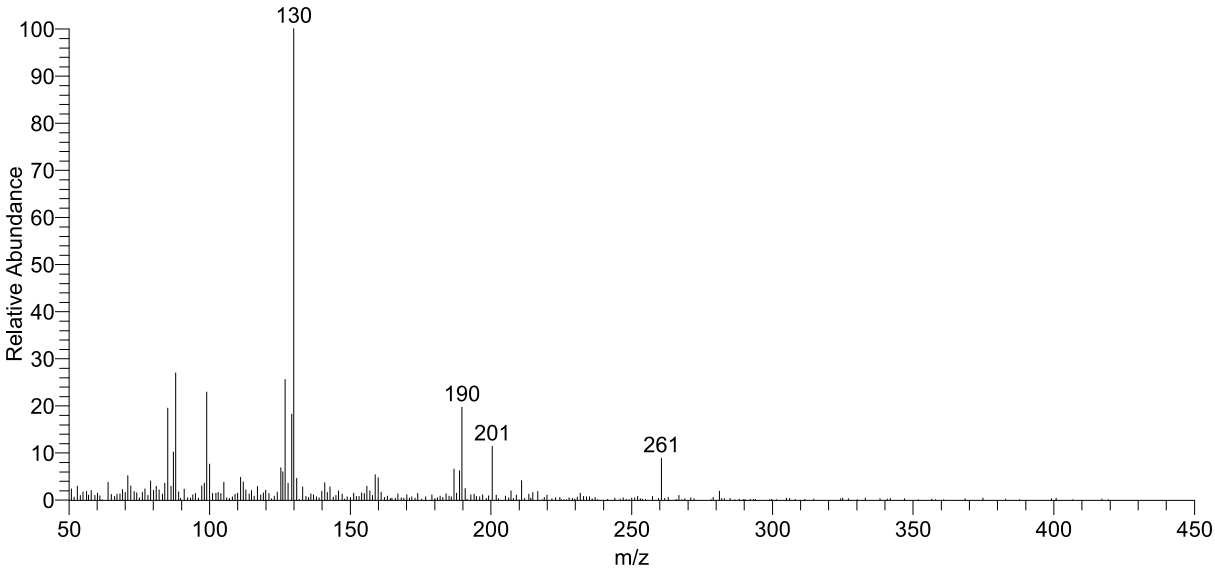


Figure 44 - EI-MS of 3-*O*-Me-Man from *B. glabrata* retention time 13.7 min (Figure 42).

All investigated snails showed exactly the same fragmentation pattern for the peak at 13.7 min. While the retention time indicates a methylated mannose, the fragmentation pattern

(Figure 44) exactly matches the indicative fragments of 3-*O*-methyl-mannose (Figure 43). No hints on the occurrence of 4-O-methylated mannose were found.

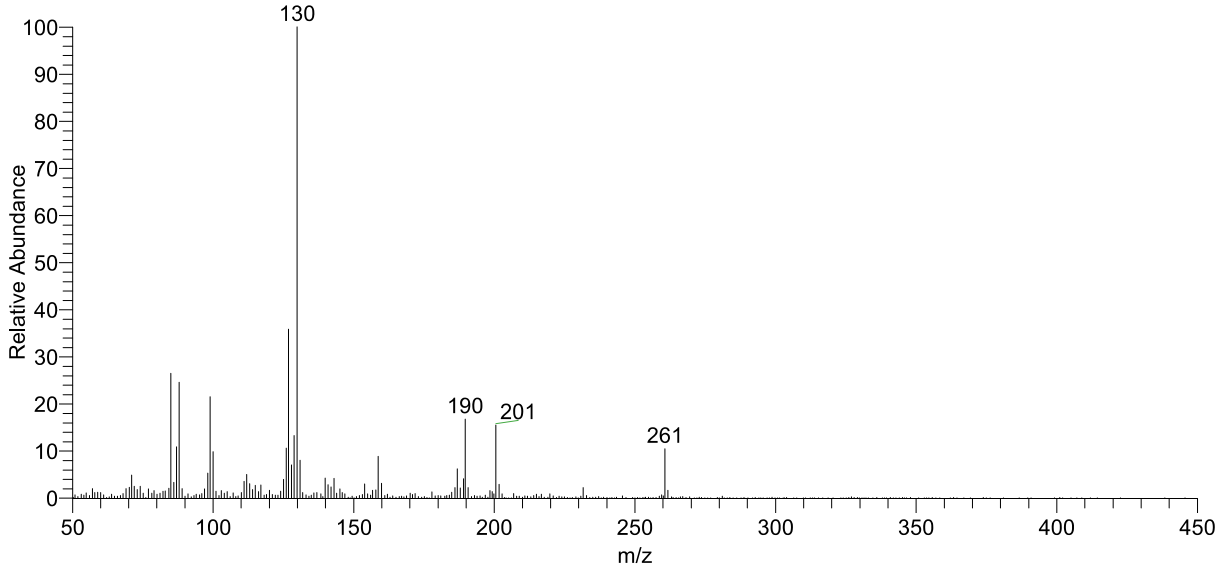


Figure 45 - EI-MS of 3-*O*-Me-Gal from *A. arbustorum* retention time 13.9 min (Figure 42).

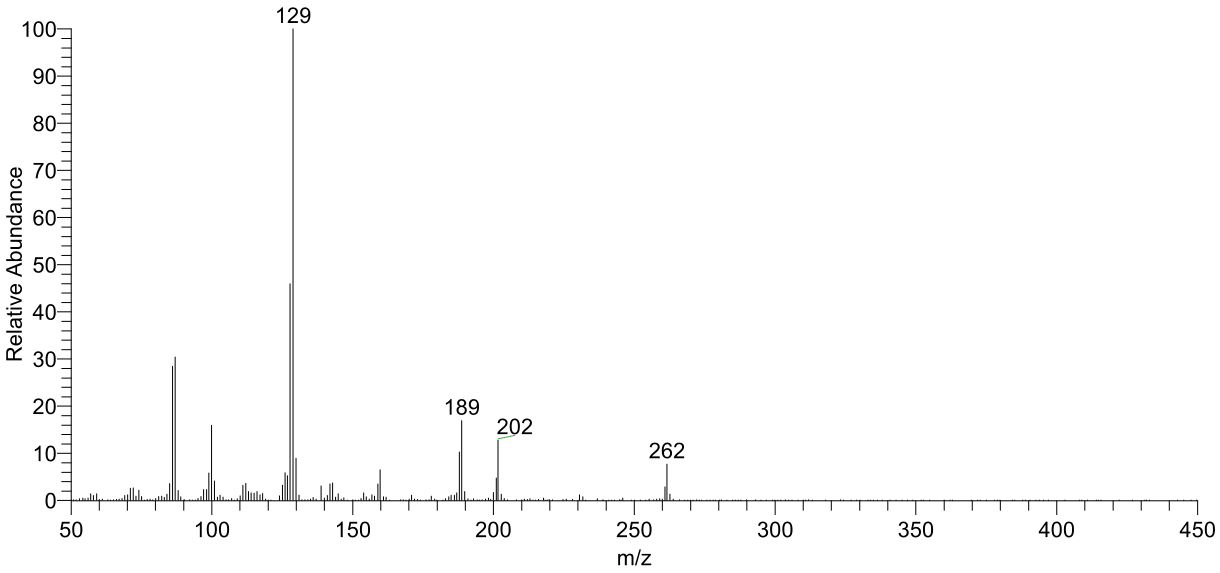


Figure 46 - EI-MS of 4-*O*-Me-Gal from *A. lusitanicus* retention time 13.9 min (Figure 42).

The analysis of the methylated galactose peak (13.9 min) though was more difficult. While *A. arbustorum* and *B. glabrata* showed spectra similar to Figure 45, the fragmentation of the methylated galactose from *A. fulica* and *A. lusitanicus* was different (Figure 46). While *A. arbustorum* and *B. glabrata* clearly show the indicative fragments (m/z of 130, 190, 201 and 261) of 3-*O*-methyl-galactose which are similar to those of 3-O-methyl-mannose (Figure 44), a mass shift of one Da due to the incorporation of the deuterium atom is found in the sample

of *A. fulica* and *A. lusitanicus*. In these samples, 4-O-methylated galactose with the indicative fragments of m/z 129, 189, 202 and 262 is present.

The three remaining species (*C. hortensis*, *H. pomatia* and *P. corneus*) showed a mixture of both methylated galactoses (Figure 47).

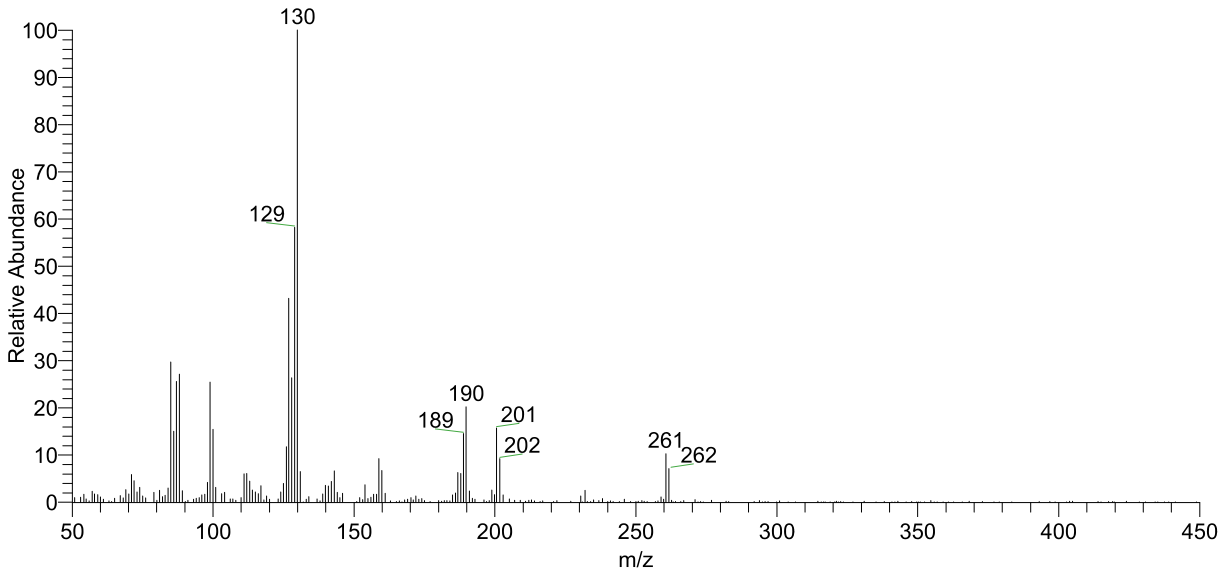


Figure 47 - EI-MS of 3-and 4-*O*-Me-Gal from *P. corneus* retention time 13.9 min (Figure 42).

Quantitative analysis of a mixture of 3- and 4-O-methylated sugar cannot be accurately accomplished by analyzing the EI-MS spectrum. Therefore no ratio between both methylated sugars is given here.

Table 15 gives a full overview of the occurrence of methylated sugars in the investigated snails. It combines GC/MS results and the findings of the HPLC analyses shown in Figure 40 and Figure 41. The small 3-*O*-Me-Gal peaks in the HPLC runs of *A. fulica* and respectively the small 4-*O*-Me-Gal peaks in the HPLC runs of *B. glabrata*, are registered as “traces” even though the GC/MS data did not explicitly prove their occurrence.

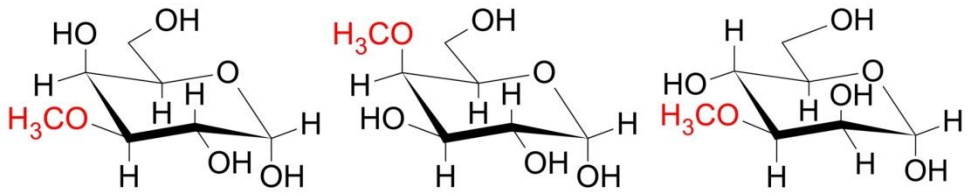
	3-*O*-methyl-galactose	4-*O*-methyl-galactose	3-*O*-methyl-mannose
			
Arianta arbustorum	+	-	+
Achatina fulica	traces	+	+
Arion lusitanicus	-	+	+
Biomphalaria glabrata	+	traces	+
Cepaea hortensis	+	+	+
Helix pomatia	+	+	+
Planorbarius corneus	+	+	+

Table 15 - Occurrence of methylated monosaccharides in selected snail species.

4.6.2 GC/MS analysis of purified glycan structures from *A. lusitanicus*

4.6.2.1 Monosaccharide analysis

Apart from GC/MS monosaccharide analysis of complete snail samples, we also did monosaccharide and linkage analyses of purified structures. While the purification method described for the ESI/MS analyses (in-gel method; 3.5.3) does not yield enough material for GC/MS analysis, we had to use a different protocol that can be used to purify much larger amounts of glycans (3.1.1.3). As a final step in this protocol, we performed multiple runs on a PGC HPLC column (3.4.2.2), collected 1-minute-fractions and screened all fractions using ESI/MS to gain clean, isolated structures.

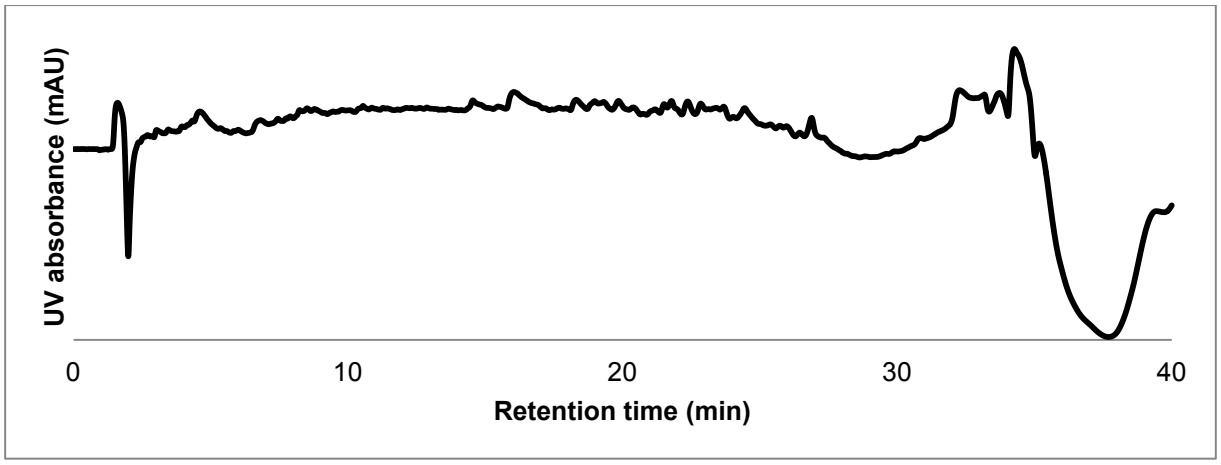


Figure 48 - PGC-HPLC fractionation of *A. lusitanicus*.

Figure 48 shows the UV absorbance at 212 nm of the underivatized β-eliminated snail glycans. O-glycans elute approximately between 10 to 20 minutes using the acetonitrile gradient described in 3.4.2.2. After purification of 100 g of *A. lusitanicus* we were able to extract sufficient amounts of four single structures to allow GC/MS analysis.

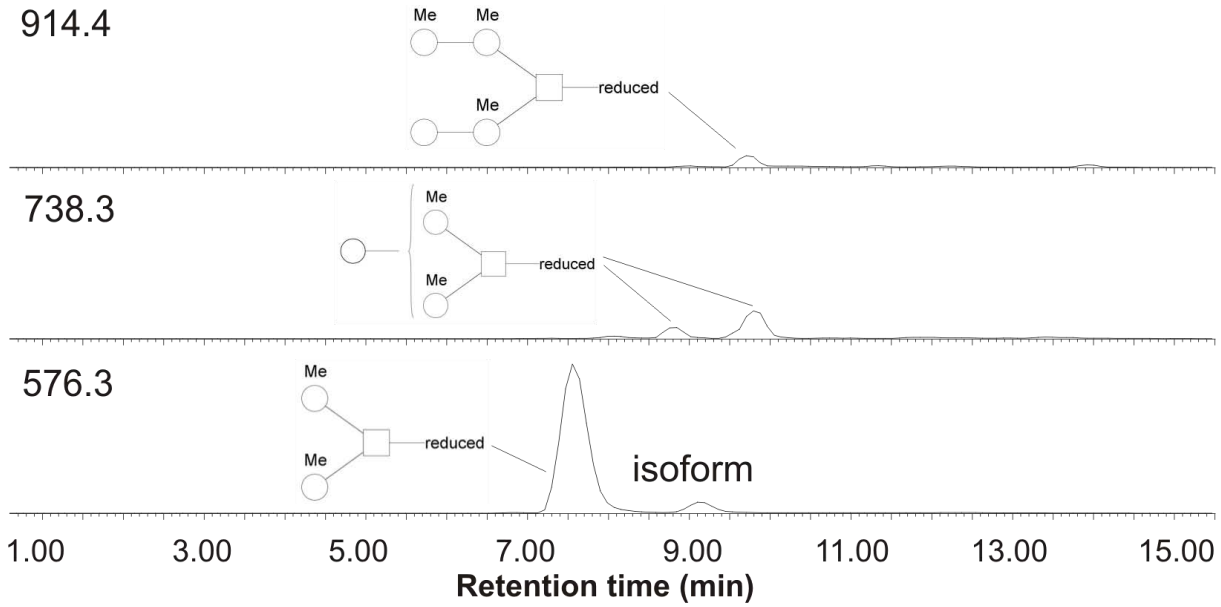


Figure 49 - Selected ion chromatograms (PGC-LC-ESI/MS) of isolated structures for GC/MS analysis.

We isolated a “core” structure and its isoform as well as two elongated forms (738.3 and 914.4 Da) shown in Figure 49.

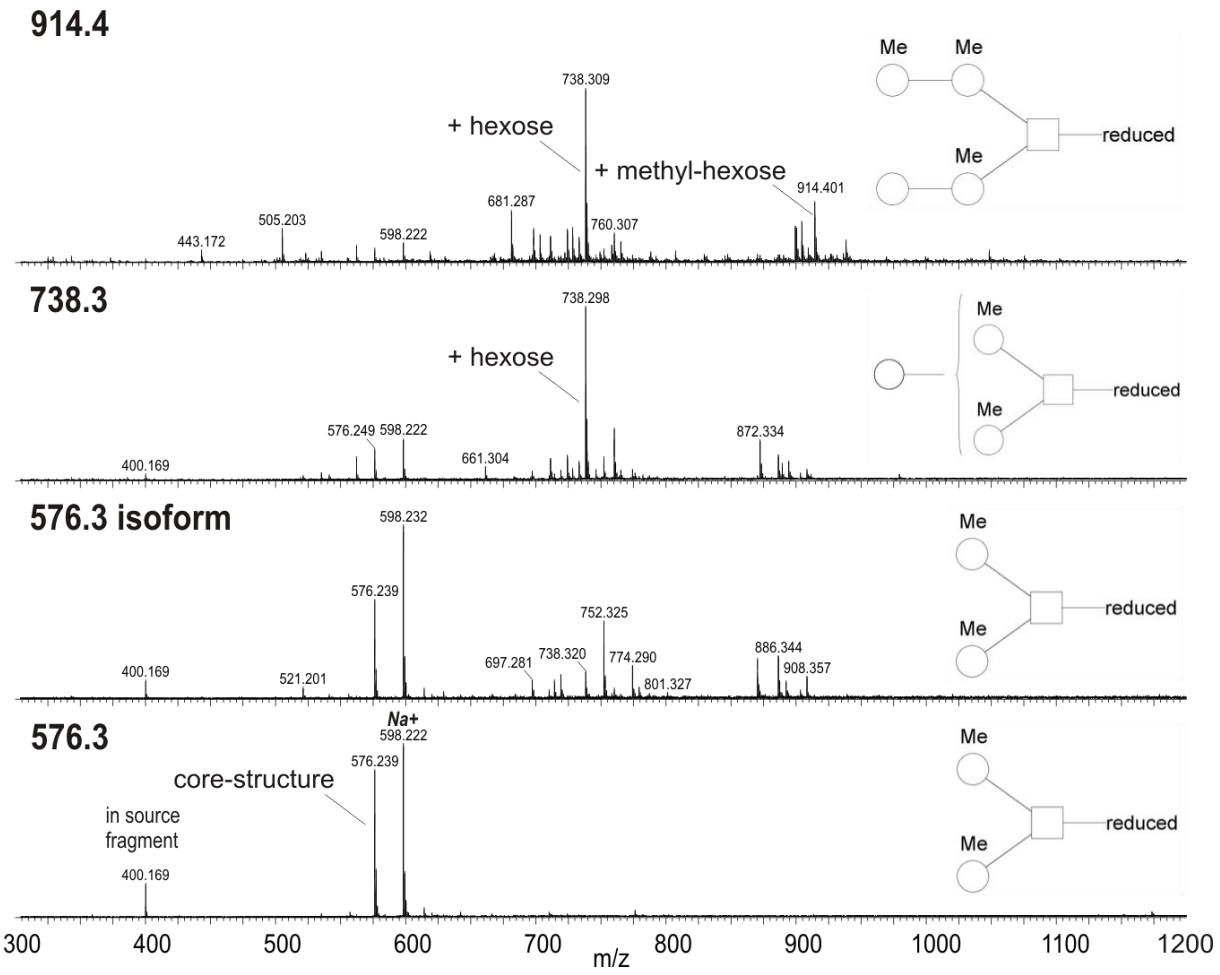


Figure 50 – MS/MS of structures shown in Figure 49

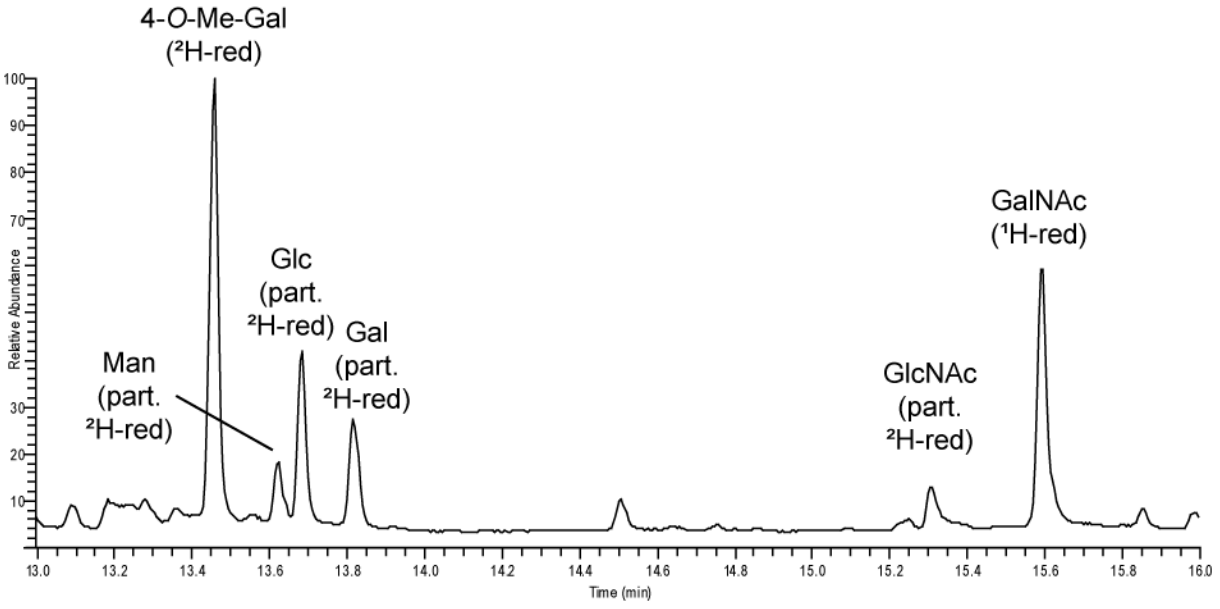


Figure 51 - GC spectrum of "core" structure (576.3 Da) of *A. lusitanicus.*

Figure 51 shows the GC spectrum of the “core” structure consisting of one N-acetylhexosamine and two methylated hexoses (Figure 63). Apart from some impurities (Man, Glc, Gal and GalNAc), two peaks are particularly striking. While all other peaks are at least partially ^{2}H-reduced due to the use of sodium borodeuteride after the hydrolysis, the GalNAc peak (15.53 min) is ^{1}H-reduced only. This is a strong indication that the “core” structure is linked to the peptide backbone via this GalNAc residue which was ^{1}H-reduced at the β-elimination step where sodium borohydride was used.

The second interesting and largest peak matches the retention time and EI-MS fragmentation pattern of 4-*O*-Me-Gal (Figure 46). This monosaccharide is deutero-reduced as expected. Since the ratio of peak areas between 4-*O*-Me-Gal and GalNAc suggest a value of about 1:2, we expect the “core” structure of *A. lusitanicus* to consist of two 4-*O*-methylated galactoses that are linked via one GalNAc residue to the protein.

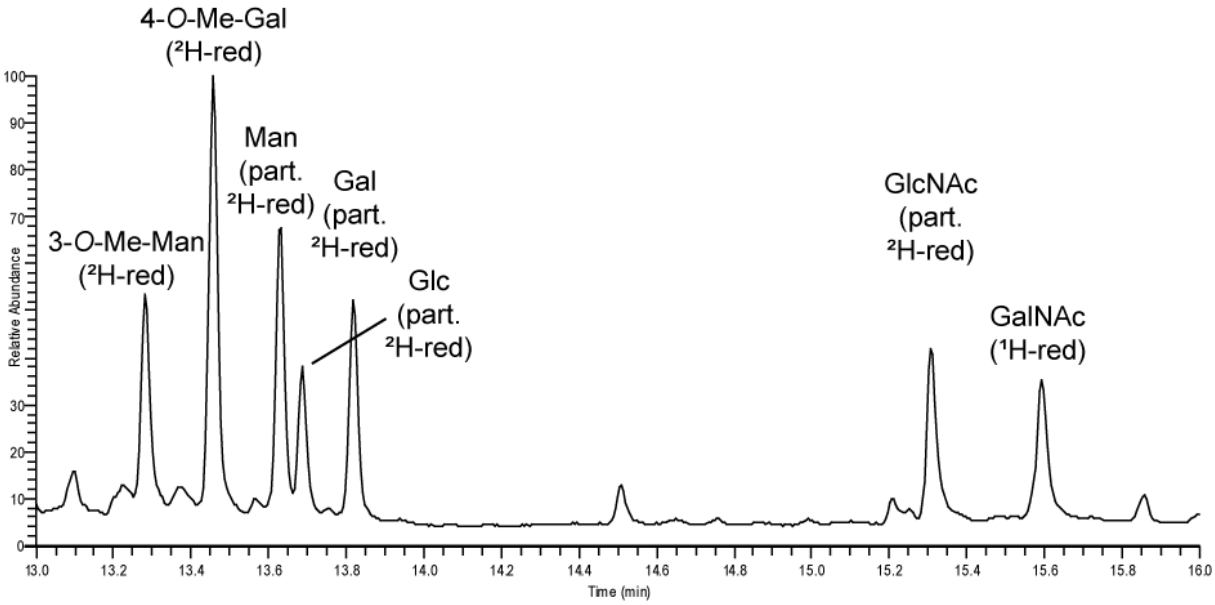


Figure 52 - GC spectrum of "core" structure isoform (576.3 Da) of *A. lusitanicus.*

Figure 52 shows the GC spectrum of the “core” isoform that can be seen as a small peak (9.1 min) eluting after the main “core” structure (7.7 min) in the PGC selected ion chromatogram (lower panel of Figure 49). Since the isoform is just a minor component, it was not possible to get a fraction completely without the “core” structure and due to the low sample amount impurities like Glc play a more dominant role in the spectrum. Nevertheless, the occurrence of mainly ^{1}H-reduced GalNAc is an indication that this residue is the inner sugar of the glycan. Apart from 4-*O*-Me-Gal, the presence of 3-*O*-Me-Man is noticeable. While it was not detectable in the “core” structure GC run (Figure 51), it seems to be a major component of this isoform. Although the incorporation of methylated mannose is quite unusual for O-glycans, we think that the isoform could consist of the inner GalNAc residue

elongated by one 4-*O*-Me-Gal and one 3-*O*-Me-Man residue. The fact that the peak ratio between the methylated sugars is not 1:1 can be explained by the fact that this fraction was not completely purified from the “core” structure.

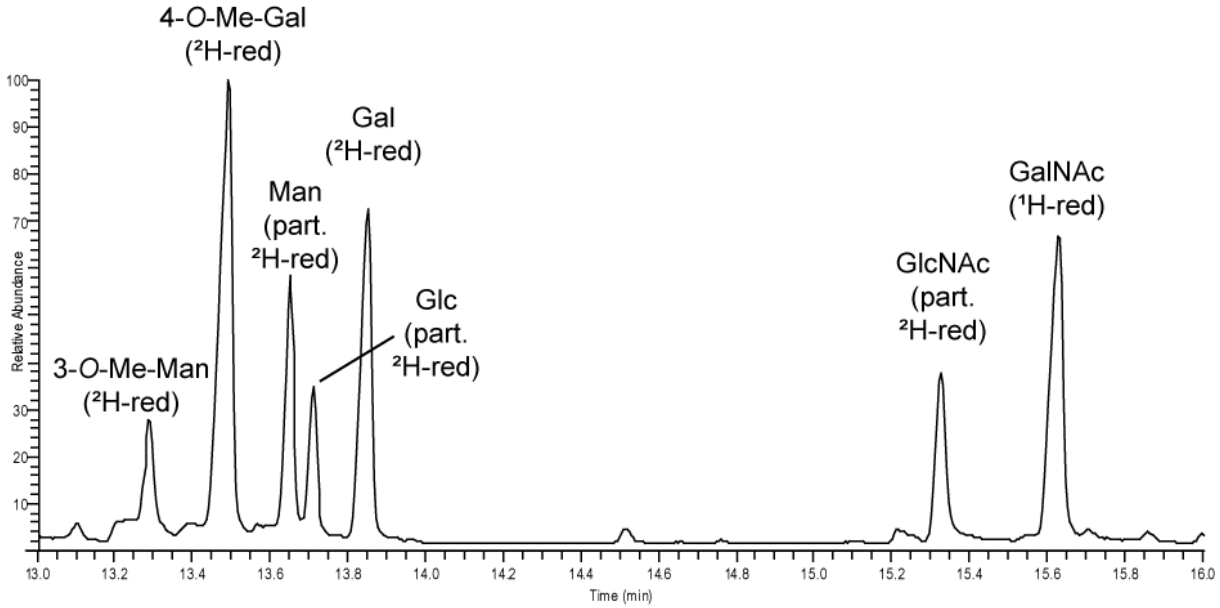


Figure 53 - GC spectrum of elongated structure (738.3 Da) of *A. lusitanicus*

The elongated structure consisting of the “core” and one additional hexose was purified as good as possible. Since some sample is lost in every additional clean-up step, we tried to find a compromise between good purification and sufficient yield. Unfortunately the GC spectrum (Figure 53) is not totally significant. While we assume that the “core” structure remains the same, the increase in deutero-reduced Gal indicates that the fourth sugar might be a non-methylated galactose. Although it has to be said that this is rather an assumption than a final proof.

Finally, the results for the fourth structure, “core” elongation enlarged by one methylated and one non-methylated hexose (914.4 Da; Figure 54) remains equally undetermined. Here again, an increase in 3-O-Me-Man and Man can be found, so that there might be a “core” modification including these two residues. Again, this has to be recognized more as an indication.

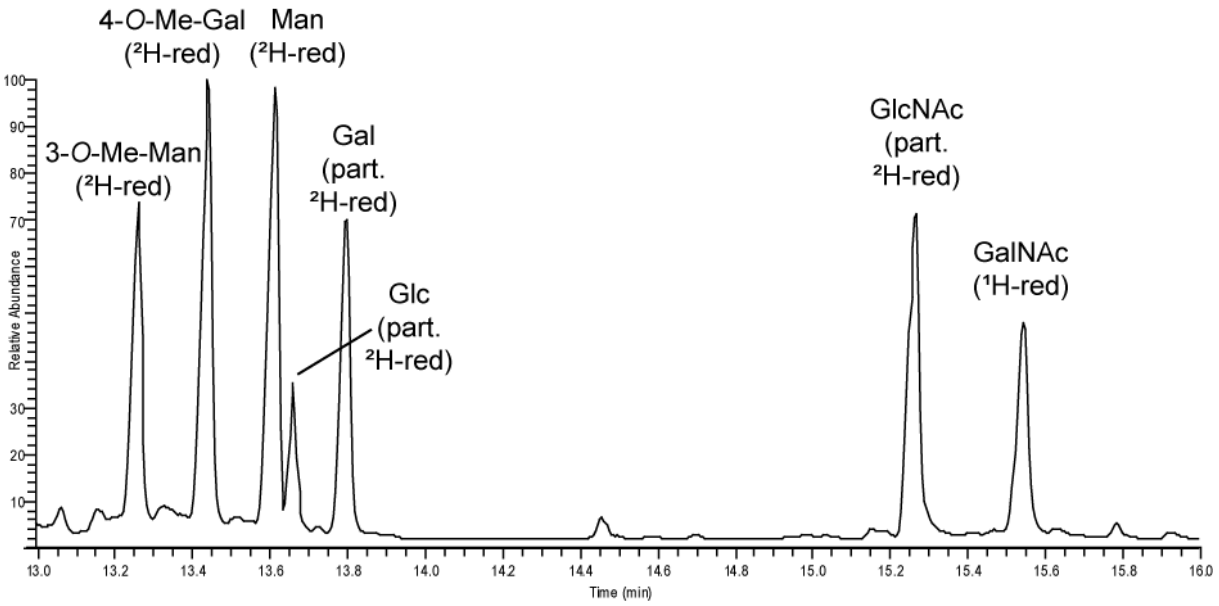


Figure 54 - GC spectrum of elongated structure (914.4 Da) of *A. lusitanicus.*

4.6.2.2 Linkage analysis of partially methylated alditol acetates (PMAA)

Linkage analysis of our snail samples was performed in two steps. First a “normal” methylation was performed using iodomethane. This makes analysis of the various fragmentation patterns much easier but disguises the natural methyl-groups in the samples. That is why a second methylation was performed using deuterated iodomethane which allows discrimination of natural and introduced methyl-groups by a mass difference of 3 Da.

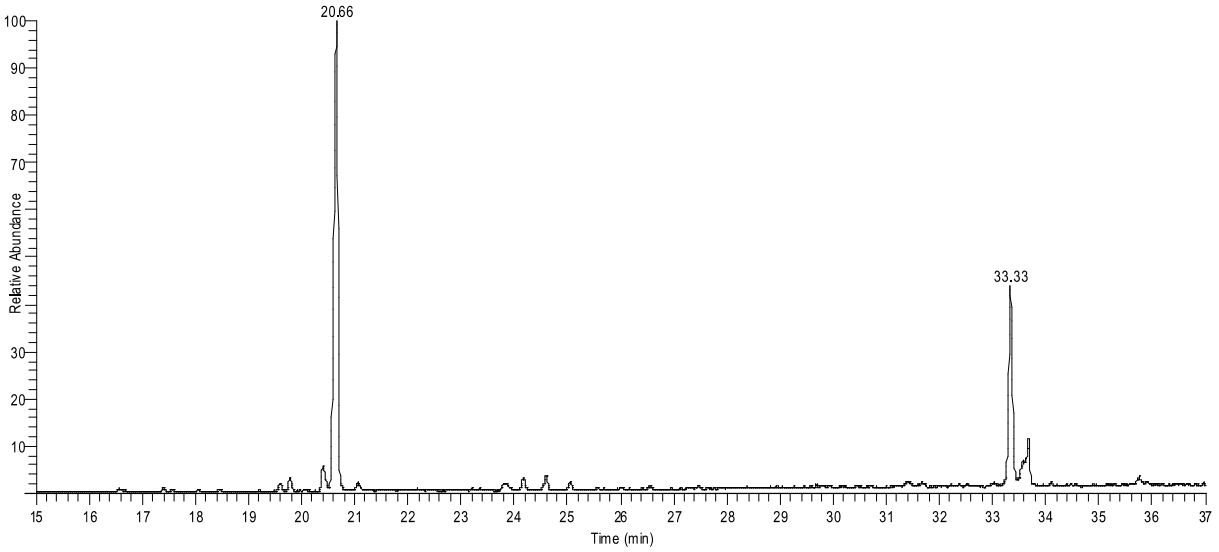


Figure 55 - GC selected ion chromatogram of the PMAAs of the "core" structure (576.3 Da).

Figure 55 shows a pure selected ion chromatogram of the PMAAs of the core structure. The EI/MS fragmentation pattern after methylation with iodomethane undoubtedly identifies the first peak (20.66 min) as terminal galactose. 2,3,4,6-Tetra-*O*-methyl-galactitol (shown in the lower panel of Figure 56) is completely methylated apart from position 1 where it was attached to the first sugar of the glycan chain and position 5 where the sugar ring was closed before hydrolysis. The second peak (33.33 min) of Figure 55 is elucidated in the upper panel of Figure 56 as 3,6-disubstituted GalNAc. 1,4,5-Tri-*O*-methyl-GalNAc-ol is methylated at position 4 as well as 1 and 5 because it was already reduced at the β-elimination step but position 3 and 6 where occupied by another sugar residue before hydrolysis.

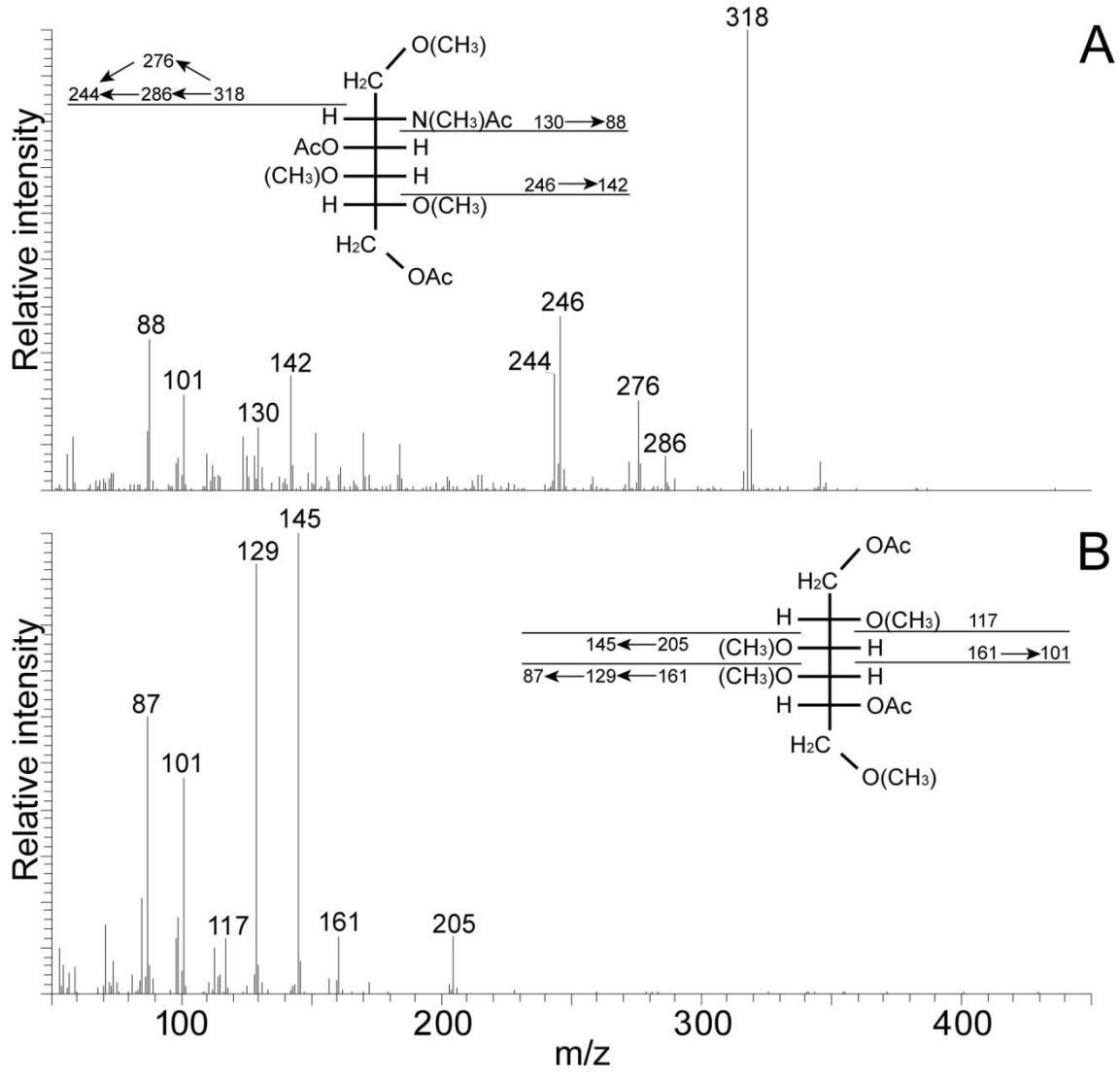


Figure 56 - EI/MS fragmentation pattern of 1,4,5-tri-*O*-methyl-GalNAc-ol (A) and 2,3,4,6-tetra-*O*-methyl-galactitol (B).

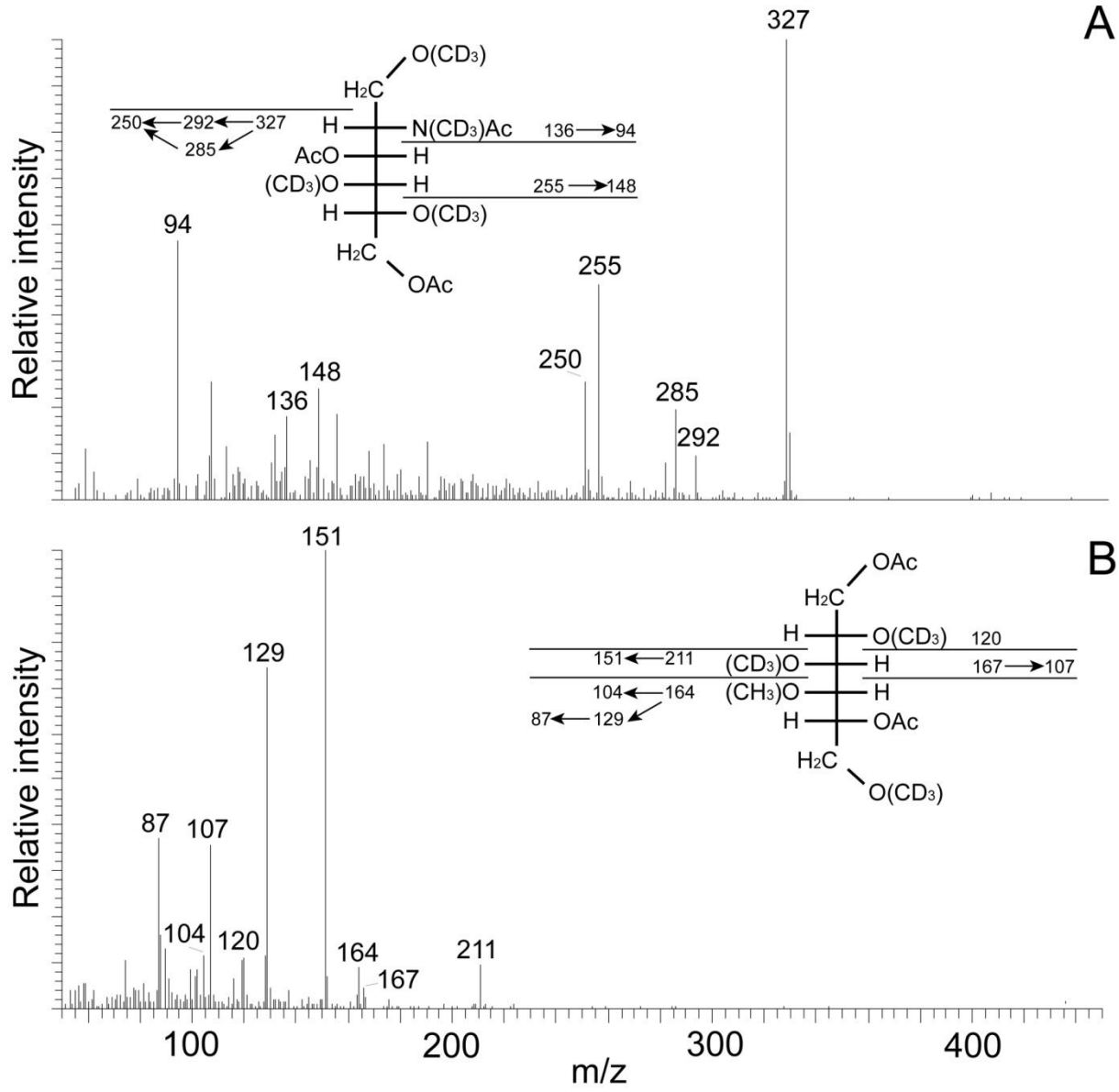


Figure 57 - EI/MS fragmentation pattern of 1,4,5-tri-*O*-deuteromethyl-GalNAc-ol (A) and terminal 4-O-methyl-2,3,6-tri-*O*-deuteromethyl-galactitol (B).

By repeating the experiment with deuterated iodomethane it was possible to confirm the previous results with the existence of a 3,6-disubstituted GalNAc as first sugar but also to show the terminal galactose residue carries a natural methyl-group at the fourth carbon atom (Figure 57).

With the clear elucidation of both types of sugar constituents and the peak-to-peak ratio of 2:1 shown in Figure 55, the structure depicted in Figure 58 can be assigned to the “core” structure that has been found to be the major O-glycan component of snails.

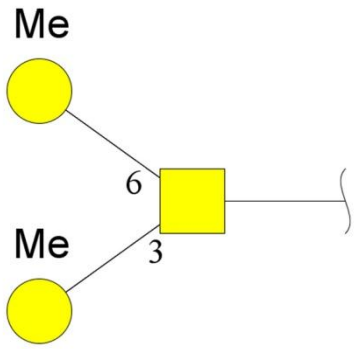


Figure 58 - Structure of the "core" consisting of one 3,6-disubstituted GalNAc (yellow square) and two terminal 4-O-methylated galactoses (yellow circles).

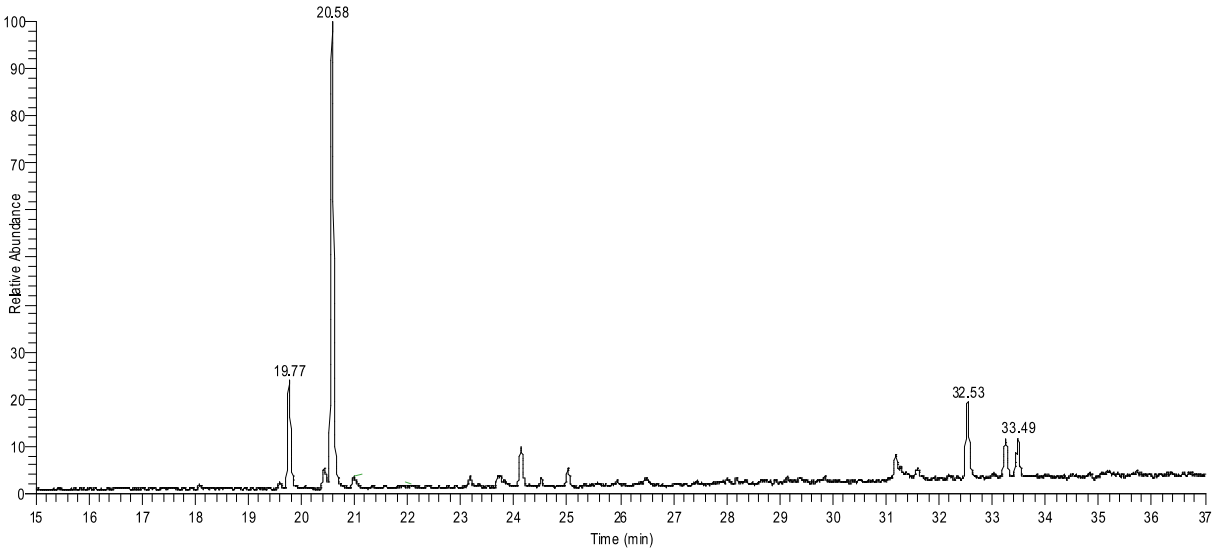


Figure 59 - GC selected ion chromatogram of the PMAAs of the "core" isoform structure (576.3 Da).

Finally, the isoform of the “core” structure (Figure 59) was analyzed. Methylation linkage analysis again revealed the occurrence of 2,3,4,6-tetra-*O*-methyl-galactitol (20.58 min) and 1,4,5-tri-*O*-methyl-GalNAc-ol (33.49 min) as well as terminal mannose (19.77 min), 4-substituted GlcNAc (24.13 min) and 4,6-disubstituted GlcNAc-ol. Especially the two GlcNAc residues are indicators for the presence of N-glycans, namely the chitobiose core. That is why we did not include these two residues in our structural considerations.

Deuteromethylation was able to verify the terminal mannose as 3-*O*-methyl-2,4,6-tri-*O*-deuteromethyl-mannositol. Together with the results from the original “core” we think that it is very likely that the “core” isoform consists of one 3,6-disubstituted inner GalNAc which is elongated with one terminal 4-*O*-methyl-galactose and one terminal 3-*O*-methyl-mannose.

4.7 LC-ESI/MS snail glycan analysis

To elucidate O-glycan structures of different snails, LC-ESI/MS analysis after in-gel release as described in 3.5.3 was employed. To check the sufficient application of snail protein, a coomassie staining (and de-staining!) can be performed before the β-elimination step (Figure 60).

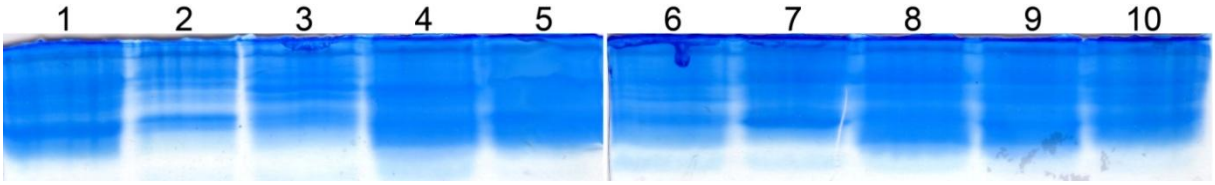


Figure 60 - Application of 200-300 µg of snail protein to SDS-PAGE: *H. pomatia* (1), *A. fulica* (2), *A. lusitanicus* (3), *L. maximus* (4), *C. hortensis* (5), *C. helena* (6), *B. glabrata* (7), *L. stagnalis* (8), *P. corneus* (9) and *A. arbustorum* (10).

LC-ESI/MS analysis was performed after separation on a PGC column. A base peak chromatogram is depicted in Figure 61. Major components of *A. lusitanicus* such as the peaks at m/z 576 or 738 were elucidated as O-glycan structures. Minor glycan components were detected by carefully screening the whole chromatogram; unassigned peaks were found to be non-glycan contaminations.

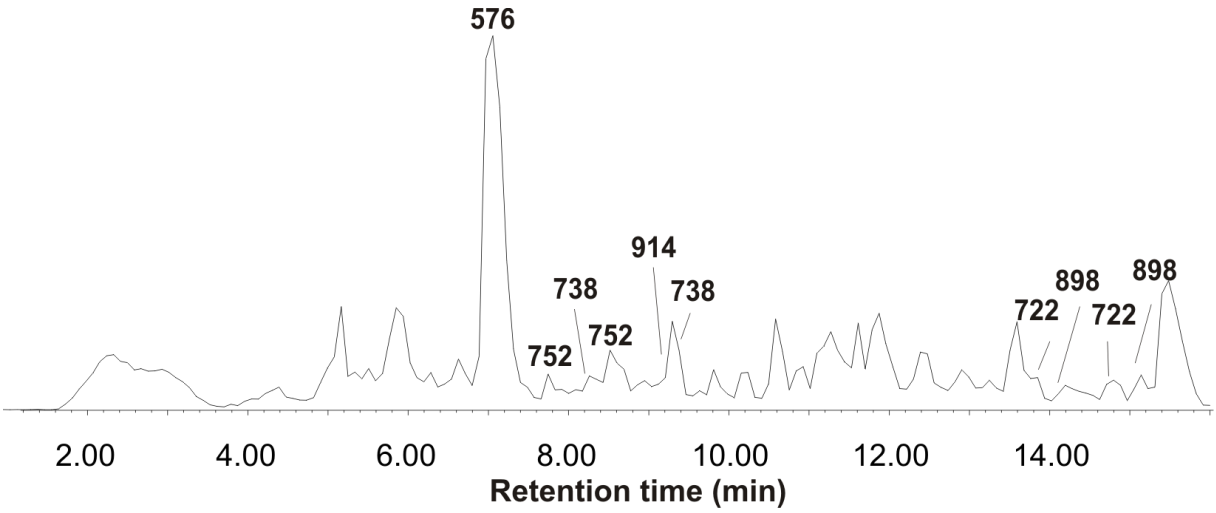


Figure 61 - LC-ESI/MS basepeak chromatogram of *A. lusitanicus*. Assigned peaks are corresponding to structures elucidated in Table 16. Minor structures were not assigned. Unassigned peaks are not corresponding to glycan structures.

Composition	Group	Mass	*Arion lusitanicus*	*Achatina fulica*	*Cepaea hortensis*	*Planorbarius corneus*	*Biomphalaria glabrata*	*Helix pomatia*
H1N1		**386.2**	0.0	6.6	0.0	0.0	0.0	0.0
mH1N1		**400.2**	5.5	0.0	2.7	0.6	0.0	1.8
H2N1		**548.2**	traces	0.0	0.0	0.0	0.5	0.0
H1mH1N1		**562.3**	9.3	0.0	0.0	11.5	18.3	25.9
mH2N1	**Core**	**576.3**	53.7	68.9	26.0	11.5	28.8	28.6
mH3N1	**MeHex** Core + additional methyl-hexoses	**752.3**	4.6	1.6	11.5	2.9	0.0	26.8
mH4N1		**928.4**	0.4	0.0	0.0	0.0	0.0	0.0
H1mH2N1	**MeHex/Hex** Core + additional methyl-hexoses and hexoses	**738.3**	14.3	16.4	17.0	16.1	44.5	11.6
H2mH2N1		**900.4**	2.1	0.0	37.3	2.3	0.0	0.9
H1mH3N1		**914.4**	2.1	4.9	5.4	0.0	5.2	3.6
mH2N1F1	**Fuc** Core + additional methyl-hexoses and fucose	**722.3**	2.1	1.6	0.0	0.1	1.3	0.4
mH3N1F1		**898.4**	5.9	0.0	0.0	0.0	0.0	0.0
H3N1	**Hex** HexNAc + additional hexoses	**710.4**	0.0	0.0	0.0	5.7	1.3	0.4
H4N1		**872.3**	0.0	0.0	0.0	45.9	0.0	0.0
H5N1		**1034.3**	0.0	0.0	0.0	2.9	0.0	0.0
H6N1		**1197.0**	0.0	0.0	0.0	0.6	0.0	0.0

Table 16 - Overview of O-glycan composition of selected snail species given in % of total O-glycans (H = hexose; mH = methylated hexose; N = N-acetylhexosamine; F = fucose). Most abundant structure of every species is marked red.

By using a short clean-up procedure it was ensured that no glycan modifications such as sulfate groups get lost during purification. As shown in Table 16, we were able to detect one "core" structure consisting of one N-acetylhexosamine (N) and two methylated hexose (mH) residues. This structure was found in all investigated species as a major component, being the most abundant structure in *A. lusitanicus*, *A. fulica* and *H. pomatia*.

C. hortensis and *B. glabrata* show the "core" structure elongated with one or respectively two non-methylated hexoses (H) as most abundant O-glycan. We grouped all found glycan structures based on the "core" in two subsets, MeHex and MeHex/Hex, depending on whether all further hexoses were methylated or some non-methylated hexoses were added. As a third group, Fuc, structures with an elongated core decorated with one fucose residue

(F) are displayed. *P. corneus* is the only found snail species which has a non-methylated structure consisting of one N-acetylhexosamine and four hexose residues as most abundant glycan and also shows a couple of other non-methylated structures (Hex-Group).

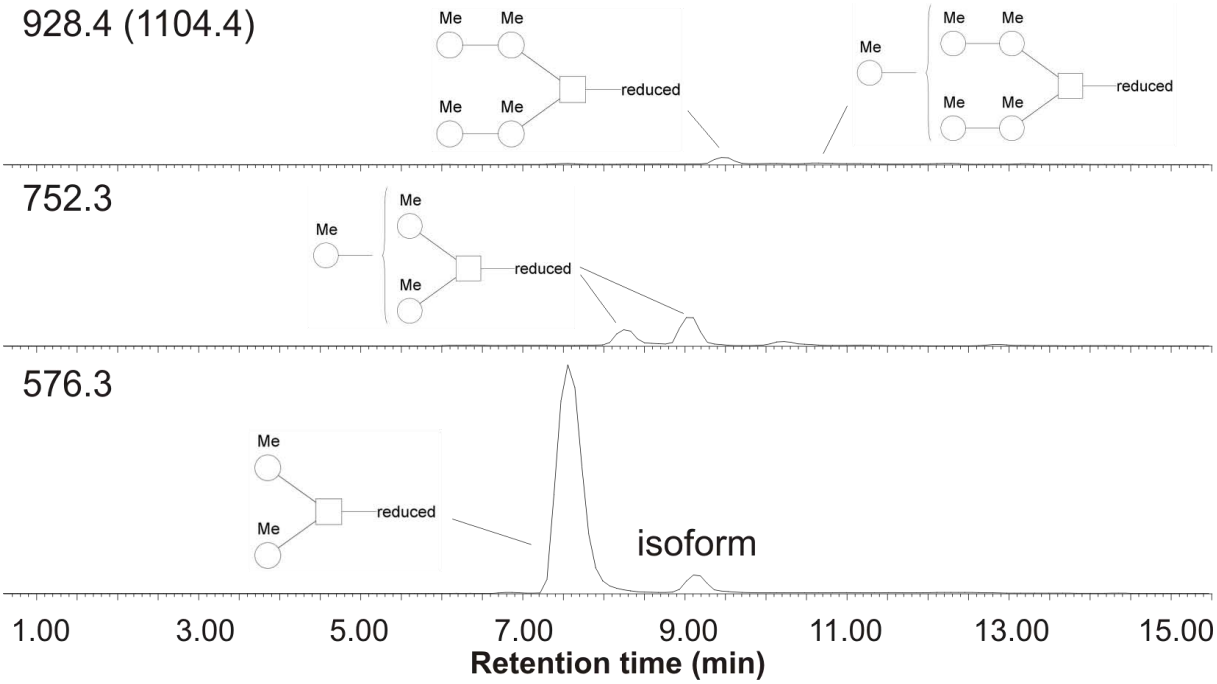


Figure 62 - Selected ion chromatogram of methylated structures (MeHex-Group).

Figure 62 shows three selected ion chromatograms of the *A. lusitanicus* MeHex-Group including the "core" structure (mass: 576.3 Da). Interestingly a second peak can be seen in the "core" chromatogram indicating that an isoform with different hexose constituents or linkages is present. The middle panel (mass: 752.3 Da) shows that the elongation with one additional methylated hexose can occur on the upper and lower arm of the glycan. The addition of two further methyl-hexoses results in two equal arms as illustrated in the upper panel.

A further elucidation of the "core" structure is shown in Figure 63. By using LC-ESI/MS/MS fragmentation techniques we were able to show that the glycan is bound to the peptide backbone via a N-acetylhexosamine residue which gets reduced during the β–elimination process. The two additional peaks with a distance of 176 Da each clearly show that two methylated hexoses are added to the N-acetylhexosamine residue. The fact that no fragment consisting of two methyl-hexoses (352 Da) can be seen is an indication that both are linked to the amino sugar rather than building an elongated structure.

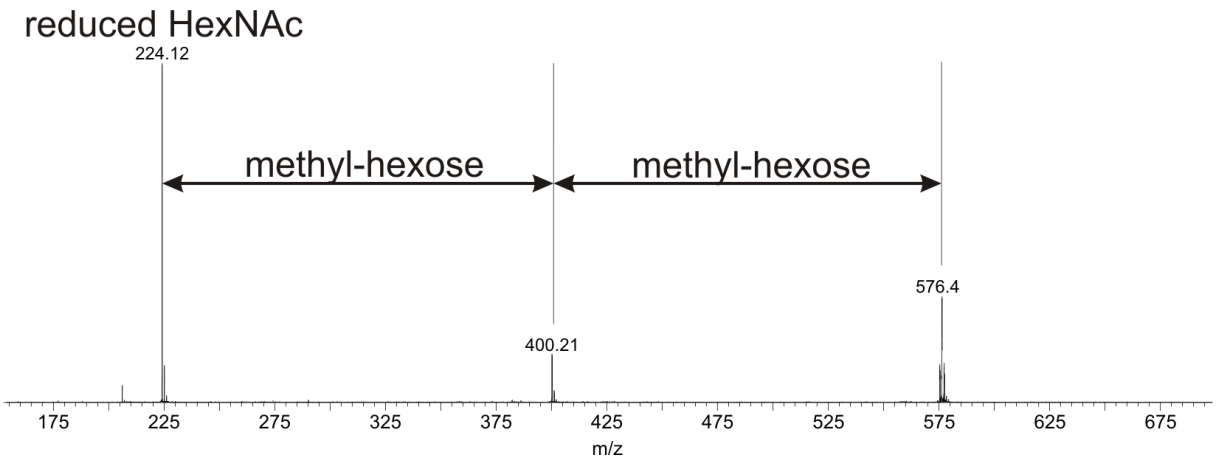


Figure 63 - LC-ESI/MS/MS spectrum of structure 576.4 Da.

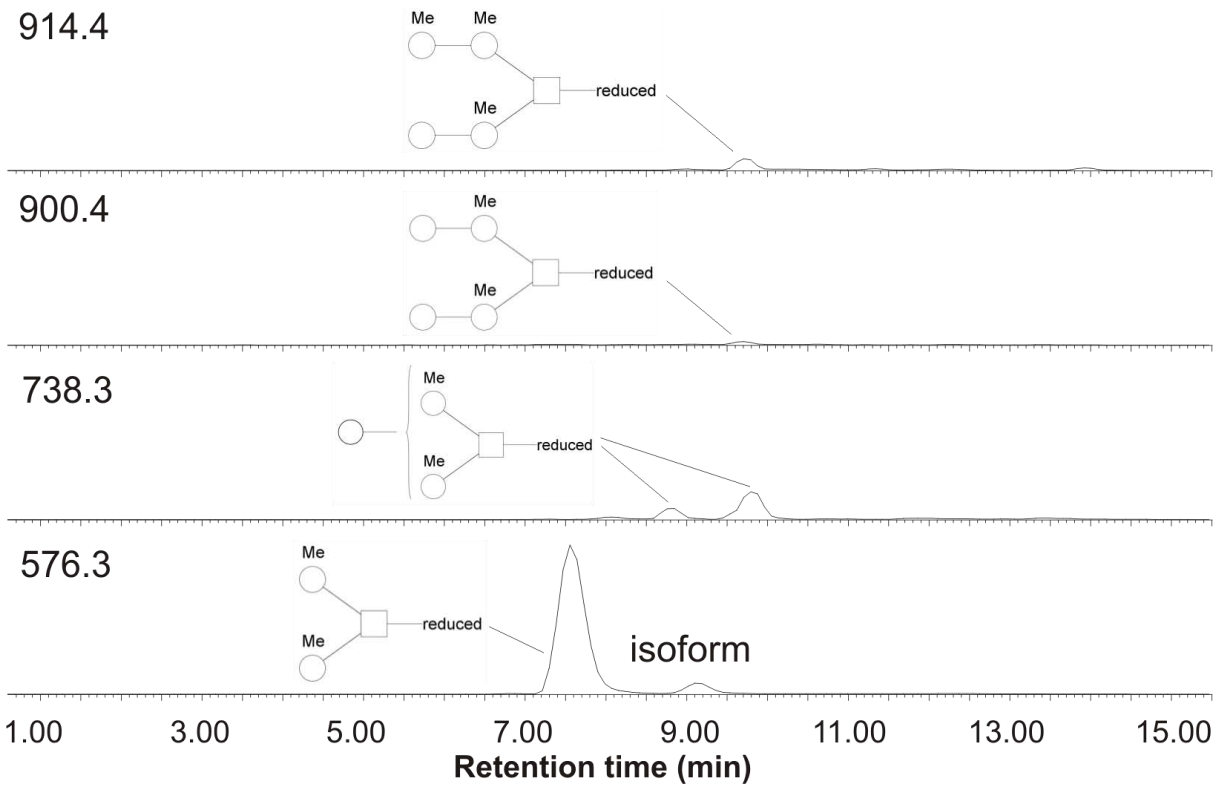


Figure 64 - Selected ion chromatogram of partially methylated structures (MeHex/Hex-Group).

Figure 64 displays the most abundant O-glycan structures of *A. lusitanicus* which include non-methylated hexoses. The MS/MS spectrum of the structure consisting of one N-acetylhexosamine, two methylated hexoses and one hexose (mass: 738.3 Da) is shown in Figure 65.

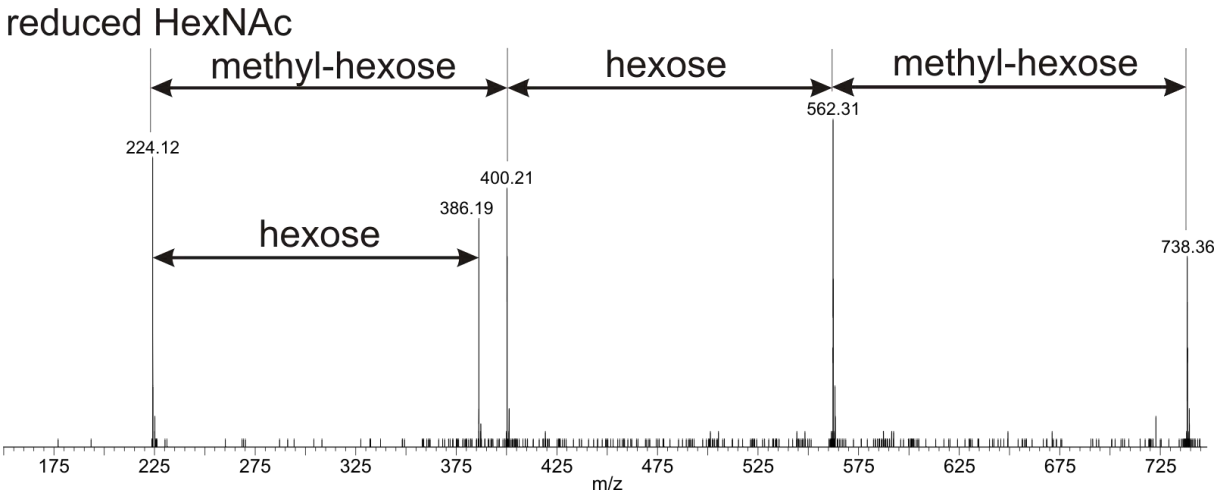


Figure 65 - LC-ESI/MS/MS spectrum of structure 738.3 Da.

Further elongation with one hexose (mass: 900.4 Da) or one methylated hexose (mass: 914.4 Da) is shown in the upper panels of Figure 64. Figure 66 clearly shows the addition of one further methylated hexose compared to the structure depicted in Figure 65.

One thing that has to be noticed here is that the fragmentation pattern of the structures containing hexoses and methylated hexoses (Figure 65 and Figure 66) both display a fragment (m/z 386.2) consisting of the reduced HexNAc and one additional hexose. This would conflict with the structural propositions of Figure 64. Obviously this could result from a lost methyl-group during the fragmentation process but also a rearrangement of smaller fragments might be a reasonable explanation. Apart from that it is certainly possible that a small percentage of the monitored structures indeed has the methylated hexose in terminal position rather than attached to the HexNAc residue.

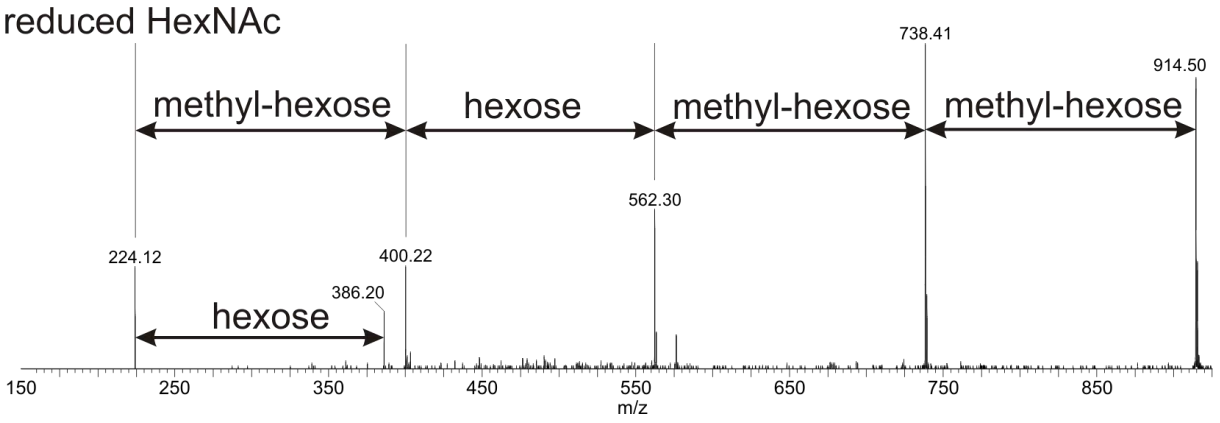


Figure 66 - LC-ESI/MS/MS spectrum of structure 914.4 Da.

Finally, fucosylated structures were analyzed (Figure 67). It was possible to detect monofucosylated “core” structures (mass: 722.3 Da) carrying the fucose residue in terminal

position (two isoforms) or attached to the N-acetylhexosamine residue. The MS/MS spectrum of this structure is shown in Figure 68. Additionally we found O-glycan structures consisting of one N-acetylhexosamine, one fucose and up to four methyl-hexose residues.

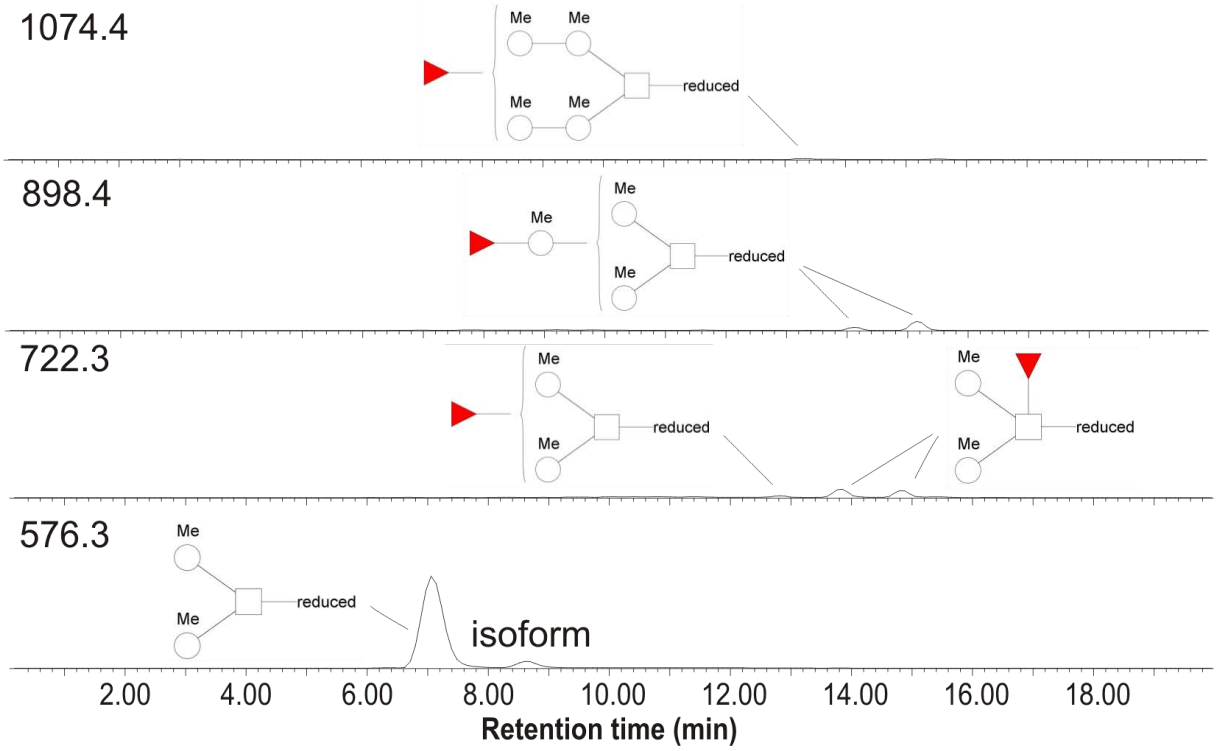


Figure 67 - Selected ion chromatogram of fucosylated structures (Fuc-Group).

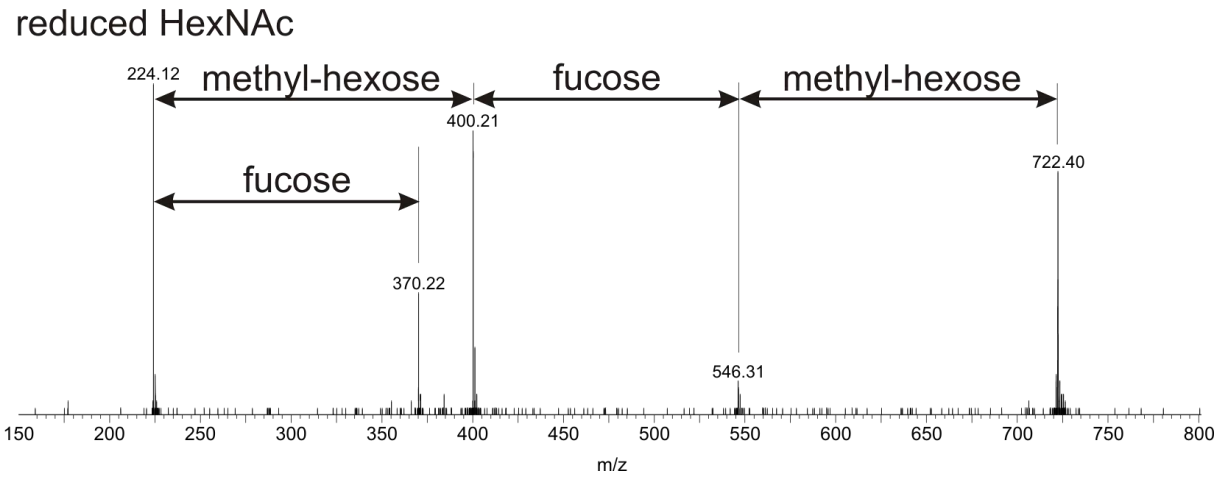


Figure 68 - LC-ESI/MS/MS spectrum of structure 722.3 Da.

5 Discussion

This work was mainly dedicated to elucidate the O-glycan structures of various different gastropods. During the last decade, our working group has gained extensive experience in snail glycan elucidation – since 1998 numerous diploma und PhD students mainly worked on the analysis of mollusk N-glycosylation and the enzymes which are involved in their biosynthesis. It has been found that the biosynthetic capacity of snails is able to produce a highly heterogeneous spectrum of glycans which includes motifs known from mammals, insects, trematodes or nematodes. Especially remarkable is the fact that a quite special carbohydrate modification seems to be very frequent in snails: methylation. While methylation of DNA for example is a common process during development of all higher organisms, methylation of carbohydrates is only known from a couple of species. It was already found in bacteria (e.g. *Bacillus anthracis, Pseudomonas syringae pv. Phaseolicola*) or plants (*Salvia officinalis L.*) where it occurs mostly within the sugar chain, and as well in terminal position of the glycan in nematodes (*Caenorhabditis elegans, Toxocara canis*) or mollusks. The findings of all resulting publications have recently been reviewed by Staudacher et al. and can be found in part 9.3 of this work.

As corollary we wanted to extend the knowledge about carbohydrates in snails by having a detailed investigation on their O-glycan structures.

While O-glycans are without doubt very important protein modifications which contribute a lot to the biochemical structure and function of many proteins, the analysis of O-glycans unfortunately poses a number of additional difficulties compared to N-glycan analysis. This might also be an explanation why the number of publications on O-glycosylation significantly lags behind the number of N-glycosylation papers.

5.1 Glycan purification from isolated glycoproteins

The first problem that arose was the question how to start the protein purification. When glycosylation analysis is performed on purified glycoproteins such as fibrin (3.1.1.1) the preparation can start with generation of glycopeptides right away. This step facilitates the access of PNGase A to the carbohydrate moieties because the protein structure is disrupted. The sample is digested with pepsin or another proteolytic enzyme and gel filtration steps are performed in order to get some separation between peptides and glycopeptides. Pooled glycopeptides are then subjected to PNGase A treatment. Unlike PNGase F which is able to cleave N-glycans from intact proteins but has its restrictions when the innermost GlcNAc residue is modified with an α-1,3-linked fucose which commonly occurs in plant or insect

proteins, PNGase A should be capable of releasing all kinds of N-glycan and is therefore often used for the work with invertebrate glycans. Although there are no limitations on the substrate specificity of this enzyme published to date and usually the majority of N-glycans is released from the peptide backbone, there might be some structural motifs or sample conditions that prevent a complete N-glycan cleavage. Extensive proteolytic digestion which produces very small peptides and cleavage sites right next to the asparagine residue that is N-glycosylated, however can limit the effectiveness of PNGase A digestion.

After the release of N-glycans from the peptides which are charged due to the amino terminus, cation exchange chromatography can be used to separate these two fractions. While glycans should not interact and elute as flow-through, peptides strongly bind to cation exchange media in an acidic environment. They are later on eluted by raising the pH value of the elution buffer.

5.2 Snail glycan purification

Certainly the method described in the previous chapter is convenient for the purification of N-glycans derived from a single glycoprotein but snail samples pose a number of additional difficulties. First of all, snails like *A. lusitanicus* or *A. fulica* produce thick viscid mucus that really troubles all following clean-up steps. Even after going through the first steps of the original protocol (ammonium sulfate precipitation, dialysis and batch anion exchange chromatography) the samples kept their high viscosity in a way that application to any kind of column chromatography was more or less impossible. Smallest amounts of sample were sufficient to completely clog the column – the necessity of a very high dilution of the samples made the purification of larger amounts practically impossible. So it was decided to wash the snails very thoroughly before dissection and homogenization. The mucus of the thawed snails was removed as good as possible by repeatedly washing and wiping it away. Water-living snails such as *B. glabrata* or *P. corneus* are easier to prepare considering the point of mucus but on the one hand side it is difficult to really deploy large amounts of snails to enable different tests and experiments and on the other hand their dissection and removal of the gut is quite hard to handle. While *B. glabrata* is so small that a clean removal of the intestinal tract is hard to ensure without losing too much snail material, the inner structure and coloring of *P. corneus* make discrimination between skin, viscera and gut very challenging.

At the beginning of this work skin and viscera were separated as described earlier and analyzed as two independent samples. The idea behind this was to avoid or at least being able to notice contaminations with foreign carbohydrates. While a contamination of the skin

might be possible due to bacterial or fungal infection, contamination of the viscera fraction might be caused by an insufficient removal of the gut and therefore by carry-over of ingested plant glycans.

During the first preparations, no really big differences between these two fractions were found and since the separation was not even possible for all snails, this step was omitted during the later work. Considering all changes and optimization steps in the protocol which had to be made, it was important to ensure a faster production of results which was certainly boosted by reducing the samples to the half amount.

This argument is also valid for the anion exchange chromatography step that was part of the original procedure. Of course the sample heterogeneity was reduced a little but again two independent preparations were to handle. Considering the availability of all the necessary equipment and supply such as columns, packing material for ion exchange, gel filtration or hydrophobic interaction chromatography, fraction collectors and so on, we were really struggling with the time consuming protocol. Taking into account that one snail was dissected into skin and viscera fraction which were then again split into flow-through and binding fraction of the ion exchange chromatography, we already had four samples derived from one snail species. Since N- and O-glycan fraction after PNGase A digestion and the subsequent cation exchange chromatography step both have to be analyzed, eight samples have to be prepared for one single species. Because we also wanted to analyze more than one species to allow a comparison between different snails, we decided to reduce the fractionation to a minimum in order to reduce the total time used for the preparation of one snail.

One of the biggest troubles during the beginning was that samples didn't seem to lose their viscosity and stickiness during the whole clean-up procedure. Since glycan carbohydrates usually can be dried without problems, we were facing the problem of unknown impurities in our samples. What we knew was that our samples did not dry in a vacuum centrifuge or during lyophilization. While this problem can be handled somehow for the analysis on HPLC, MALDI-TOF/MS or derivatization for GC/MS really deserve dry samples. Also some further experiments such as permethylation are impeded drastically. The two possible explanations were contamination with some form of lipid or amino acid. In order to remove fatty components, an additional chromatography step using a hydrophobic interaction media was introduced. Unfortunately, the HIC step was very time consuming and we did not have the feeling that the overall sample quality was improved.

Finally, the breakthrough was achieved by using different kinds of precipitation. By using acetone and methanol precipitation at several stages of the purification, it was possible to get dry samples that can be easily used for further analysis. As a very pleasant side effect, we

got rid of all time consuming gel filtration and most ion exchange chromatography steps. Since many samples can be precipitated and centrifuged in parallel which cannot be done when column chromatography has to be used, this was a major time saving change.

To avoid any contamination of samples due to improperly cleaned columns, all media (except the cation exchange chromatography media) were bought as cartridges and used for one single extraction step only. PGC and RP-C18 cartridges were used in different sizes to accommodate every sample batch size.

5.3 Release of O-glycans

One major barrier which impedes the analysis of O-glycans is that so far there is no enzyme available which allows specific cleavage of all kinds of O-glycans from the peptide backbone. The only available enzyme is an O-glycanase (O-glycopeptide endo-D-galactosyl-N-acetyl-α-galactosaminohydrolase) which cleaves the disaccharide Gal-β(1-3)-GalNAc-α from serine or threonine and therefore matches the structure of core 1 mucin-type O-glycans. Nevertheless various further modifications such as sialylation prevent liberation and other O-glycan types are not affected at all.

Apart from enzymatic hydrolysis, chemical methods may be applied. The two most commonly cited methods are hydrazinolysis and different kinds of β-elimination. In theory, the process of hydrazinolysis can be conveniently used to cleave O-glycans separately from N-glycans or under slightly different conditions release of both fractions in one single step. One advantage of this method is that the reducing end of the glycans stays intact and further derivatization steps are not hindered. This is of big importance if a following HPLC separation is desired in order to purify various structures or to the reduce heterogeneity of the full glycan spectrum. Also mass spectrometric analysis (MALDI-TOF/MS) of small glycans consisting of two to four carbohydrate residues is difficult or nearly impossible if the total mass is too low and cannot increased by adding high molecular mass labels.

Unfortunately, hydrazine is explosive when exposed to oxygen and so the work is hazardous and the handling is quite difficult because all working procedures have to be performed in an argon atmosphere. Nevertheless we performed the experiment once and compared the N- and O-glycan fractions after derivatization with anthranilic acid or 2-aminopyridine. Both fractions were analyzed and the found differences were not very convincing. The peaks displayed by the O-glycan fraction were eluting very early right after the dead volume on a normal phase-HPLC column. The N-glycan fraction showed completely the same peaks at the very beginning of the chromatogram and additionally some more peaks right behind. Since retention times on a normal phase column are size dependent, small molecules elute

at the beginning while larger structures show stronger interaction. Because of the very short retention times of the majority of the peaks we did not have the impression that these peaks were corresponding to typical glycan structures. After these not very convincing results and because of the difficult handling we decided not to optimize the conditions.

Nowadays β-elimination seems to be the most commonly used method for the cleavage of O-glycans. The advantages are the reliability as well as the fast, cheap and easy protocol. The disadvantage is that the reducing end of the first carbohydrate gets lost and cannot be used for further derivatization. Therefore this method is actually only helpful if MS analysis is available. HPLC analysis with UV or fluorescent detection like applied after PA or AA labeling is not possible anymore. As workaround for this problem a non-reductive β-elimination protocol was published some years ago. This method should combine the reliable release of O-glycans with the conservation of the reducing end. Although this sounds very promising, we were not able to generate labeled glycans after non-reductive β-elimination following the protocol given. Probably we tried this method with early, rather crude snail purifications and it would work better with cleaner samples. Colleagues from other groups had similar problems but most recently two publications suggested the combination of non-reductive β-elimination with PMP-labeling in one reaction pot [120, 121]. This might be a new approach to label O-glycans for HPLC analysis.

However, during this work conventional β-elimination protocols were used after the decision to do the further analyses using ESI/MS and GC/MS which do not require a labeled glycan was made. The difference between the two methods described in the methods part is that the one published by Spiro and Bhoyroo releases less N-glycans when glycoproteins are used that were not previously subjected to PNGase digestion [115]. Iyer and Carlson on the other hand side published a protocol that cleaves fast and reliable all O-glycans with some N-glycans contaminations [114].

At the beginning of this work the approach was to remove the N-glycans from the glycoproteins and get a clean O-glycoprotein or O-glycopeptide fraction. This would have been a good starting point for monosaccharide analysis, making the reliable comparison of N- and O-glycans possible. However, all results indicate that neither PNGase A nor F are capable of cleaving all N-glycans from the peptide backbone of the snail derived glycoproteins. Multiple digestions with the same enzyme as well as subsequent digestion with PNGase A and F have been tried but MALDI-TOF/MS still showed characteristic N-glycan structures with the core consisting of two N-acetylglucosamine and three mannose residues. Throughout many different approaches it was tried to figure out how to improve the N-glycan cleavage. Finally, we had to admit that we were not able to get an O-glycan fraction that does not include at least some N-glycans. While PNGase digestion obviously leaves

some N-glycans behind which are released during β-elimination later on, even the mild β-elimination published by Spiro and Bhoyroo splits off some N-glycans. Removing the N-glycan impurities with affinity chromatography columns, e.g. Concanavalin A, was considered as well but since these lectins are also do not bind all kinds of N-glycans, it was finally decided not to prolong the protocol.

To make the best out of this situation, the protocol was shortened to a minimum by removing all steps that are not totally efficient. As a matter of fact the final snail purification protocol does not include any kind of PNGase digestion, just the β-elimination step. In accordance with the planned analysis on LC-ESI/MS using porous graphitized carbon columns which allows a discrimination of N- and O-glycans by their retention time and of course by their mass and fragmentation pattern, we suggest the protocol described in 3.1.1.3.

5.4 Storage carbohydrates

Another problem that is frequently encountered at the point of monosaccharide analysis is that large peaks of glucose or galactose occur. These monosaccharides are obviously not coming from protein-bound glycans but from storage carbohydrates. Glucose chains are a common way for all kinds of animals to store energy. Many of our monosaccharide analyses showed huge glucose peaks and MALDI-TOF/MS/MS results confirmed that our carbohydrate samples were not only containing glycan carbohydrates but also carbohydrates from another origin. Some samples showed a complete set of hexose chains ranging from three up to more than ten hexose residues. To remove these storage carbohydrates we tested various different exo- and endoglycosidases. Finally we found that α-glucosidase from rice is very effectively degrading the storage carbohydrates found in our samples. MALDI-TOF/MS results comparing a sample before and after α-glucosidase digestion clearly show that hexose chains are removed. The subsequent use of a cation exchange chromatography step removes the small digested residues.

5.5 HPLC monosaccharide analysis

Apart from all the optimization work that was done concerning the snail purification protocol, the labeling protocol of monosaccharides for subsequent HPLC analysis was also improved. Most commonly two methods are used: derivatization with anthranilic acid combined with fluorescent detection or labeling with PMP followed by UV detection. Both methods have their advantages and limitations and since retention time on a HPLC column cannot be used for the undoubtful identification of a compound, both methods were used to complement each another. Of course it is possible that an analyte that has not been analyzed so far co-

elutes with one of the reference substances. If just one method was applied this may result in a false positive conclusion but if these two methods are applied it is very unlikely that the result of both labeling techniques will match.

The big advantages of AA labeling are most certainly the fast and easy protocol as well as the very sensitive detection method. After hydrolysis, samples can be derivatized in one hour and are ready for analysis right away. Especially when large amounts of samples have to be checked, every additional pipetting step or liquid-liquid extraction step sums up to a significant increase of time that has to be used for sample preparation. However, for this method samples are just resolved and mixed with the AA reaction mixture. After incubation at 80 °C for one hour, samples are diluted and ready to be injected into the HPLC system. This is only possible because there are no substances in the reaction mixture that have to be removed before analysis.

When this method was first used following the guidelines of well known publications, it became obvious that the published method is designed for much larger sample amounts than those we usually are working with. Since the detection mechanism is able to recognize picomolar amounts of labeled sugar, it is a waste to use milligram amounts of sample. Although our analysis of small sample amounts did work, one problem was to solve: since the excessive AA which is not used in the labeling process is not removed before the analysis, a huge peak of AA arises when low sample amounts are derivatized. This is not only an optical problem but peaks eluting close to the AA peak might be hidden and the column might be overloaded. In a first step, derivatized monosaccharide samples in the range from 250-1000 µg were derivatized and showed that even when 1 mg of sample was applied the size of the AA did not significantly decrease. In a series of optimization steps the total volume of the reaction mixture was lowered to one tenth of the original protocol, and the amount of AA used to one hundredth. By doing this we kept the total volume (15 µl) in an easy to handle range and allowed the derivatization of samples in the range from 5-300 nmol while still having a good peak-to-peak ratio. It is possible to analyze even lower amounts of sugar but this was not really necessary in case of our samples. After agreeing on the new derivatization procedure, the reliability of the quantification of samples was tested. Obviously, there is a significant difference between hexoses, amino hexose or for example methylated hexoses. Especially those sugars that have any kind of modification on their second carbon atom seem to be less susceptible for derivatization. This is valid for amino sugars as well as for sugars that have a methyl group at the second ring carbon atom. We think that the spatial proximity of the modification (amino or methyl group to the locus of the reductive amination (first carbon atom) somehow impedes the reaction. This certainly has to be concerned when samples are quantified in order to get a ratio between the different glycan sugars.

To allow the assignment of as many peaks of our snail samples as possible, we determined the retention times of 24 reference sugars which are possibly occurring in glycan structures on our RP-HPLC column. Unfortunately, not many methylated sugars were available. Since previous studies have shown that snails are able to incorporate methylated forms of mannose and galactose, it would have been good to have these derivatives available. While Prof. Paul Kosma was kindly supplying us with 2-O- and 4-O-methylated galactose, we were lacking 3-*O*-methyl-galactose as well as methylated forms of mannose. So, when analyzing our samples we were constantly confronted with unknown peaks of significant size that could not been elucidated until we were able to compare our HPLC and GC/MS results. Since the availability of methylated sugars is different in various snails, finally all different forms could be discriminated in the HPLC spectrum. Even sugars where no proper reference substances were available were assigned to retention times by this approach.

In order to finally conclude the introduction of the modified protocol, some statistical work on the detection and quantitation limits was done. By using the guidelines for their determination published in the European Pharmacopeia, we calculated a limit of quantitation for AA labeled sugars of 220 fmol and a limit of detection as low as 65 fmol.

As described earlier in this section, the analysis of a substance based just on one single retention time is not possible. A more reliable result is achieved if the retention times of two different experiments following two different labeling procedures are compared and matched. Therefore the PMP labeling system was chosen as second labeling procedure. In contrast to the AA labeling method, PMP derivatization is much more time consuming. This is mostly caused by the necessity to remove the excess PMP reagent before analysis. PMP is added on a vast excess to the sample and would spoil the analysis if not removed beforehand. This is achieved by a liquid-liquid extraction performed five times where an organic solvent is used to extract the remaining PMP from the aqueous sample. While three extraction steps might be sufficient, five steps are recommended in order to get a really clean sample. Apart from that, some tests resulted in diethyl ether to be the best extraction liquid of all tested organic solvents.

AA labeled monosaccharides allow a rough estimation of their chemical structure based on their retention times. While amino acids strictly elute before the AA peak, hexoses elute just after it and methylated hexoses in general have the longest retention times. For the PMP labeled sugars this statement is not valid. Here all kinds of sugars, amino sugars and methylated sugars are scattered throughout the whole chromatogram. It also has to be kept in mind that UV detection does not allow detection limits as low as fluorescent detection. Especially the large baseline drift throughout the whole runtime and the peak broadening are dramatically pushing the limits of detection and quantitation. We have calculated that sugars

that elute quite early such as glucosamine have a limit of detection of 0.2 pmol and a limit of quantitation of about 0.7 pmol. This is about the three-fold amount that has been stated for the AA labeling method and can be considered as quite good result because of the different sensitivity of the two detection methods. Nevertheless these limits do not apply to all sugars. Galactose, for example, which has a retention time of 18.5 min has a calculated limit of detection of 3.2 pmol and a limit of quantitation of 10.7 pmol. These limits are about 15-times higher than those established for glucosamine and are caused by the typical peak broadening that increases with retention time on the one hand side, and on the other hand side by the increase of the noise value. This noise was calculated as six times the standard deviation of the linear regression of the drift and therefore is strongly influenced by a rising baseline.

5.6 GC/MS monosaccharide analysis

The use of GC/MS for monosaccharide analysis was a major break-through of this work. Before, we knew that somehow digestions with exoglycosidases do not work as expected and that there obviously are unknown sugar constituents in the HPLC monosaccharide analysis. With the help of GC/MS analysis it was possible to elucidate the unknown monosaccharides but also to find an explanation for the untypical behavior of our glycan samples.

The electron impact mass spectra of our samples were unambiguously displaying the fragmentation pattern of methylated sugars. While we had expected that to some extent because of the previously gained N-glycan structures, we were surprised of the ratio between methylated and non-methylated hexoses. Obviously in some snails a majority of hexoses are methylated. The experience and reference substances of the laboratory of Prof. Geyer who has worked with GC/MS analysis of glycans for more than 30 years, were truly essential for the further work. Methylated mannose and galactose purified by preparative GC were used as standards to compare the retention times of our methylated snail sugars. It was therefore possible to elucidate that both, methylated mannose and galactose, are occurring in snail glycans. With an additional analysis, reduction of monosaccharides using sodium borodeuteride, it was possible to confirm that the methylated mannose is 3-*O*-methyl-mannose while two forms of methylated galactose are available: 3-*O*- and 4-*O*-methyl-galactose. 3-*O*-Me-Man was found in all investigated samples – and all samples showed at least one form of methylated galactose. While some species display both forms, others just show 3-O- or 4-O-methylated galactose.

The occurrence of 3-O-methylated hexoses had been previously published; on the contrary the occurrence of 4-O-methylated galactose was “quite unknown”. It has been briefly mentioned in a book as a minor constituent of *Helix pomatia* hemocyanin glycans but did not appear in any publications hereafter [180].

5.7 LC-ESI/MS of O-glycan structures

Another big milestone was the analysis of our snail glycans using LC-ESI/MS. The coupling of a porous graphitized carbon column to mass spec analysis allows analysis of samples that are eventually containing some N-glycans because they can easily be discriminated from their O-glycan counterparts due to their retention time as well as due to their specific size and fragmentation pattern. Since we have struggled unsuccessfully for long times to separate these two classes of glycans, finally a way to analyze these samples without being impeded by the fact that there were some impurities was found. With the analyses made by Dr. Martin Pabst of Prof. Friedrich Altmann’s group we were able to gain some O-glycan structure compositions. With the use of the in-gel purification protocol which reduces all clean-up steps to a minimum, first results can be conveniently achieved in a very short time. Of course the low amounts of sample that are purified using this approach are not sufficient to enable further analysis by GC/MS or even NMR, but MS/MS experiments are possible by all means. Due to the results gained from retention time, mass and fragmentation pattern we were able to postulate first O-glycan structures (4.7). Most strikingly we found a structure consisting of two methylated hexoses and one N-acetylhexosamine residue. This structure was called “core” because it seems to be the major building block for all other O-glycan structures. Apart from some minor components that are smaller, all other O-glycans can be lead back to this “core” structure. Some snails showed the “core” as most abundant structures while others tend to elongate the “core” with additional hexoses or methyl-hexoses to be the most prominent structure. Only one snail, *P. corneus*, shows a completely non-methylated structure as most abundant O-glycan (Table 16). While these results were very promising and giving a boost to the whole work, LC-ESI/MS is not really capable of elucidating the linkage between sugars in a glycan structure and cannot elucidate the exact type of involved sugar.

We therefore purified a large amount of *A. lusitanicus* glycans following our new snail glycan purification protocol and fractionated it blindly on a PGC-HPLC column. ESI/MS was then used to find the fractions with the most abundant structures which were then re-chromatographed multiple times to gain pools containing just one single structure. These pools of the “core” structure and its isoform as well as two elongated forms where then

subjected to monosaccharide and linkage analysis on GC/MS to fully elucidate all components.

5.8 GC/MS linkage analysis

Apart from NMR elucidation which still has quite large sample demands, GC/MS linkage analysis is the method of choice for the full structural analysis of glycans. The purified sample was therefore methylated, hydrolyzed and acetylated. The retention times and fragmentation patterns of these partially methylated alditol acetates (PMAA) not only allow discrimination of the respective sugar but also give additional information on their linkage. The monosaccharide analysis of our “core” structure resulted that there was GalNAc as well as 4-*O*-methyl-galactose in the sample. The peak ratio of both substances as well as the ESI/MS fragmentation pattern of the whole structure suggested that the three sugar residues were consisting of two methylated hexoses and one N-acetylhexosamine residue. The linkage of the three constituents had to be determined. The analysis of the PMAA clearly showed that the two 4-*O*-methyl-galactose residues are terminal and that they were attached to another sugar instead of the peptide backbone. On the contrary, it was found for the GalNAc residue that it must have been attached to the peptide backbone. Since the β-elimination was performed with sodium borohydride and the reduction after methylation was induced by sodium borodeuteride, the mass difference of 1 Da clearly indicates which sugar was attached to the protein and which was linked to another sugar within the glycan chain. Additionally the GalNAc residue showed that position 3 and 6 had been occupied by another sugar before hydrolysis. Taking all this into account, only one structural element can be drawn: One N-acetylgalactosamine residue which has one 3- and one 6-linked 4-*O*-methyl-galactose attached to it.

While the analysis of the “core” isoform is not as conclusive as for the “core” itself, it still seems to be significant enough to postulate the exchange of one 4-*O*-methyl-galactose by a 3-*O*-methyl-mannose residue.

5.9 Final overview

As conclusion of this work, it is important to point out that snails are a challenging as well as rewarding field for glycobiologists. They are capable of producing very different glycan structures, some that are known from other species and also others that are quite unique. The frequent use of methylation as modification on mannose and galactose residues has been documented across several different snail species. So far, all investigated snails show glycan methylation regardless if they are land- or water-living snails, shell-carrying snails or

slugs. Together with the fact that snails are rather easy to breed or to collect, it makes them a wonderful model organism for the study on non-vertebrate glycosylation pathways. Of course there was a lot of work necessary to adapt the clean-up protocol to deal with the rather tough meat and the viscid mucus that is found in a lot of snails but we think that the finally established protocol is a good way to deal with this specialty.

So far we have no information on the special function of methylation and no data on how these glycans are built up. Maybe some future work will elucidate if the methyl group is added to the final glycan or if methylated sugar nucleotides are involved in the biosynthesis. About the function we can also just bring up some ideas: One possibility is that the methyl groups are used as some kind of protection against glycan degrading enzymes. We have seen that common exoglycosidases are not able to digest the majority of snail glycans and so this protective function could be very important for animals that are highly dependent on thick and viscid mucus as outer barrier.

The combination of GC/MS and LC-ESI/MS analysis methods supported by HPLC results was able to provide a great insight into the so far rather unknown field of snail O-glycosylation.

6 References

1. Varki, A., *Essentials of glycobiology*. 2nd ed. 2009, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press. xxix, 784 p.
2. Dove, A., *The bittersweet promise of glycobiology.* Nat Biotechnol, 2001. **19**(10): p. 913-7.
3. Fuster, M.M. and J.D. Esko, *The sweet and sour of cancer: glycans as novel therapeutic targets.* Nat Rev Cancer, 2005. **5**(7): p. 526-42.
4. Gagneux, P. and A. Varki, *Evolutionary considerations in relating oligosaccharide diversity to biological function.* Glycobiology, 1999. **9**(8): p. 747-55.
5. Varki, A., *Biological roles of oligosaccharides: all of the theories are correct.* Glycobiology, 1993. **3**(2): p. 97-130.
6. Varki, A., *Nothing in glycobiology makes sense, except in the light of evolution.* Cell, 2006. **126**(5): p. 841-5.
7. Hansen, J.E., et al., *O-GLYCBASE version 2.0: a revised database of O-glycosylated proteins.* Nucleic Acids Res, 1997. **25**(1): p. 278-82.
8. Elhammer, A.P., et al., *The specificity of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase as inferred from a database of in vivo substrates and from the in vitro glycosylation of proteins and peptides.* J Biol Chem, 1993. **268**(14): p. 10029-38.
9. Hansen, J.E., et al., *Prediction of O-glycosylation of mammalian proteins: specificity patterns of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase.* Biochem J, 1995. **308 (Pt 3)**: p. 801-13.
10. Wopereis, S., et al., *Mechanisms in protein O-glycan biosynthesis and clinical and molecular aspects of protein O-glycan biosynthesis defects: a review.* Clin Chem, 2006. **52**(4): p. 574-600.
11. Spiro, R.G., *Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds.* Glycobiology, 2002. **12**(4): p. 43R-56R.
12. Hang, H.C. and C.R. Bertozzi, *The chemistry and biology of mucin-type O-linked glycosylation.* Bioorg Med Chem, 2005. **13**(17): p. 5021-34.
13. Ten Hagen, K.G., et al., *Functional characterization and expression analysis of members of the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase family from Drosophila melanogaster.* J Biol Chem, 2003. **278**(37): p. 35039-48.

14. Van den Steen, P., et al., *Concepts and principles of O-linked glycosylation.* Crit Rev Biochem Mol Biol, 1998. **33**(3): p. 151-208.

15. Tian, E. and K.G. Ten Hagen, *Recent insights into the biological roles of mucin-type O-glycosylation.* Glycoconj J, 2009. **26**(3): p. 325-34.

16. Schwientek, T., et al., *Functional conservation of subfamilies of putative UDP-N-acetylgalactosamine:polypeptide N-acetylgalactosaminyltransferases in Drosophila, Caenorhabditis elegans, and mammals. One subfamily composed of l(2)35Aa is essential in Drosophila.* J Biol Chem, 2002. **277**(25): p. 22623-38.

17. Tian, E. and K.G. Ten Hagen, *Expression of the UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase family is spatially and temporally regulated during Drosophila development.* Glycobiology, 2006. **16**(2): p. 83-95.

18. Hagen, F.K. and K. Nehrke, *cDNA cloning and expression of a family of UDP-N-acetyl-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase sequence homologs from Caenorhabditis elegans.* J Biol Chem, 1998. **273**(14): p. 8268-77.

19. Bennett, E.P., et al., *A novel human UDP-N-acetyl-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase, GalNAc-T7, with specificity for partial GalNAc-glycosylated acceptor substrates.* FEBS Lett, 1999. **460**(2): p. 226-30.

20. Ten Hagen, K.G., et al., *Cloning and characterization of a ninth member of the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase family, ppGaNTase-T9.* J Biol Chem, 2001. **276**(20): p. 17395-404.

21. Ten Hagen, K.G., et al., *Characterization of a UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase that displays glycopeptide N-acetylgalactosaminyltransferase activity.* J Biol Chem, 1999. **274**(39): p. 27867-74.

22. Tetaert, D., et al., *Glycopeptide N-acetylgalactosaminyltransferase specificities for O-glycosylated sites on MUC5AC mucin motif peptides.* Biochem J, 2001. **357**(Pt 1): p. 313-20.

23. Fukuda, M., *Roles of mucin-type O-glycans synthesized by core2beta1,6-N-acetylglucosaminyltransferase.* Methods Enzymol, 2006. **416**: p. 332-46.

24. Kozarsky, K., D. Kingsley, and M. Krieger, *Use of a mutant cell line to study the kinetics and function of O-linked glycosylation of low density lipoprotein receptors.* Proc Natl Acad Sci U S A, 1988. **85**(12): p. 4335-9.

25. Oh-eda, M., et al., *O-linked sugar chain of human granulocyte colony-stimulating factor protects it against polymerization and denaturation allowing it to retain its biological activity.* J Biol Chem, 1990. **265**(20): p. 11432-5.

26. Neustroev, K.N., et al., *Effect of modification of carbohydrate component on properties of glucoamylase.* FEBS Lett, 1993. **316**(2): p. 157-60.

27. Carraway, K.L. and S.R. Hull, *Cell surface mucin-type glycoproteins and mucin-like domains.* Glycobiology, 1991. **1**(2): p. 131-8.

28. Strous, G.J. and J. Dekker, *Mucin-type glycoproteins.* Crit Rev Biochem Mol Biol, 1992. **27**(1-2): p. 57-92.

29. Jentoft, N., *Why are proteins O-glycosylated?* Trends Biochem Sci, 1990. **15**(8): p. 291-4.

30. Shogren, R., T.A. Gerken, and N. Jentoft, *Role of glycosylation on the conformation and chain dimensions of O-linked glycoproteins: light-scattering studies of ovine submaxillary mucin.* Biochemistry, 1989. **28**(13): p. 5525-36.

31. Corfield, A.P., et al., *Ocular mucins: Purification, metabolism and functions.* Progress in Retinal and Eye Research, 1997. **16**(4): p. 627-656.

32. Guzman-Aranguez, A. and P. Argueso, *Structure and biological roles of mucin-type O-glycans at the ocular surface.* Ocul Surf, 2010. **8**(1): p. 8-17.

33. Zhang, Q., et al., *Novel genes dramatically alter regulatory network topology in amphioxus.* Genome Biol, 2008. **9**(8): p. R123.

34. Guerardel, Y., et al., *The nematode Caenorhabditis elegans synthesizes unusual O-linked glycans: identification of glucose-substituted mucin-type O-glycans and short chondroitin-like oligosaccharides.* Biochem J, 2001. **357**(Pt 1): p. 167-82.

35. Previato, J.O., et al., *O-glycosidically linked N-acetylglucosamine-bound oligosaccharides from glycoproteins of Trypanosoma cruzi.* Biochem J, 1994. **301 (Pt 1)**: p. 151-9.

36. Todeschini, A.R., et al., *Alpha-N-acetylglucosamine-linked O-glycans of sialoglycoproteins (Tc-mucins) from Trypanosoma cruzi Colombiana strain.* Mem Inst Oswaldo Cruz, 2009. **104 Suppl 1**: p. 270-4.

37. Dieckmann-Schuppert, A., E. Bause, and R.T. Schwarz, *Studies on O-glycans of Plasmodium-falciparum-infected human erythrocytes. Evidence for O-GlcNAc and O-GlcNAc-transferase in malaria parasites.* Eur J Biochem, 1993. **216**(3): p. 779-88.

38. Haltiwanger, R.S., et al., *Glycosylation of nuclear and cytoplasmic proteins is ubiquitous and dynamic.* Biochem Soc Trans, 1992. **20**(2): p. 264-9.

39. Nyame, K., R.D. Cummings, and R.T. Damian, *Schistosoma mansoni synthesizes glycoproteins containing terminal O-linked N-acetylglucosamine residues.* J Biol Chem, 1987. **262**(17): p. 7990-5.

40. Khoo, K.H., et al., *Characterization of nematode glycoproteins: the major O-glycans of Toxocara excretory-secretory antigens are O-methylated trisaccharides.* Glycobiology, 1991. **1**(2): p. 163-71.

41. North, S.J., et al., *Glycomic studies of Drosophila melanogaster embryos.* Glycoconj J, 2006. **23**(5-6): p. 345-54.

42. Schwientek, T., et al., *A serial lectin approach to the mucin-type O-glycoproteome of Drosophila melanogaster S2 cells.* Proteomics, 2007. **7**(18): p. 3264-77.

43. ten Hagen, K.G., et al., *Glycobiology on the fly: developmental and mechanistic insights from Drosophila.* Glycobiology, 2009. **19**(2): p. 102-11.

44. Garenaux, E., et al., *Structural characterization of complex O-linked glycans from insect-derived material.* Carbohydr Res, 2011. **346**(9): p. 1093-104.

45. Koles, K., et al., *Sialylation in protostomes: a perspective from Drosophila genetics and biochemistry.* Glycoconj J, 2009. **26**(3): p. 313-24.

46. Urai, M., et al., *Structural analysis of O-glycans of mucin from jellyfish (Aurelia aurita) containing 2-aminoethylphosphonate.* Carbohydr Res, 2009. **344**(16): p. 2182-7.

47. Guerardel, Y., et al., *Glycomic survey mapping of zebrafish identifies unique sialylation pattern.* Glycobiology, 2006. **16**(3): p. 244-57.

48. Guerardel, Y., et al., *O-glycan variability of egg-jelly mucins from Xenopus laevis: characterization of four phenotypes that differ by the terminal glycosylation of their mucins.* Biochem J, 2000. **352 Pt 2**: p. 449-63.

49. Li, B., et al., *Structure determination by MALDI-IRMPD mass spectrometry and exoglycosidase digestions of O-linked oligosaccharides from Xenopus borealis egg jelly.* Glycobiology, 2011. **21**(7): p. 877-94.

50. Gutternigg, M., et al., *Neutral N-glycans of the gastropod Arion lusitanicus.* Eur J Biochem, 2004. **271**(7): p. 1348-56.

51. Gutternigg, M., et al., *Neutral N-glycan patterns of the gastropods Limax maximus, Cepaea hortensis, Planorbarius corneus, Arianta arbustorum and Achatina fulica.* Glycoconj J, 2007. **24**(8): p. 475-89.

52. Staudacher, E., H. Stepan, and M. Gutternigg, *Protein N-Glycosylation of Gastropods.* Curr Top Biochem Res, 2009. **11**(2): p. 29-39.

53. Dolashka-Angelova, P., et al., *Characterization of the carbohydrate moieties of the functional unit RvH1-a of Rapana venosa haemocyanin using HPLC/electrospray ionization MS and glycosidase digestion.* Biochem J, 2003. **374**(Pt 1): p. 185-92.

54. Hall, R.L., et al., *3-O-methyl sugars as constituents of glycoproteins. Identification of 3-O-methylgalactose and 3-O-methylmannose in pulmonate gastropod haemocyanins.* Biochem J, 1977. **165**(1): p. 173-6.

55. Hori, T., et al., *Isolation and characterization of a 4-O-methylglucuronic acid-containing glycosphingolipid from spermatozoa of a fresh water bivalve, Hyriopsis schlegelii.* J Biol Chem, 1983. **258**(4): p. 2239-45.

56. Lehr, T., et al., *Structural characterization of N-glycans from the freshwater snail Biomphalaria glabrata cross-reacting with Schistosoma mansoni glycoconjugates.* Glycobiology, 2007. **17**(1): p. 82-103.

57. Marxen, J.C., et al., *The major soluble 19.6 kDa protein of the organic shell matrix of the freshwater snail Biomphalaria glabrata is an N-glycosylated dermatopontin.* Biochim Biophys Acta, 2003. **1650**(1-2): p. 92-8.

58. Puanglarp, N., et al., *Structure of the N-linked oligosaccharides from tridacnin, a lectin found in the haemolymph of the giant clam Hippopus hippopus.* Eur J Biochem, 1995. **232**(3): p. 873-80.

59. Stoeva, S., et al., *Carbohydrate content and monosaccharide composition of Rapana thomasiana grosse (Gastropoda) hemocyanin and its structural subunits. Comparison with gastropodan hemocyanins.* Comp Biochem Physiol B Biochem Mol Biol, 1995. **110**(4): p. 761-5.

60. Stepan, H., et al., *Determination of 3-O- and 4-O-methylated monosaccharide constituents in snail glycans.* Carbohydr Res, 2010. **345**(10): p. 1504-7.

61. Humphries, J.E. and T.P. Yoshino, *Schistosoma mansoni excretory-secretory products stimulate a p38 signalling pathway in Biomphalaria glabrata embryonic cells.* Int J Parasitol, 2006. **36**(1): p. 37-46.

62. Theron, A. and C. Coustau, *Are Biomphalaria snails resistant to Schistosoma mansoni?* J Helminthol, 2005. **79**(3): p. 187-91.

63. Steinmann, P., et al., *Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk.* Lancet Infect Dis, 2006. **6**(7): p. 411-25.

64. Cummings, R.D. and A.K. Nyame, *Schistosome glysoconjugates.* Biochim Biophys Acta, 1999. **1455**(2-3): p. 363-74.

65. Nyame, K., et al., *Complex-type asparagine-linked oligosaccharides in glycoproteins synthesized by Schistosoma mansoni adult males contain terminal beta-linked N-acetylgalactosamine.* J Biol Chem, 1989. **264**(6): p. 3235-43.

66. Srivatsan, J., D.F. Smith, and R.D. Cummings, *The human blood fluke Schistosoma mansoni synthesizes glycoproteins containing the Lewis X antigen.* J Biol Chem, 1992. **267**(28): p. 20196-203.

67. Srivatsan, J., D.F. Smith, and R.D. Cummings, *Schistosoma mansoni synthesizes novel biantennary Asn-linked oligosaccharides containing terminal beta-linked N-acetylgalactosamine.* Glycobiology, 1992. **2**(5): p. 445-52.

68. Nyame, K., R.D. Cummings, and R.T. Damian, *Characterization of the N- and O-linked oligosaccharides in glycoproteins synthesized by Schistosoma mansoni schistosomula.* J Parasitol, 1988. **74**(4): p. 562-72.

69. Geyer, H., et al., *Identification and characterization of keyhole limpet hemocyanin N-glycans mediating cross-reactivity with Schistosoma mansoni.* J Biol Chem, 2005. **280**(49): p. 40731-48.

70. Lehr, T., et al., *N-Glycosylation patterns of hemolymph glycoproteins from Biomphalaria glabrata strains expressing different susceptibility to Schistosoma mansoni infection.* Exp Parasitol, 2010. **126**(4): p. 592-602.

71. Alves-Brito, C.F., et al., *Analysis of anti-keyhole limpet haemocyanin antibody in Brazilians supports its use for the diagnosis of acute schistosomiasis mansoni.* Trans R Soc Trop Med Hyg, 1992. **86**(1): p. 53-6.

72. Mansour, M.M., et al., *Serological differentiation of acute and chronic schistosomiasis mansoni by antibody responses to keyhole limpet hemocyanin.* Am J Trop Med Hyg, 1989. **41**(3): p. 338-44.

73. Marino, K., et al., *A systematic approach to protein glycosylation analysis: a path through the maze.* Nat Chem Biol, 2010. **6**(10): p. 713-23.

74. Geyer, H. and R. Geyer, *Strategies for analysis of glycoprotein glycosylation.* Biochim Biophys Acta, 2006. **1764**(12): p. 1853-69.

75. Mechref, Y. and M.V. Novotny, *Structural investigations of glycoconjugates at high sensitivity.* Chem Rev, 2002. **102**(2): p. 321-69.

76. Jensen, P.H., D. Kolarich, and N.H. Packer, *Mucin-type O-glycosylation--putting the pieces together.* Febs J, 2010. **277**(1): p. 81-94.

77. Cummings, R.D., *Use of lectins in analysis of glycoconjugates.* Methods Enzymol, 1994. **230**: p. 66-86.

78. Bundy, J.L. and C. Fenselau, *Lectin and carbohydrate affinity capture surfaces for mass spectrometric analysis of microorganisms.* Anal Chem, 2001. **73**(4): p. 751-7.

79. Sharon, N. and H. Lis, *Lectins as cell recognition molecules.* Science, 1989. **246**(4927): p. 227-34.

80. Yang, Z. and W.S. Hancock, *Approach to the comprehensive analysis of glycoproteins isolated from human serum using a multi-lectin affinity column.* J Chromatogr A, 2004. **1053**(1-2): p. 79-88.

81. Huang, M., J. Plocek, and M.V. Novotny, *Hydrolytically stable cellulose-derivative coatings for capillary electrophoresis of peptides, proteins and glycoconjugates.* Electrophoresis, 1995. **16**(3): p. 396-401.

82. Kakehi, K., et al., *Capillary electrophoresis of sialic acid-containing glycoprotein. Effect of the heterogeneity of carbohydrate chains on glycoform separation using an alpha1-acid glycoprotein as a model.* Anal Chem, 2001. **73**(11): p. 2640-7.

83. Kinoshita, M., et al., *Comparative studies on the analysis of glycosylation heterogeneity of sialic acid-containing glycoproteins using capillary electrophoresis.* J Chromatogr A, 2000. **866**(2): p. 261-71.

84. Sottani, C., M. Fiorentino, and C. Minoia, *Matrix performance in matrix-assisted laser desorption/ionization for molecular weight determination in sialyl and non-sialyl oligosaccharide proteins.* Rapid Commun Mass Spectrom, 1997. **11**(8): p. 907-13.

85. Bonfichi, R., et al., *Preliminary investigation of glycosylated proteins by capillary electrophoresis and capillary electrophoresis/mass spectrometry using electrospray ionization and by matrix-assisted laser desorption ionization/time-to-flight mass spectrometry.* Rapid Commun Mass Spectrom, 1995. **Spec No**: p. S95-106.

86. Nagy, K., et al., *Electrospray ionization fourier transform ion cyclotron resonance mass spectrometry of human alpha-1-acid glycoprotein.* Anal Chem, 2004. **76**(17): p. 4998-5005.

87. Faid, V., et al., *Site-specific glycosylation analysis of the bovine lysosomal alpha-mannosidase.* Glycobiology, 2006. **16**(5): p. 440-61.

88. Reinders, J., et al., *Challenges in mass spectrometry-based proteomics.* Proteomics, 2004. **4**(12): p. 3686-703.

89. Wuhrer, M., et al., *Protein glycosylation analyzed by normal-phase nano-liquid chromatography--mass spectrometry of glycopeptides.* Anal Chem, 2005. **77**(3): p. 886-94.

90. Annesley, T.M., *Ion suppression in mass spectrometry.* Clin Chem, 2003. **49**(7): p. 1041-4.

91. Alvarez-Manilla, G., et al., *Tools for glycoproteomic analysis: size exclusion chromatography facilitates identification of tryptic glycopeptides with N-linked glycosylation sites.* J Proteome Res, 2006. **5**(3): p. 701-8.

92. Tajiri, M., S. Yoshida, and Y. Wada, *Differential analysis of site-specific glycans on plasma and cellular fibronectins: application of a hydrophilic affinity method for glycopeptide enrichment.* Glycobiology, 2005. **15**(12): p. 1332-40.

93. Wada, Y., M. Tajiri, and S. Yoshida, *Hydrophilic affinity isolation and MALDI multiple-stage tandem mass spectrometry of glycopeptides for glycoproteomics.* Anal Chem, 2004. **76**(22): p. 6560-5.

94. Xiong, L. and F.E. Regnier, *Use of a lectin affinity selector in the search for unusual glycosylation in proteomics.* J Chromatogr B Analyt Technol Biomed Life Sci, 2002. **782**(1-2): p. 405-18.

95. Geng, M., et al., *Proteomics of glycoproteins based on affinity selection of glycopeptides from tryptic digests.* J Chromatogr B Biomed Sci Appl, 2001. **752**(2): p. 293-306.

96. Wang, L., et al., *Concanavalin A-captured glycoproteins in healthy human urine.* Mol Cell Proteomics, 2006. **5**(3): p. 560-2.

97. Hirabayashi, J., *Lectin-based structural glycomics: glycoproteomics and glycan profiling.* Glycoconj J, 2004. **21**(1-2): p. 35-40.

98. Yang, Z. and W.S. Hancock, *Monitoring glycosylation pattern changes of glycoproteins using multi-lectin affinity chromatography.* J Chromatogr A, 2005. **1070**(1-2): p. 57-64.

99. Ohta, M., et al., *Selective glycopeptide mapping of erythropoietin by on-line high-performance liquid chromatography--electrospray ionization mass spectrometry.* J Chromatogr A, 2001. **910**(1): p. 1-11.

100. Zhang, B., et al., *Rapid determination of the binding affinity and specificity of the mushroom Polyporus squamosus lectin using frontal affinity chromatography coupled to electrospray mass spectrometry.* Glycobiology, 2001. **11**(2): p. 141-7.

101. Huddleston, M.J., M.F. Bean, and S.A. Carr, *Collisional fragmentation of glycopeptides by electrospray ionization LC/MS and LC/MS/MS: methods for selective detection of glycopeptides in protein digests.* Anal Chem, 1993. **65**(7): p. 877-84.

102. Wuhrer, M., et al., *Glycoproteomics based on tandem mass spectrometry of glycopeptides.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **849**(1-2): p. 115-28.

103. Wuhrer, M., C.H. Hokke, and A.M. Deelder, *Glycopeptide analysis by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry reveals novel features of horseradish peroxidase glycosylation.* Rapid Commun Mass Spectrom, 2004. **18**(15): p. 1741-8.

104. Tretter, V., F. Altmann, and L. Marz, *Peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase F cannot release glycans with fucose attached alpha 1----3 to the asparagine-linked N-acetylglucosamine residue.* Eur J Biochem, 1991. **199**(3): p. 647-52.

105. O'Neill, R.A., *Enzymatic release of oligosaccharides from glycoproteins for chromatographic and electrophoretic analysis.* J Chromatogr A, 1996. **720**(1-2): p. 201-15.

106. Yosizawa, Z., T. Sato, and K. Schmid, *Hydrazinolysis of alpha-1-acid glycoprotein.* Biochim Biophys Acta, 1966. **121**(2): p. 417-20.

107. Kuraya, N. and S. Hase, *Release of O-linked sugar chains from glycoproteins with anhydrous hydrazine and pyridylamination of the sugar chains with improved reaction conditions.* J Biochem, 1992. **112**(1): p. 122-6.

108. Mizuochi, T., *Microscale sequencing of N-linked oligosaccharides of glycoproteins using hydrazinolysis, Bio-Gel P-4, and sequential exoglycosidase digestion.* Methods Mol Biol, 1993. **14**: p. 55-68.

109. Takasaki, S., T. Mizuochi, and A. Kobata, *Hydrazinolysis of asparagine-linked sugar chains to produce free oligosaccharides.* Methods Enzymol, 1982. **83**: p. 263-8.

110. Anumula, K.R., *Unique anthranilic acid chemistry facilitates profiling and characterization of Ser/Thr-linked sugar chains following hydrazinolysis.* Anal Biochem, 2008. **373**(1): p. 104-11.

111. Patel, T., et al., *Use of hydrazine to release in intact and unreduced form both N- and O-linked oligosaccharides from glycoproteins.* Biochemistry, 1993. **32**(2): p. 679-93.

112. Merry, A.H., et al., *Recovery of intact 2-aminobenzamide-labeled O-glycans released from glycoproteins by hydrazinolysis.* Anal Biochem, 2002. **304**(1): p. 91-9.

113. Carlson, D.M., *Structures and immunochemical properties of oligosaccharides isolated from pig submaxillary mucins.* J Biol Chem, 1968. **243**(3): p. 616-26.

114. Iyer, R.N. and D.M. Carlson, *Alkaline borohydride degradation of blood group H substance.* Arch Biochem Biophys, 1971. **142**(1): p. 101-5.

115. Spiro, R.G. and V.D. Bhoyroo, *Structure of the O-glycosidically linked carbohydrate units of fetuin.* J Biol Chem, 1974. **249**(18): p. 5704-17.

116. Debray, H., G. Strecker, and J. Montreuil, *Effect of alkalis on N-glycosidic linkages of glycoproteins.* Biochem Soc Trans, 1984. **12**(4): p. 611-2.

117. Pabst, M., et al., *Comparison of fluorescent labels for oligosaccharides and introduction of a new postlabeling purification method.* Anal Biochem, 2009. **384**(2): p. 263-73.

118. Chai, W., et al., *Nonreductive release of O-linked oligosaccharides from mucin glycoproteins for structure/function assignments as neoglycolipids: application in the detection of novel ligands for E-selectin.* Glycobiology, 1997. **7**(6): p. 861-72.

119. Huang, Y., et al., *Matrix-assisted laser desorption/ionization mass spectrometry compatible beta-elimination of O-linked oligosaccharides.* Rapid Commun Mass Spectrom, 2002. **16**(12): p. 1199-204.

120. Wang, C., et al., *One-pot nonreductive O-glycan release and labeling with 1-phenyl-3-methyl-5-pyrazolone followed by ESI-MS analysis.* Proteomics, 2011. **11**(21): p. 4229-42.

121. Zauner, G., et al., *Mass spectrometric O-glycan analysis after combined O-glycan release by beta-elimination and 1-phenyl-3-methyl-5-pyrazolone labeling.* Biochim Biophys Acta, 2011. **[epub ahead of print]**.

122. Anumula, K.R., *Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates.* Anal Biochem, 2006. **350**(1): p. 1-23.

123. Bigge, J.C., et al., *Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid.* Anal Biochem, 1995. **230**(2): p. 229-38.

124. Chiesa, C. and R.A. O'Neill, *Capillary zone electrophoresis of oligosaccharides derivatized with various aminonaphthalene sulfonic acids.* Electrophoresis, 1994. **15**(8-9): p. 1132-40.

125. Guttman, A., F.T. Chen, and R.A. Evangelista, *Separation of 1-aminopyrene-3,6,8-trisulfonate-labeled asparagine-linked fetuin glycans by capillary gel electrophoresis.* Electrophoresis, 1996. **17**(2): p. 412-7.

126. Alpert, A.J., *Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds.* J Chromatogr, 1990. **499**: p. 177-96.

127. Guile, G.R., et al., *A rapid high-resolution high-performance liquid chromatographic method for separating glycan mixtures and analyzing oligosaccharide profiles.* Anal Biochem, 1996. **240**(2): p. 210-26.

128. Blumberg, K., et al., *Fractionation of oligosaccharides containing N-acetyl amino sugars by reverse-phase high-pressure liquid chromatography.* Anal Biochem, 1982. **119**(2): p. 407-12.

129. Cheetham, N.W. and V.E. Dube, *Preparation of lacto-N-neotetraose from human milk by high-performance liquid chromatography.* J Chromatogr, 1983. **262**: p. 426-30.

130. Kuraya, N. and S. Hase, *Analysis of pyridylaminated O-linked sugar chains by two-dimensional sugar mapping.* Anal Biochem, 1996. **233**(2): p. 205-11.

131. Fan, J.Q., et al., *High-performance liquid chromatography of glycopeptides and oligosaccharides on graphitized carbon columns.* Anal Biochem, 1994. **219**(2): p. 224-9.

132. Pabst, M. and F. Altmann, *Influence of electrosorption, solvent, temperature, and ion polarity on the performance of LC-ESI-MS using graphitic carbon for acidic oligosaccharides.* Anal Chem, 2008. **80**(19): p. 7534-42.

133. Packer, N.H., et al., *A general approach to desalting oligosaccharides released from glycoproteins.* Glycoconj J, 1998. **15**(8): p. 737-47.

134. Kawasaki, N., et al., *Analysis of carbohydrate heterogeneity in a glycoprotein using liquid chromatography/mass spectrometry and liquid chromatography with tandem mass spectrometry.* Anal Biochem, 1999. **269**(2): p. 297-303.

135. Lipniunas, P.H., et al., *Separation of biosynthetic oligosaccharide branch isomers using high-performance liquid chromatography on a porous two-dimensional graphite stationary phase.* Anal Biochem, 1996. **243**(2): p. 203-9.

136. Pabst, M., et al., *Mass + retention time = structure: a strategy for the analysis of N-glycans by carbon LC-ESI-MS and its application to fibrin N-glycans.* Anal Chem, 2007. **79**(13): p. 5051-7.

137. Ruhaak, L.R., A.M. Deelder, and M. Wuhrer, *Oligosaccharide analysis by graphitized carbon liquid chromatography-mass spectrometry.* Anal Bioanal Chem, 2009. **394**(1): p. 163-74.

138. Simpson, R.C., et al., *Adaptation of a thermospray liquid chromatography/mass spectrometry interface for use with alkaline anion exchange liquid chromatography of carbohydrates.* Anal Chem, 1990. **62**(3): p. 248-52.

139. Cummings, R.D. and S. Kornfeld, *Fractionation of asparagine-linked oligosaccharides by serial lectin-Agarose affinity chromatography. A rapid, sensitive, and specific technique.* J Biol Chem, 1982. **257**(19): p. 11235-40.

140. Bunkenborg, J., et al., *Screening for N-glycosylated proteins by liquid chromatography mass spectrometry.* Proteomics, 2004. **4**(2): p. 454-65.

141. Wang, Y., S.L. Wu, and W.S. Hancock, *Approaches to the study of N-linked glycoproteins in human plasma using lectin affinity chromatography and nano-HPLC coupled to electrospray linear ion trap--Fourier transform mass spectrometry.* Glycobiology, 2006. **16**(6): p. 514-23.

142. Geyer, H., et al., *Structural analysis of glycoconjugates by on-target enzymatic digestion and MALDI-TOF-MS.* Anal Chem, 1999. **71**(2): p. 476-82.

143. Edge, C.J., et al., *Fast sequencing of oligosaccharides: the reagent-array analysis method.* Proc Natl Acad Sci U S A, 1992. **89**(14): p. 6338-42.

144. Jackson, P., *The use of polyacrylamide-gel electrophoresis for the high-resolution separation of reducing saccharides labelled with the fluorophore 8-aminonaphthalene-1,3,6-trisulphonic acid. Detection of picomolar quantities by an imaging system based on a cooled charge-coupled device.* Biochem J, 1990. **270**(3): p. 705-13.

145. Jackson, P., *High-resolution polyacrylamide gel electrophoresis of fluorophore-labeled reducing saccharides.* Methods Enzymol, 1994. **230**: p. 250-65.

146. Jackson, P., *Polyacrylamide gel electrophoresis of reducing saccharides labeled with the fluorophore 2-aminoacridone: subpicomolar detection using an imaging system based on a cooled charge-coupled device.* Anal Biochem, 1991. **196**(2): p. 238-44.

147. Jackson, P., *Fluorophore-assisted carbohydrate electrophoresis: a new technology for the analysis of glycans.* Biochem Soc Trans, 1993. **21**(1): p. 121-5.

148. Klausen, N.K. and T. Kornfelt, *Analysis of the glycoforms of human recombinant factor VIIa by capillary electrophoresis and high-performance liquid chromatography.* J Chromatogr A, 1995. **718**(1): p. 195-202.

149. Yim, K., J. Abrams, and A. Hsu, *Capillary zone electrophoretic resolution of recombinant human bone morphogenetic protein 2 glycoforms. An investigation into the separation mechanisms for an exquisite separation.* J Chromatogr A, 1995. **716**(1-2): p. 401-12.

150. Knuver-Hopf, J. and H. Mohr, *Differences between natural and recombinant interleukin-2 revealed by gel electrophoresis and capillary electrophoresis.* J Chromatogr A, 1995. **717**(1-2): p. 71-4.

151. Jorgenson, J.W., *Electrophoresis.* Anal Chem, 1986. **58**(7): p. 743A-760A.

152. Gennaro, L.A., et al., *Capillary electrophoresis/electrospray ion trap mass spectrometry for the analysis of negatively charged derivatized and underivatized glycans.* Rapid Commun Mass Spectrom, 2002. **16**(3): p. 192-200.

153. Hermentin, P., et al., *A strategy for the mapping of N-glycans by high-performance capillary electrophoresis.* Anal Biochem, 1994. **221**(1): p. 29-41.

154. Hillenkamp, F., et al., *Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers.* Anal Chem, 1991. **63**(24): p. 1193A-1203A.

155. Tsarbopoulos, A., et al., *Comparative mapping of recombinant proteins and glycoproteins by plasma desorption and matrix-assisted laser desorption/ionization mass spectrometry.* Anal Chem, 1994. **66**(13): p. 2062-70.

156. Gusev, A.I., et al., *Direct quantitative analysis of peptides using matrix assisted laser desorption ionization.* Anal Bioanal Chem, 1996. **354**(4): p. 455-63.

157. Pfenninger, A., et al., *Matrix optimization for matrix-assisted laser desorption/ionization mass spectrometry of oligosaccharides from human milk.* J Mass Spectrom, 1999. **34**(2): p. 98-104.

158. Mock, K.K., C.W. Sutton, and J.S. Cottrell, *Sample immobilization protocols for matrix-assisted laser-desorption mass spectrometry.* Rapid Commun Mass Spectrom, 1992. **6**(4): p. 233-8.

159. Stadlmann, J., et al., *Analysis of immunoglobulin glycosylation by LC-ESI-MS of glycopeptides and oligosaccharides.* Proteomics, 2008. **8**(14): p. 2858-71.

160. Wuhrer, M., et al., *Normal-phase nanoscale liquid chromatography-mass spectrometry of underivatized oligosaccharides at low-femtomole sensitivity.* Anal Chem, 2004. **76**(3): p. 833-8.

161. Geyer, R. and H. Geyer, *Saccharide linkage analysis using methylation and other techniques.* Methods Enzymol, 1994. **230**: p. 86-108.

162. Levery, S.B. and S. Hakomori, *Microscale methylation analysis of glycolipids using capillary gas chromatography-chemical ionization mass fragmentography with selected ion monitoring.* Methods Enzymol, 1987. **138**: p. 13-25.

163. Duus, J., C.H. Gotfredsen, and K. Bock, *Carbohydrate structural determination by NMR spectroscopy: modern methods and limitations.* Chem Rev, 2000. **100**(12): p. 4589-614.

164. Mechref, Y., P. Kang, and M.V. Novotny, *Solid-phase permethylation for glycomic analysis.* Methods in molecular biology (Clifton, N.J.), 2009. **534**: p. 53-64.

165. Peyer, C., P. Bonay, and E. Staudacher, *Purification and characterization of a beta-xylosidase from potatoes (Solanum tuberosum).* Biochim Biophys Acta, 2004. **1672**(1): p. 27-35.

166. Pabst, M., et al., *Nucleotide and nucleotide sugar analysis by liquid chromatography-electrospray ionization-mass spectrometry on surface-conditioned porous graphitic carbon.* Anal Chem, 2010. **82**(23): p. 9782-8.

167. Huang, Y., Y. Mechref, and M.V. Novotny, *Microscale nonreductive release of O-linked glycans for subsequent analysis through MALDI mass spectrometry and capillary electrophoresis.* Anal Chem, 2001. **73**(24): p. 6063-9.

168. Ansorge, W., *Fast and sensitive detection of protein and DNA bands by treatment with potassium permanganate.* J Biochem Biophys Methods, 1985. **11**(1): p. 13-20.

169. Anumula, K.R., *High-sensitivity and high-resolution methods for glycoprotein analysis.* Anal Biochem, 2000. **283**(1): p. 17-26.

170. Anumula, K.R., *Quantitative determination of monosaccharides in glycoproteins by high-performance liquid chromatography with highly sensitive fluorescence detection.* Anal Biochem, 1994. **220**(2): p. 275-83.

171. Honda, S., et al., *High-performance liquid chromatography of reducing carbohydrates as strongly ultraviolet-absorbing and electrochemically sensitive 1-phenyl-3-methyl-5-pyrazolone derivatives.* Anal Biochem, 1989. **180**(2): p. 351-7.

172. Fu, D. and R.A. O'Neill, *Monosaccharide composition analysis of oligosaccharides and glycoproteins by high-performance liquid chromatography.* Anal Biochem, 1995. **227**(2): p. 377-84.

173. Hakomori, S., *A Rapid Permethylation of Glycolipid, and Polysaccharide Catalyzed by Methylsulfinyl Carbanion in Dimethyl Sulfoxide.* J Biochem, 1964. **55**: p. 205-8.

174. Kolarich, D. and F. Altmann, *N-Glycan analysis by matrix-assisted laser desorption/ionization mass spectrometry of electrophoretically separated nonmammalian proteins: application to peanut allergen Ara h 1 and olive pollen allergen Ole e 1.* Anal Biochem, 2000. **285**(1): p. 64-75.

175. Kuster, B., et al., *Structural determination of N-linked carbohydrates by matrix-assisted laser desorption/ionization-mass spectrometry following enzymatic release within sodium dodecyl sulphate-polyacrylamide electrophoresis gels: application to species-specific glycosylation of alpha1-acid glycoprotein.* Electrophoresis, 1998. **19**(11): p. 1950-9.

176. Rendic, D., et al., *Adaptation of the "in-gel release method" to N-glycome analysis of low-milligram amounts of material.* Electrophoresis, 2007. **28**(23): p. 4484-4492.

177. Grass, J., et al., *Discovery and structural characterization of fucosylated oligomannosidic N-glycans in mushrooms.* J Biol Chem, 2011. **286**(8): p. 5977-84.

178. Toegel, S., et al., *Phenotype-related differential alpha-2,6- or alpha-2,3-sialylation of glycoprotein N-glycans in human chondrocytes.* Osteoarthritis Cartilage, 2010. **18**(2): p. 240-8.

179. Stepan, H. and E. Staudacher, *Optimization of monosaccharide determination using anthranilic acid and 1-phenyl-3-methyl-5-pyrazolone for gastropod analysis.* Anal Biochem, 2011. **418**(1): p. 24-9.

180. Montreuil, J., J.F.G. Vliegenthart, and H. Schachter, *Glycoproteins II*. 1997, Amsterdam ; New York: Elsevier. xxvi, 652 p.

7 List of figures

8 List of tables

9 Appendix

Carbohydrate Research 345 (2010) 1504–1507

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Note

Determination of 3-O- and 4-O-methylated monosaccharide constituents in snail glycans

Herwig Stepan [a], Christina Bleckmann [b], Hildegard Geyer [b], Rudolf Geyer [b], Erika Staudacher [a,*]

[a] Department of Chemistry, University of Natural Resources and Applied Life Sciences, Vienna, Austria
[b] Institute of Biochemistry, Faculty of Medicine, University of Giessen, Germany

ARTICLE INFO

Article history:
Received 14 January 2010
Received in revised form 12 March 2010
Accepted 19 March 2010
Available online 21 March 2010

Keywords:
Gastropods
Snail
Glycan
Methylation

ABSTRACT

The N- and O-glycans of *Arianta arbustorum*, *Achatina fulica*, *Arion lusitanicus* and *Planorbarius corneus* were analysed for their monosaccharide pattern by reversed-phase HPLC after labelling with 2-aminobenzoic acid or 3-methyl-1-phenyl-2-pyrazolin-5-one and by gas chromatography–mass spectrometry. Glucosamine, galactosamine, mannose, galactose, glucose, fucose and xylose were identified. Furthermore, three different methylated sugars were detected: 3-*O*-methyl-mannose and 3-*O*-methyl-galactose were confirmed to be a common snail feature; 4-*O*-methyl-galactose was detected for the first time in snails.

The more non-mammalian glycan structures are investigated, the more new and unexpected structural features are found. One of these features is the methylation of sugar residues. Methylation is a frequent modification of some amino acids and especially of DNA molecules. Linked to sugar residues it is quite a rare modification. So far it has been found in some cases in bacteria (*Bacillus anthracis*,[1] *Pseudomonas syringae pv. Phaseolicola*,[2]) algae[3–5] and plants (*Salvia officinalis L.*[6]) mostly within the sugar chain, and in nematodes (*Caenorhabditis elegans*,[7] *Toxocara canis*[8]) and mollusc glycans the methylation is present mainly in terminal position of the sugar chains. Methylation in gastropod glycans (3-*O*-methyl-mannose, 3-*O*-methyl-galactose) was first reported by Hall and Wood 1977 in *Helix pomatia*.[9] Later findings in other snails (*Rapana thomasiana* and *Rapana venosa*,[10,11] *Biomphalaria glabrata*[12,13]), our studies[14,15] and other molluscs (giant clam *Hippopus hippopus*,[16] fresh water bivalve *Hyriopsis schlegelii*[17]) followed. Methylation therefore seems to be a very important and frequent modification of snail glycans.

Our current analysis of four more species (*Arion lusitanicus*, *Arianta arbustorum*, *Achatina fulica* and *Planorbarius corneus*) confirms the occurence of 3-*O*-methyl-mannose and 3-*O*-methyl-galactose in snail glycans. Furthermore, 4-*O*-methyl-galactose was detected for the first time in snails.

Protein preparations of four different snail species (*A. arbustorum*, *A. fulica*, *A. lusitanicus* and *P. corneus*) were isolated, subjected to proteolytic digestion and incubated with PNGase A to release the N-glycan fraction. In further purification steps the N-glycans were separated from the remaining peptides containing the O-linked glycans. The N-glycan pool, as well as the O-glycopeptide pool, was hydrolysed and the monosaccharides were labelled for HPLC analysis or subjected to gas chromatography–mass spectrometry. Comparing them with standard sugars we could easily identify in all of our three analytical systems glucosamine, galactosamine, mannose, galactose, glucose, fucose and xylose. In Figure 1 the GC patterns of *A. arbustorum*, *A. fulica* and *A. lusitanicus* O-glycans are given as examples.

The mass spectra of the peaks labelled with A, B and C in Figure 1 are given in Figure 2. Due to their retention times in gas chromatography and their fragmentation patterns obtained by electron impact mass spectrometry[13], these signals were identified to be 3-*O*-methyl-mannose, 4-*O*-methyl-galactose and 3-*O*-methyl-galactose plus 4-*O*-methyl-galactose, respectively. In agreement with previous studies on snail glycoproteins,[18,19] monosaccharides present are assumed to have D-configuration. According to the literature and our own experiments we had expected the existence of 3-O-methylated mannose and 3-*O*-methyl-galactose. These kinds of modified sugars had been found in snails before. The additional 4-O-methylated galactose is new for snail tissues. It has been detected so far only in the nematode *T. canis*.[8] The GC–MS results were supported by labelling the monosaccharides with 2-aminobenzoic acid and 3-methyl-1-phenyl-2-pyrazolin-5-one, respectively, and subjecting them to HPLC-analysis. Here, the 4-O-methylated galactose was also clearly visible (Figs. 3 and 4).

All four investigated snail species contained 3-O-methylated mannose and mostly 4-O-methylated galactose in their N- and

* Corresponding author. Tel.: +43 1 47654 6063; fax: +43 1 47654 6059.
E-mail address: erika.staudacher@boku.ac.at (E. Staudacher).

0008-6215/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2010.03.027

O-glycans. *A. arbustorum* and *P. corneus* showed in addition 3-O-methylated galactose. For details on distribution see Table 1.

1. Experimental

1.1. Materials

A. arbustorum and *A. lusitanicus* were collected by the authors under the supervision of Dr. Manfred Pintar (Department of Integrative Biology and Biodiversity Research, Institute of Zoology, University of Natural Resources and Applied Life Sciences, Vienna) in areas close to Vienna. *P. corneus* and *A. fulica* were bred in the laboratory at room temperature. All animals were frozen at −80 °C immediately after collection.

1.2. Preparation of proteins

Thawed slugs were washed to remove the extraneous mucous components and were dissected into three fractions; the skin and inner organs (viscera) were pooled separately, while the intestinal

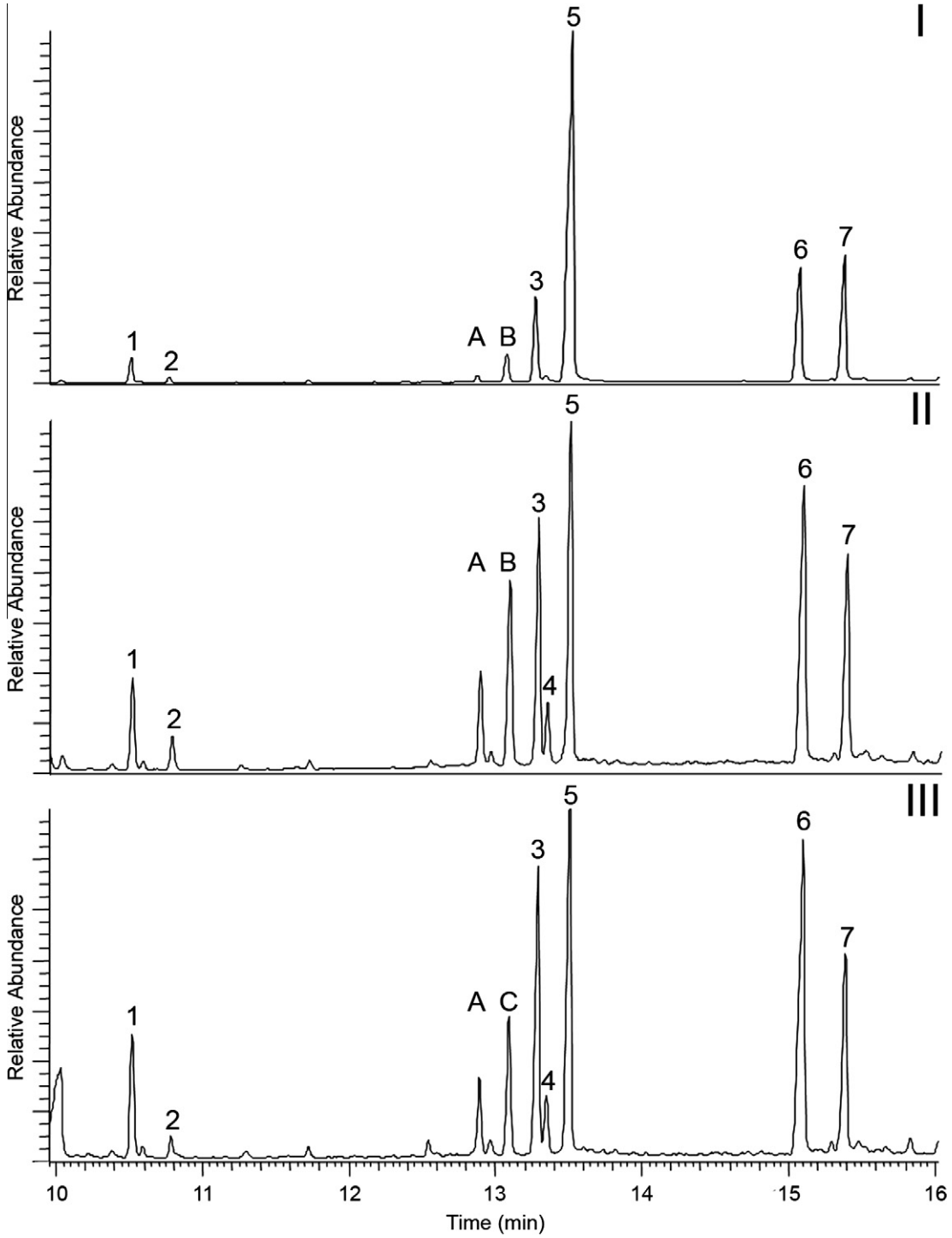


Figure 1. Monosaccharide patterns of *Achatina fulica* viscera and skin (I), *Arion lusitanicus* viscera and skin (II) and *Arianta arbustorum* viscera and skin (III) O-glycans obtained by gas chromatography of the corresponding alditol acetates. Fucose (1), xylose (2), mannose (3), glucose (4), galactose (5), *N*-acetyl-glucosamine (6) and *N*-acetyl-galactosamine (7). Unknown sugars A, B and C are elucidated in Figure 2.

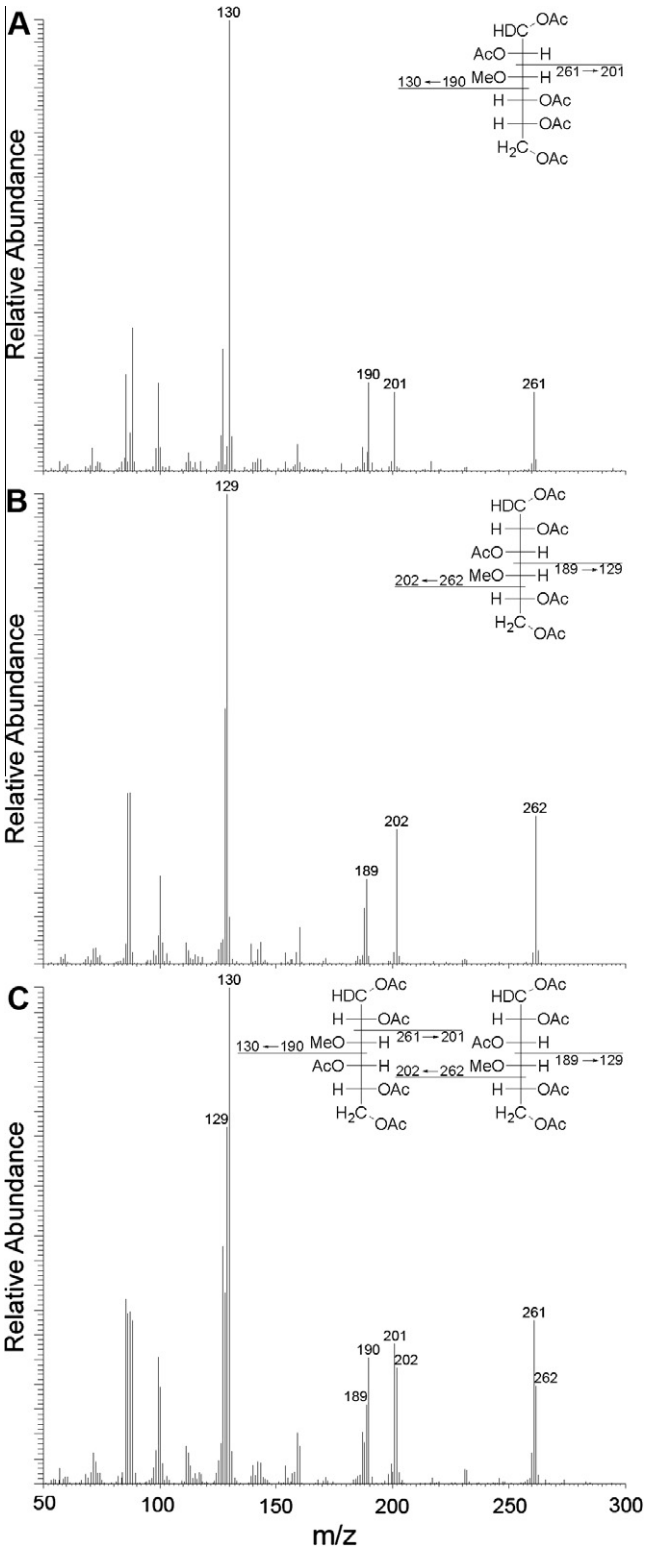


Figure 2. Electron impact mass spectra of unknown sugars (labelled A, B and C in Fig. 1) obtained by GC–MS analysis. Diagnostically relevant primary fragments are indicated in corresponding insets. 3-O-Methylated mannose (A), 4-O-methylated galactose (B), 3-O- and 4-O-methylated galactose (C).

tract was discarded. About 15–30 g of material was homogenised in 0.05 M Tris–HCl buffer, pH 7.5 containing 3 mM dithiothreitol by an IKA Ultra Turrax T25 (IKA Labortechnik, Janke und Kunkel GmbH, Staufen, Germany) at 15,000 rpm for 2×20 s. Then an ammonium sulfate precipitation was performed by increasing the salt concentration to 30%. Samples were centrifuged at 15,000*g* for 30 min and the precipitate was discarded. Ammonium sulfate concentration was then increased to 80% and the samples were centrifuged at 25,000*g* for 30 min. The precipitate was dissolved in approximately 150 mL of 10 mM sodium phosphate buffer, pH 7.0 containing 1.2 M ammonium sulfate and subjected to hydrophobic interaction chromatography using octyl Sepharose CL4B.[20] To remove ammonium sulfate, the samples were dialysed.

1.3. Preparation of N-glycans and O-glycopeptides

Proteolytic digests with pepsin and the release of N-glycans from the glycopeptides by peptide:N-glycanase A were carried out as described in detail by Gutternigg et al.[14] The released N-glycans were separated by cation exchange chromatography (AG 50W x 2, Bio-Rad-Laboratories).

1.4. Monosaccharide analysis

Monosaccharide analysis was carried out by hydrolysis of the glycans with 4 M trifluoroacetic acid at 100 °C followed by (a) labelling with 2-aminobenzoic acid and reversed-phase HPLC with fluorescence detection according to Anumula,[21] (b) labelling with

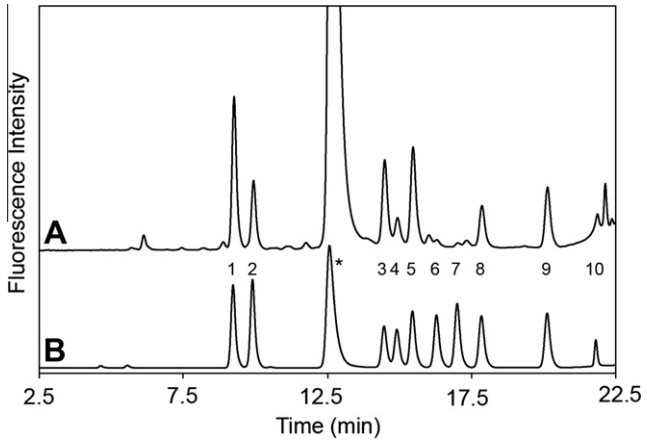


Figure 3. Complete monosaccharide pattern of (A) *Arion lusitanicus* viscera, (B) standard monosaccharides after labelling with 2-aminobenzoic acid and separation by reversed-phase HPLC. Glucosamine (1), galactosamine (2), galactose (3), mannose (4), glucose (5), arabinose (6), xylose (7), fucose (8), 4-O-methylated galactose (9), 2-O-methylated galactose (10) and excess reagent (*).

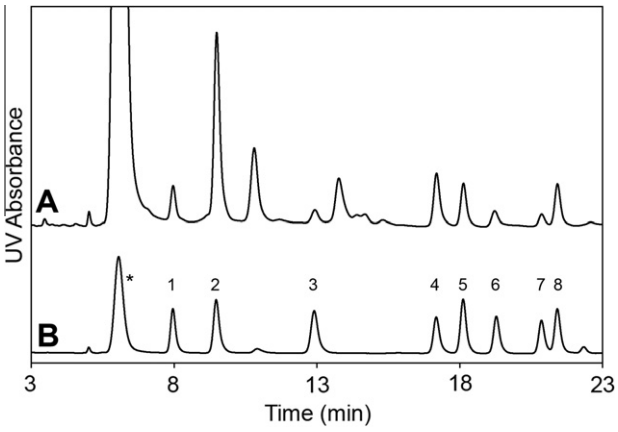


Figure 4. Complete monosaccharide pattern of (A) *Arion lusitanicus* viscera, (B) standard monosaccharides after labelling with 3-methyl-1-phenyl-2-pyrazolin-5-one and separation by reversed-phase HPLC. Mannose (1), glucosamine (2), galactosamine (3), glucose (4), galactose (5), xylose (6), fucose (7), 4-O-methylated galactose (8) and excess reagent (*).

Table 1
Distribution of methylated sugar residues in N- and O-glycans of snails

	3-*O*-Methyl-mannose		3-*O*-Methyl-galactose		4-*O*-Methyl-galactose	
	N-Glycans	O-Glycans	N-Glycans	O-Glycans	N-Glycans	O-Glycans
Achatina fulica	+	+	–	–	+	+
Arianta arbustorum	+	+	+	+	–	+
Arion lusitanicus	+	+	–	–	+	+
Planorbarius corneus	+	+	+	+	+	+

3-methyl-1-phenyl-2-pyrazolin-5-one and reversed-phase HPLC with UV detection at 245 nm,[22] or (c) conversion of the monosaccharides into their corresponding alditol acetates which were analysed by gas chromatography/mass spectrometry. To this end, the monosaccharides were reduced with an excess of sodium borodeuteride to obtain the corresponding alditols. The use of sodium borodeuteride instead of sodium borohydride, which leads to an incorporation of a deuterium atom at the reducing end, enables discrimation between 3-O-methylated and 4-O-methylated sugar isomers. Afterwards acetylation was performed by the addition of acetic anhydride and alditol acetates were analysed by gas chromatography–mass spectrometry using a VF 5 ms capillary column (60 m, 0.25 mm inner diameter, 0.1 mm film thickness; Varian, Darmstadt Germany) and 2.5 mL/min helium as a carrier gas. Temperature was maintained at 40 °C for 1.5 min, raised to 130 °C with 40 °C/min and to 290 °C with 8 °C/min and was finally maintained at 290 °C for 5 min. Monosaccharide derivatives were registered by electron impact (EI) mass spectrometry in the positive ion mode using an PolarisQ instrument (ThermoQuest Analytical Systems).[23]

Acknowledgements

This study was supported by the Austrian Fonds zur Förderung der wissenschaftlichen Forschung, project number P 20393-B11. The authors thank Professor Paul Kosma (Department of Chemistry, University of Natural Resources and Applied Life Sciences, Vienna) for providing 2-O-methylated- and 4-O-methylated galactose and Peter Kaese (Institute of Biochemistry, University of Giessen) for expert technical assistance.

References

1. Fox, A.; Black, G. E.; Fox, K.; Rostovtseva, S. *J. Clin. Microbiol.* **1993**, *31*, 887–894.
2. Zdorovenko, E. L.; Ovod, V. V.; Zatonsky, G. V.; Shashkov, A. S.; Kocharova, N. A.; Knirel, Y. A. *Carbohydr. Res.* **2001**, *330*, 505–510.
3. Chiovitti, A.; Bacic, A.; Craik, D. J.; Kraft, G. T.; Liao, M. L. *Carbohydr. Res.* **2004**, *339*, 459–466.
4. Andriamanantoanina, H.; Chambat, G.; Rinaudo, M. *Carbohydr. Polym.* **2007**, *68*, 77–88.
5. Rodríguez, M. C.; Matulewicz, M. C.; Noseda, M.; Ducatti, D. R.; Leonardi, P. I. *Bioresour. Technol.* **2009**, *100*, 1435–1441.
6. Capek, P. *Carbohydr. Res.* **2008**, *343*, 1390–1393.
7. Guérardel, Y.; Balanzino, L.; Maes, E.; Leroy, Y.; Coddeville, B.; Oriol, R.; Strecker, G. *Biochem. J.* **2001**, *357*, 167–182.
8. Khoo, K. H.; Maizels, R. M.; Page, A. P.; Taylor, G. W.; Rendell, N. B.; Dell, A. *Glycobiology* **1991**, *1*, 163–171.
9. Hall, R. L.; Wood, E. J.; Kamerling, J. P.; Gerwig, G. J.; Vliegenthart, J. F. G. *Biochem. J.* **1977**, *165*, 173–176.
10. Stoeva, S.; Rachev, R.; Severov, S.; Voelter, W.; Genov, N. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* **1995**, *110*, 761–765.
11. Dolashka-Angelova, P.; Beck, A.; Dolashki, A.; Beltramini, M.; Stevanovic, S.; Salvato, B.; Voelter, W. *Biochem. J.* **2003**, *374*, 185–192.
12. Marxen, J. C.; Nimtz, M.; Becker, W.; Mann, K. *Biochim. Biophys. Acta* **2003**, *1650*, 92–98.
13. Lehr, T.; Geyer, H.; Maaß, K.; Doenhoff, M. J.; Geyer, R. *Glycobiology* **2007**, *17*, 82–103.
14. Gutternigg, M.; Ahrer, K.; Grabher-Meier, H.; Bürgmayr, S.; Staudacher, E. *Eur. J. Biochem.* **2004**, *271*, 1348–1356.
15. Gutternigg, M.; Bürgmayr, S.; Pöltl, G.; Rudolf, J.; Staudacher, E. *Glycoconjugate J.* **2007**, *24*, 475–489.
16. Puanglarp, N.; Oxley, D.; Currie, G. J.; Bacic, A.; Craik, D. J.; Yellowlees, D. *Eur. J. Biochem.* **1995**, *232*, 873–880.
17. Hori, T.; Sugita, M.; Ando, S.; Tsukada, K.; Shiota, K.; Tsuzuki, M.; Itasaka, O. *J. Biol. Chem.* **1983**, *258*, 2239–2245.
18. Lommrse, J. P.; Thomas-Oates, J. E.; Gielens, C.; Préaux, G.; Kamerling, J. P.; Vliegenthart, J. F. *Eur. J. Biochem.* **1997**, *249*, 195–222.
19. Kuik, J. A.; Sijbesma, R. P.; Kamerling, J. P.; Vliegenthart, J. F.; Wood, E. J. *Eur. J. Biochem.* **1987**, *169*, 399–411.
20. Peyer, C.; Bonay, P.; Staudacher, E. *Biochem. Biophys. Acta* **2004**, *1672*, 27–35.
21. Anumula, K. R. *Anal. Biochem.* **1994**, *220*, 275–283.
22. Altmann, F.; Lomonossoff, G. P. *J. Gen. Virol.* **2000**, *81*, 1111–1114.
23. Geyer, R.; Geyer, H. *Methods Enzymol.* **1994**, *230*, 86–108.

Analytical Biochemistry 418 (2011) 24–29

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Optimization of monosaccharide determination using anthranilic acid and 1-phenyl-3-methyl-5-pyrazolone for gastropod analysis

H. Stepan, E. Staudacher *

Department of Chemistry, University of Natural Resources and Life Sciences, A-1190 Vienna, Austria

ARTICLE INFO

Article history:
Received 1 February 2011
Received in revised form 21 June 2011
Accepted 5 July 2011
Available online 14 July 2011

Keywords:
Monosaccharide analysis
Anthranilic acid
1-Phenyl-3-methyl-5-pyrazolone (PMP)
Snail glycans

ABSTRACT

The protein-linked glycomes and, thereby, the range of individual monosaccharides of invertebrates differ from those of mammals due to a number of special modifications; therefore, it is necessary to adapt methods for monosaccharide analysis in order to cover these. We optimized the labeling procedure for anthranilic acid (AA) and 1-phenyl-3-methyl-5-pyrazolone (PMP) and the subsequent separation of the labeled monosaccharides on high-performance liquid chromatography (HPLC), with the result that we were able to identify 26 different monosaccharides. The detection limit for anthranilic acid derivatives obtained was 65 fmol, and a reliable quantification of samples was possible up to 200 nmol under the tested conditions. PMP derivatives showed a significantly higher detection limit but allow quantification of larger sample amounts. Applying these methods on snails, their impressive set of monosaccharide constituents, including methylated sugars, was shown.

Glycans play important roles in many recognition processes such as fertilization and development, allergies, pathological events, and cell death. Therefore, their detailed analysis is an important issue to get a closer view of these processes. As a first step, usually monosaccharide constituent analysis is performed. Several methods have been established employing liquid chromatography, gas chromatography, or capillary electrophoresis. However, each of these methods has its limitations in terms of selectivity, separation, detection, sensitivity, and/or effort for sample preparation. To detect monosaccharides following liquid chromatography or capillary electrophoresis, it is necessary to label them by a fluorescent or ultraviolet (UV)[1] tag. 2-Aminobenzoic acid, also known as anthranilic acid (AA), and 1-phenyl-3-methyl-5-pyrazolone (PMP) are the most commonly used tags. Both are highly reproducible and accurate. The reaction scheme and detailed chemistry for the derivatization with AA were well described in Refs. [1,2], and the derivatization with PMP was described in Ref. [3]. So far, most of the studies have concentrated on the analysis of GalN, GlcN, Man, Gal, Glc, and Fuc due to their frequent occurrence in mammalian glycans. If plant material is analyzed, Xyl and Ara are also included. However, increasingly the focus is on the glycosylation capacities of microorganisms, parasites, or invertebrates that may contain uncommon monosaccharide constituents. Snails, for example, show methylated mannoses and galactoses [4]. Therefore, we expanded the spectrum of the usual monosaccharides for high-performance liquid chromatography (HPLC)-based analyses by methylated sugars and other rare sugars to examine up to 26 different monosaccharides, thereby aiding studies on uncommon glycan structures. Furthermore, we improved and optimized these well-established methods in terms of detection limit, minimal sample amount, and separation capacity to make them applicable for the analysis of gastropod glycoconjugates.

Materials and methods

Materials

Cepaea hortensis, *Planorbarius corneus*, *Arion lusitanicus*, *Helix pomatia*, and *Arianta arbustorum* were collected by the authors under the supervision of Manfred Pintar (Department of Integrative Biology and Biodiversity Research, Institute of Zoology, University of Natural Resources and Life Sciences, Vienna, Austria) in areas close to Vienna. *Achatina fulica* and *Biomphalaria glabrata* were bred in the laboratory at 25 °C. All animals were frozen at −80 °C immediately after collection. 2- and 4-*O*-Methylated Gal were provided by Paul Kosma (Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria), and 3-*O*-methylated GlcN and 3-*O*-methylated GlcNAc were provided by Athanassios Giannis (Institute of Organic Chemistry, University of Leipzig, Leipzig, Germany). All chemicals purchased were of the highest quality available from Sigma or Fluka.

* Corresponding author. Fax: +43 1 47654 6059.
E-mail address: erika.staudacher@boku.ac.at (E. Staudacher).

[1] *Abbreviations used:* UV, ultraviolet; AA, 2-aminobenzoic acid (anthranilic acid); PMP, 1-phenyl-3-methyl-5-pyrazolone; HPLC, high-performance liquid chromatography; LC, liquid chromatography; EDTA, ethylenediaminetetraacetic acid; Me, methyl.

0003-2697/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2011.07.005

Derivatization with AA and separation on reversed phase HPLC

The derivatization was modified and downscaled from Ref. [5] to optimize the ratio between reagent and monosaccharide peaks, especially in the case of samples containing low amounts of carbohydrate. The whole assay was scaled down to 1/10 of the original protocol, whereas the amount of reagent could be reduced to 1/100 in total. Dry monosaccharides (180 nmol each for standard procedure) were dissolved in 5 μl of sodium acetate trihydrate solution (80 mg/ml) by mixing vigorously with a vortex mixer. Then 10 μl of AA reagent solution (3 mg of AA and 30 mg of sodium cyanoborohydride in 1 ml of 2% [w/v] boric acid in methanol) was added and incubated for 60 min at 80 °C. Samples were diluted 1:500 prior to HPLC analysis. Analysis was carried out on an Agilent 1200 LC (liquid chromatography) system at a constant flow rate of 1 ml/min with fluorometric detection at 360 nm excitation and 425 nm emission using a reversed phase C18 column (ODS Hypersil, 5 μm, 250 × 4 mm, Thermo Scientific, part no. 30105-254030). Solvent A was composed of 1.0% tetrahydrofuran, 0.5% phosphoric acid, and 0.2% 1-butylamine in water. Solvent B was a mixture of equal amounts of solvent A and acetonitrile. After injection of 5 μl of sample, a linear gradient of 5–25% B was applied over 17 min. For the next 6 min, another gradient up to 100% B was applied. To wash and reequilibrate the column, the run was continued by an isocratic elution with 100% B for 6 min, followed by a gradient of 100 to 5% B over 1 min. Finally, 5 min of initial conditions was applied. For quantitative analysis, samples in the range from 0.5 to 350 nmol of monosaccharide (Gal, Man, GlcN, GalN, Xyl, and Fuc) were derivatized according to the standard protocol. The limits of detection (corresponding to a signal-to-noise ratio of 3) and quantitation (corresponding to a signal-to-noise ratio of 10) were calculated according to the guidelines of the European Pharmacopoeia [6]. Background noise was determined as 6 times the standard deviation of the linear regression of the drift in a chromatogram obtained after application of a blank by the Agilent ChemStation for LC 3D Systems software (version B.04.01 SP1). Ranges for background noise calculation were set as the distance of 20 times the width at half-height of the corresponding reference peak distributed equally around respective retention times.

All experiments were carried out at least in triplicate.

Derivatization with PMP and separation on reversed phase HPLC

The derivatization was modified from Ref. [7]. Dry monosaccharides (180 nmol each for standard procedure) were dissolved in 25 μl of a solution of 0.5 M PMP in methanol. After adding 15 μl of 0.5 M NaOH and 10 μl of water, the mixture was incubated for 120 min at 70 °C. For neutralization, 20 μl of 0.5 M HCl was added. The mixture was extracted 5 times by the addition of 500 μl of organic solvent (for standards, diethyl ether was used and dichloromethane and butylether were also tried), followed by vigorous mixing and centrifugation at 3000*g* for 1 min. The organic layer was removed carefully. In contrast to the previous publication [7], the final aqueous layer was evaporated to dryness and then redissolved in 1 ml of water, yielding a standardized final volume. HPLC analysis was carried out on a reversed phase C18 column (ODS Hypersil, 5 μm, 250 × 4 mm) on an Agilent 1200 LC system at a constant flow rate of 1.5 ml/min. Solvent A was composed of 8% acetonitrile in 0.1 M ammonium acetate buffer (pH 5.5), and solvent B was composed of 30% acetonitrile in 0.1 M ammonium acetate buffer (pH 5.5). After injection of 5 μl of sample, a linear gradient of 45–50% B was applied over 10 min. Next, a gradient of 50–85% B was applied over 16 min. Finally, 5 min of initial conditions was applied to reequilibrate the column for further analyses. Detection was carried out at 245 nm. For quantitative analysis, samples in the range from 0.5 to 350 nmol of monosaccharide (Gal, Man, GlcN, GalN, Xyl, and Fuc) were derivatized according to the standard protocol. Limits of detection and quantification were determined similar to the AA method. All experiments were carried out at least in triplicate.

Preparation of proteins from snail origin

Snails were dissected as described previously [8], and proteins were purified according to Ref. [9] with minor modifications. Wet tissue (10 g) was homogenized in 50 ml of Chaps-based lysis buffer (0.5% [w/v] Chaps, 150 mM NaCl, 20 mM Tris/HCl, 2.5 mM sodium pyrophosphate, 1 mM ethyleneglycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.5] and incubated for 60 min at 4 °C. Insoluble compounds were removed by a centrifugation step of 30 min at 10,000*g*. Next, 2.5 ml of 100 mM dithiothreitol was added to 50 ml of supernatant and incubated for 45 min at 56 °C. After that, 2.5 ml of 55 mM iodoacetamide was added and incubated for 30 min at room temperature in the dark. The samples were dialyzed against water overnight and lyophilized.

Hydrolysis and analysis of snail-derived monosaccharides

An aliquot of the purified (glyco)proteins containing approximately 50 μg of carbohydrate was dissolved in 300 μl of 4 M trifluoroacetic acid and hydrolyzed for 120 min at 115 °C. The samples were dried under reduced pressure prior to further 3-fold reevaporation from 500 μl of 30% (v/v) methanol. The dried samples were subject to the derivatization and separation protocols described above for AA and PMP.

Results and discussion

To be able to identify a broad range of monosaccharides by their retention times, two independent derivatization reagents that cause different elution behaviors of the labeled monosaccharides on reversed phase HPLC were chosen. The combined evaluation of the data of both methods enables a precise identification of each monosaccharide.

Derivatization with AA

Labeling of monosaccharides with the fluorescent marker AA is known for its high sensitivity and the easy and fast derivatization chemistry [1,2]. An advantage of this method is that no re-*N*-acetylation is necessary after hydrolysis. Method validation for five sugars has been done previously [10]. Nevertheless, HPLC analysis of samples with low carbohydrate content sometimes causes problems due to the large peak of excess reagent and the relatively small monosaccharide peaks. Attempts to increase the signals of the labeled sugars in contrast to the reagent peak by optimizing the wavelengths of excitation and the emission did not succeed. However, following further experiments regarding the derivatization procedure, we were able to scale down the method of Anumula [11] in order to fit the requirements for gastropod monosaccharide analysis. Using the original protocol, almost similar peak heights for the reagent peak were obtained within a range of 0.5–5 μmol of sugar, whereas the sugar peak increased proportionally (data not shown). Because our usual amount of monosaccharides does not exceed 0.5 μmol, we reduced the total reagent volume by a factor of 10, dissolving the sample in 5 μl of sodium acetate solution and adding only 10 μl of reaction solution. In addition, we reduced the amount of AA from 30 to 3 mg per milliliter of AA reaction solution while keeping the amount of sodium cyanoborohydride constant. This improved the ratio of the sugar and

AA peaks and kept the concentration of the reaction mixture high enough to ensure proper derivatization of small amounts of sugar (Fig. 1). Under our conditions, we did not observe any epimerization from GlcN to ManN during derivatization as reported previously [10]. The limits of detection (65 fmol) and quantitation (220 fmol) were calculated according to the guidelines of the European Pharmacopoeia [6]. However, we recommend a working limit of 350 fmol, which is in accordance with previous publications [12].

Separation of 26 AA-labeled monosaccharides

A great advantage of AA-labeled monosaccharides is the different elution behavior of amino sugars as opposed to the other monosaccharides on reversed phase HPLC. They are clearly distinguishable by their elution before (amino sugars) or after (all other monosaccharides analyzed so far) the reagent peak. Methylated monosaccharides elute significantly later than all other sugars. The separation capacity of the chosen gradient is optimal. Even with the high number of sugars, we were able to attain a run time of 35 min rather than 2 h as published previously [10]. Five pairs of sugars that cannot be distinguished by this method were found: allose/Man, Ara/altrose, Rha/Fuc, 4-*O*-Me-Gal/3-*O*-Me-Glc, and 3-*O*-Me-GlcNAc/2-*O*-Me-Gal (where Me represents methyl). Even changes in the solvents or the gradient did not improve their separation. However, these pairs are effectively separated in the PMP method; therefore, the identification of these sugars is unambiguous. For a standard run of AA-labeled sugars, see Fig. 2. The retention times are listed in Table 1.

Quantification of sugars labeled with AA

Calibration curves have been established for GlcN, GalN, Man, Gal, Xyl, and Fuc by derivatizing different amounts (from 0.1 to 100 μg, which equates to an approximate range of 0.5–500 nmol depending on the particular sugar) of the monosaccharides under standard conditions. The curves obtained show a linear range for the derivatization of amino sugars from 0.5 to 100 nmol and for the other sugars from 0.5 to 200 nmol (Fig. 3A). Whereas the peak areas of Gal and Man showed comparable values at certain amounts of derivatized sugar, Xyl and Fuc showed slightly higher response factors and GlcN and GalN showed significantly lower response factors. Because we also found that the derivatization efficiency of 2-*O*-methylated galactose is significantly lower than that of 4-*O*-methylated galactose, we presume that additional groups at the C2 position of the sugar ring negatively interfere with the derivatization at the reducing end.

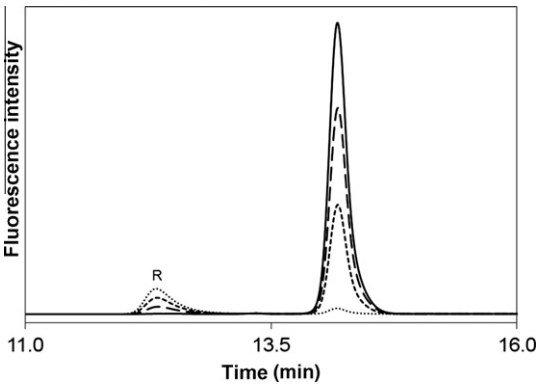


Fig.1. Derivatization of different amounts of Gal (..., 5 nmol; - - -, 90 nmol; — —, 180 nmol; —, 300 nmol) with equal amounts of AA.

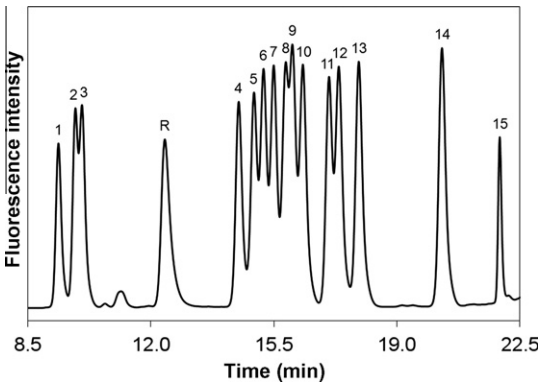


Fig.2. Monosaccharides labeled with AA and separated by reversed phase HPLC. Shown is an application of equal amounts (180 nmol) of GlcN (1), ManN (2), GalN (3), Gal (4), Man (5), idose (6), Glc (7), gulose (8), Rib (9), Ara (10), Xyl (11), glucuronic acid (12), Fuc (13), 4-*O*-Me-Gal (14), 2-*O*-Me-Gal (15), and reagent (R).

Table 1
Retention times of monosaccharides on reversed phase HPLC after labeling with AA or PMP.

Monosaccharide	Retention time after AA labeling (min)	Retention time after PMP labeling (min)
Standard		
Glucosamine	9.17	9.90
Mannosamine	9.65	10.24
Galactosamine	9.84	13.45
Galactose	14.23	18.54
Talose	14.49	10.92
Allose	14.62*	11.28
Mannose	14.66*	8.25
Idose	14.92	17.22
Glucose	15.20	17.57
Galacturonic acid	15.33	14.99
Deoxyribose	15.46	23.59
Gulose	15.53	8.81
Ribose	15.73	11.05*
Arabinose	16.01*	19.61*
Altrose	16.03*	18.84
Xylose	16.73	19.69*
Glucuronic acid	17.03	13.82
Rhamnose	17.55*	11.12*
Fucose	17.56*	21.29
3-*O*-Me-GlcN	19.06	10.79
4-*O*-Me-Gal	19.84*	21.84
3-*O*-Me-Glc	19.89*	18.38
3-*O*-Me-GlcNAc	21.67*	16.96
2-*O*-Me-Gal	21.70*	22.75
Determined monosaccharide		
3-*O*-Me-Gal	21.59	20.07
3-*O*-Me-Man	21.85	14.76

Note: Peaks labeled with an asterisk (*) coelute with another sugar in the same method.

Derivatization with PMP

Labeling with the UV tag PMP is also a relatively simple process, and previous optimizations of this method have been described by Honda and coworkers [3] and Fu and O'Neill [7]. Similar to the AA method, it also does not require re-*N*-acetylation after hydrolysis. However, in contrast to AA labeling, the sample needs to undergo further purification after the PMP derivatization because the excess reagent must be extracted by organic solvent. Diethyl ether, dichloromethane, and butylether were tried. For standard preparation, diethyl ether was chosen because in our experiments it gave the best ratio between sugar and reagent peak. Previous publications have

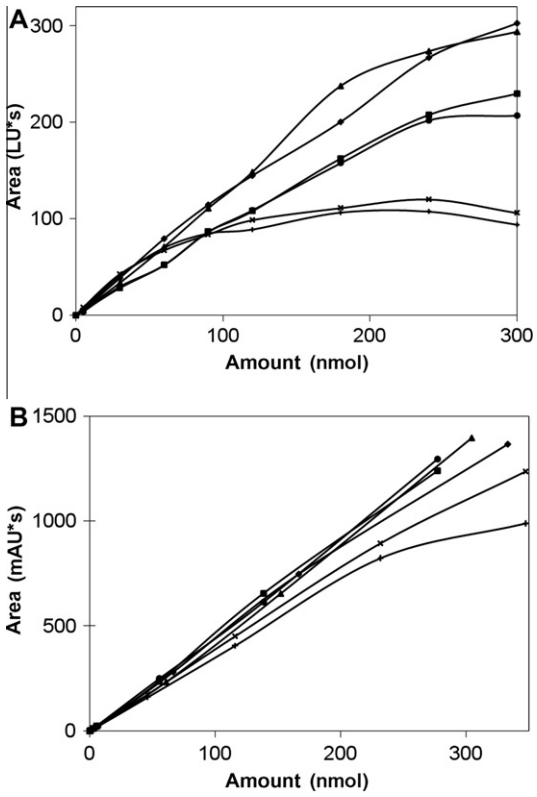


Fig.3. (A) Calibration curves of GlcN, GalN, Man, Gal, Xyl, and Fuc labeled with AA. Area is given in "light units multiplied by second" (LU*s). (B) Calibration curves of GlcN, GalN, Man, Gal, Xyl, and Fuc labeled with PMP. Area is given in "milliabsorbance units multiplied by second" (mAU*s). ♦, Xyl; ▲, Fuc; ■, Gal; ●, Man; ×, GalN; +, GlcN.

suggested performing the extraction two or three times, which reduces the reagent peak drastically. We found that five extraction steps are necessary to remove excess reagent completely. Limits of detection and quantitation were determined as described above. Due to an increased baseline drift toward the end of the run, signal-to-noise ratios vary significantly between early and late eluting sugars. Therefore, limits of detection and quantitation vary between 0.2 pmol (detection)/0.7 pmol (quantitation) for the early eluting glucosamine (at 9.9 min) and 3.2/10.7 pmol, respectively, for the late eluting galactose (at 18.5 min).

Separation of 26 PMP-labeled monosaccharides

In contrast to the AA method, retention times on HPLC after PMP labeling allow no easy prediction of the nature of a sugar. Amino sugars, as well as methylated sugars, are scattered throughout the whole chromatogram. All sugar peaks elute after the reagent peak starting with Man. Again some monosaccharides cannot be separated, but because they form two pairs (Ara/Xyl and Rib/Rha) that are clearly distinguishable after AA labeling, their identification proves to be unambiguous. For a standard run, see Fig. 4. The retention times of all sugars are listed in Table 1.

Quantification of sugars labeled with PMP

Calibration curves have been established for GlcN, GalN, Man, Gal, Xyl, and Fuc by derivatizing different amounts (from 0.1 to 100 μg, which equates to an approximate range of 0.5–500 nmol depending on the particular sugar) of the monosaccharides under standard conditions. For Gal, Man, Fuc, and Xyl, the method showed a linear range throughout our test assay. For amino sugars,

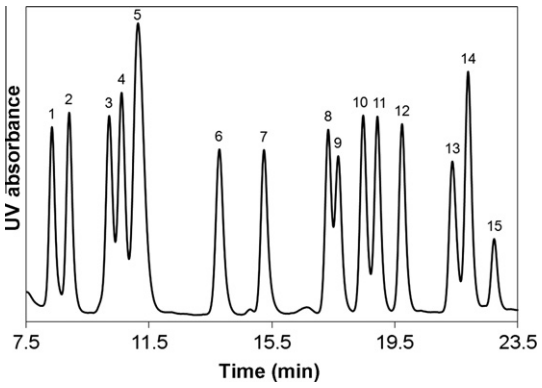


Fig.4. Monosaccharides labeled with PMP and separated by reversed phase HPLC. Shown is an application of equal amounts (180 nmol) of Man (1), gulose (2), GlcN (3), ManN (4), Rib (5), GalN (6), galacturonic acid (7), idose (8), Glc (9), 3-O-Me-Glc (10), altrose (11), Xyl (12), Fuc (13), 4-O-Me-Gal (14), and 2-O-Me-Gal (15).

the linear range was significantly smaller, just up to approximately 300 nmol. In contrast to the AA derivatization method, where the amount of AA used limits the maximum amount of sugar that can be derivatized, PMP is used in high excess and, therefore, needs to be removed before HPLC analysis. Similar to AA derivatization, peak areas for amino sugars were significantly smaller than those for other sugars when the same amount of monosaccharide was derivatized. The other sugars—Man, Gal, Xyl, and Fuc—show comparable results, but it should be noted that late eluting sugars show peak broadening, and therefore significant lower peak heights, for comparable peak area values. For the linear regions of the calibration curves, see Fig. 3B. Our data for Gal, Glc, Xyl, and Fuc correlate well with those published previously [7], but our curves for the amino sugars were suboptimal. This is due to the different methodical approaches. In the previous publication, the curves represent dilution series of one derivatized sample, where 10 nmol of each sugar was derivatized and varying amounts of the single final product were analyzed by HPLC. We performed separate derivatizations of different amounts of each sugar to obtain the points of the calibration curves. Therefore, our calibration curves include all derivatization and purification effects using these different concentrations. We believe that this methodical strategy correlates better with a broader range of sample types.

Monosaccharide analysis of snail glycans

Monosaccharide analysis using the two labeling techniques described above was carried out with seven species of snails: two water snails (*B. glabrata* and *P. corneus*), one land living slug (*A. lusitanicus*), and four land living snails carrying shells (*A. arbustorum*, *A. fulica*, *C. hortensis*, and *H. pomatia*). All of them contain an impressive set of monosaccharides. Besides those occurring frequently in nearly all higher organisms, snails also contain methylated variants of hexoses, typically 3-*O*-methylated Man, 3-*O*-methylated Gal, and 4-*O*-methylated Gal. Those have been detected and identified by gas chromatography/mass spectrometry in detail for some species recently [4].

For the snail tissues, the combination of these two methods allows a precise identification of all major peaks. Of course, the analysis of many compounds in quite short runs may result in rather small differences in elution times. Here we used the second system to resolve pairs whose identification was ambiguous. For example, AA-labeled Rha and Fuc elute at 17.55 and 17.56 min, respectively, and cannot be distinguished; however, when labeled with PMP, they show rather divergent elution times (11.11 min for Rha and 21.29 min for Fuc), making it easy to prove that the snails contain Fuc but do not contain any Rha. Some of our snail tissues contain

remarkable amounts of ribose and deoxyribose. This may be due to the developmental stage of the cells and the RNA and DNA content, respectively. Also in the monosaccharide patterns of microorganisms during the logarithmic reproduction stage, the amount of ribose and deoxyribose is strongly increased (data not shown).

Methylated sugars labeled with AA elute in the last part of the chromatogram, whereas those labeled with PMP are distributed over the whole run. A problem in the analysis of methylated sugars was the poor availability of standard monosaccharides. Whereas a 4-*O*-methylated Gal standard could be obtained, there was no source for the 3-*O*-methylated Man or 3-*O*-methylated Gal available. However, in comparing the previous quantification data from the gas chromatography analysis with the HPLC patterns, we were able to identify the appropriate signals. Most of our snail patterns contained two obvious peaks that did not comigrate with any of our other 24 standard monosaccharides and that appeared close to other methylated sugars in AA analysis. From gas chromatography/mass spectrometry analysis, we knew that there should be significant amounts of 3-*O*-methylated Gal and 3-*O*-methylated Man in most of the snails as well as some quantitative differences in some others [4]. Both *B. glabrata* and *A. fulica* show a significant amount of 3-*O*-methylated Man. 3-*O*-Methylated Gal is clearly present in *B. glabrata*, whereas it is just a minor compound in *A. fulica*. This indicated that the last peak of the AA pattern (21.85 min) is 3-*O*-methylated Man, whereas the previous one (21.59 min) is 3-*O*-methylated Gal. In the PMP pattern, 3-*O*-methylated Man elutes at 14.76 min and 3-*O*-methylated Gal elutes at 20.06 min. Those findings were confirmed by the analysis of *H. pomatia*, which shows a clearly larger 3-*O*-methylated Gal peak compared with the 3-*O*-methylated Man signal (Figs. 5 and 6). All of these data correlate with the amounts obtained by gas chromatography/mass spectrometry analysis [4]. Therefore, we are confident that these two sugars could be included in our table of retention times.

The monosaccharide patterns of *A. lusitanicus*, *A. fulica*, and *H. pomatia* shown in Figs. 5 and 6 are typical of the results for snail material. All of the commonly found sugars (GalN, GlcN, Man, Glc, Gal, and Fuc) are clearly present; the amount of Xyl and methylated hexoses (3-*O*-Me-Man, 3-*O*-Me-Gal, and 4-*O*-Me-Gal) varies with the species. No special similarities were found to distinguish between land- and water-living animals or between those with and without shells.

With two different labeling techniques (using AA and PMP), we could separate 26 different monosaccharides on reverse phase HPLC, including several methylated sugars that were not examined previously by these systems. The short run times (<40 min) also present a significant optimization and so are applicable for routine use. Because retention times may shift slightly by using a column from another supplier or batch and also with changes in the temperature, standard runs before and after the sample runs are essential. Because the order of the peaks remains constant, the system is a valuable analytical tool for the identification of monosaccharides. Those monosaccharides coeluting in one method can be clearly separated with the other method. Each sugar examined can be completely resolved from every other sugar by at least one of the two methods. The combination of these two methods provides a reliable detection system for identification as well as quantification. Whereas fluorescent detection of the AA tag facilitates analysis of very small sample amounts after a fast single step derivatization, the more time-consuming PMP derivatization technique is a valuable addition for the quantification of larger sample amounts.

In optimizing both derivatization protocols, we were able to detect 65 fmol of sugar using the AA method and to quantify sample amounts up to 300 nmol of sugar using the PMP method. Applying this system to gastropod monosaccharide analysis confirmed the broad spectrum of monosaccharides in snails as well as the presence of methylation as a feature of glycoconjugates of all snail species investigated.

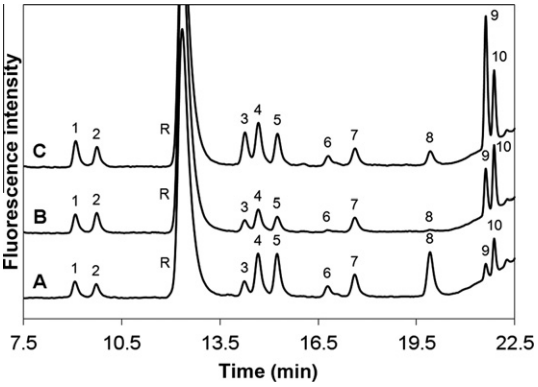


Fig.5. Monosaccharide pattern of *A. fulica* (trace A), *B. glabrata* (trace B), and *H. pomatia* (trace C) after labeling with AA and separation by reversed phase HPLC. Shown are GlcN (1), GalN (2), Gal (3), Man (4), glucose (5), Xyl (6), Fuc (7), 4-*O*-Me-Gal (8), 3-*O*-Me-Gal (9), 3-*O*-Me-Man (10), and reagent (R).

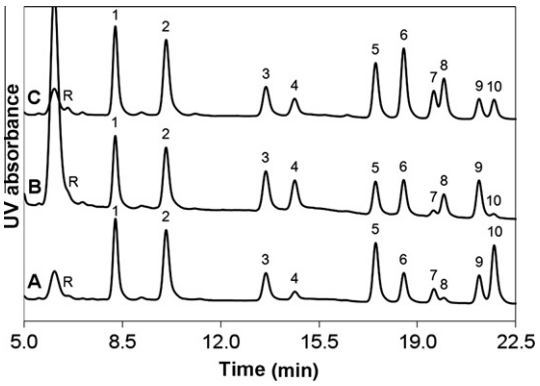


Fig.6. Monosaccharide pattern of *A. fulica* (trace A), *B. glabrata* (trace B), and *H. pomatia* (trace C) after labeling with PMP and separation by reversed phase HPLC. Shown are Man (1), GlcN (2), GalN (3), 3-*O*-Me-Man (4), Glc (5), Gal (6), Xyl (7), 3-*O*-Me-Gal (8), Fuc (9), 4-*O*-Me-Gal (10), and reagent (R).

Acknowledgments

This study was supported by the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (Project P20393-B11). The authors thank Prof. Paul Kosma (Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria) for providing 2- and 4-*O*-methylated Gal and thank Prof. Athanassios Giannis (Institute of Organic Chemistry, University of Leipzig, Leipzig, Germany) for providing 3-*O*-methylated GlcN and 3-*O*-methylated GlcNAc. The assistance of Stefanie Brezina is highly appreciated. We thank Prof. Iain Wilson for reading the manuscript.

References

[1] K.R. Anumula, High-sensitivity and high-resolution methods for glycoprotein analysis, Anal. Biochem. 283 (2000) 17–26.
[2] K.R. Anumula, Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates, Anal. Biochem. 350 (2006) 1–23.
[3] S. Honda, E. Akao, S. Suzuki, M. Okuda, K. Kakehi, J. Nakamura, High-performance liquid chromatography of reducing carbohydrates as strongly ultraviolet-absorbing and electrochemically sensitive 1-phenyl-3-methyl-5-pyrazolone derivatives, Anal. Biochem. 180 (1989) 351–357.

[4] H. Stepan, C. Bleckmann, H. Geyer, R. Geyer, E. Staudacher, Determination of 3-*O*- and 4-*O*-methylated monosaccharide constituents in snail glycans, Carbohydr. Res. 345 (2010) 1504–1507.
[5] K.R. Anumula, Quantitative determination of monosaccharides in glycoproteins by high-performance liquid chromatography with highly sensitive fluorescence detection, Anal. Biochem. 220 (1994) 275–283.
[6] Council of Europe, Chromatographic separation techniques, in: European Pharmacopoeia, 5th ed., Council of Europe, Strassbourg, France, 2005 (Chapter 2.2.46).
[7] D. Fu, R.A. O'Neill, Monosaccharide composition analysis of oligosaccharides and glycoproteins by high-performance liquid chromatography, Anal. Biochem. 227 (1995) 377–384.
[8] M. Gutternigg, S. Bürgmayr, G. Pöltl, J. Rudolf, E. Staudacher, Neutral *N*-glycan patterns of the gastropods *Limax maximus*, *Cepaea hortensis*, *Planorbarius corneus*, *Arianta arbustorum*, and *Achatina fulica*, Glycoconj. J. 24 (2007) 475–489.
[9] Y. Mechref, P. Kang, M.V. Novotny, Solid-phase permethylation for glycomic analysis, in: N.H. Packer, N.G. Karlsson (Eds.), Glycomics: Methods and Protocols, Methods in Molecular Biology, vol. 534, Humana, Totowa, NJ, 2009, pp. 53–64.
[10] K. Račaitytė, S. Kiessig, F. Kálmán, Application of capillary zone electrophoresis and reversed-phase high-performance liquid chromatography in the biopharmaceutical industry for the quantitative analysis of the monosaccharides released from a highly glycosylated therapeutic protein, J. Chromatogr. A 1079 (2005) 354–365.
[11] K.R. Anumula, Unique anthranilic acid chemistry facilitates profiling and characterization of Ser/Thr-linked sugar chains following hydrazinolysis, Anal. Biochem. 373 (2008) 104–111.
[12] K.R. Anumula, P. Du, Characterization of carbohydrates using highly fluorescent 2-aminobenzoic acid tag following gel electrophoresis of glycoproteins, Anal. Biochem. 275 (1999) 236–242.

Current Topics in Biochemical Research

Review

Protein N-glycosylation of gastropods

Erika Staudacher*, Herwig Stepan and Martin Gutternigg

University of Natural Resources and Applied Life Sciences, Vienna Department of Chemistry, Division of Biochemistry, Glycobiology, Muthgasse 18, A-1190 Vienna, Austria

ABSTRACT

Glycosylation plays an important role in several types of recognition processes associated with fertilisation and development, allergies, pathological events and cell death. Whereas the amino acid sequence of a protein is fixed by the DNA, the glycosylation abilities depend on enzymes and substrates currently present in the cell. During the last decades our knowledge on glycosylation – the structure of glycans as well as the corresponding biochemical pathways including the responsible enzymes - especially on glycans of mammalian origin increased enormously. The glycosylation capabilities of other species were under investigation only if their glycans were for any reason connected to human life (e.g. some recognition processes of pathogens or allergy on food or plant glycans) or if they were potent candidates for cell culture systems for the expression of therapeutic agents (some insect, yeast and plant cells). However, in the meantime there is an increasing interest also in invertebrate glycosylation. Snails in particular show a broad spectrum of glycosylation abilities within their N-glycosylation pattern. In one case this has been shown to be involved in an intermediate host – parasite recognition process. For other snail species, it was found that they share many structural elements of N-glycans with mammals, plants, insects or nematodes. Sometimes several of these elements are present within one single structure. Here we present an overview of the current knowledge of N-glycosylation of snails, the glycan structures and the corresponding enzymes involved in the biosynthetic glycosylation pathway.

KEYWORDS: gastropod, snail, N-glycan, glycosyltransferase

*erika.staudacher@boku.ac.at

INTRODUCTION

The structure of protein-linked glycans determines recognition events, decides about binding or not and influences the speed or quality of biological processes. Differences within the oligosaccharide structures appear not only between different species but also between individuals of the same species. Furthermore the current glycosylation pattern highly depends on the developmental and physiological status of the cell.

So, in medicine, the microheterogeneity of glycans has to be kept in mind in transfusion and transplantation events, especially, of course, in xenotransplantation. Since changes in the glycosylation pattern can be observed in the course of several diseases, this can be used for diagnoses or as control of therapy success. Different glycosylation features also play an important role in allergic processes against plant extracts, pollen or some food [1-3].

A number of pharmaceutically relevant products are already produced in cell-culture. Whereas the amino acid sequence of a protein is fixed by the cloned DNA, the posttranslational modifications depend on the current presence of enzymes and substrates in the expression cell-line. Amongst all possible modifications, the glycosylation pattern of the recombinant glycoprotein has to be analysed especially carefully.

Glycosylation contributes not only to physical properties, such as conformational stability, protease resistance, charge or hydrophilicity, but glycans may also function as recognition determinants in host-pathogen relationships, protein targeting and cell-cell interactions [4, 5]. Incorrect glycosylation may result, in the worst case, in a complete loss of activity or in the formation of allergenic structures [1, 6]. Economic reasons force industry more and more to establish easier and cheaper cell culture systems for the production of recombinant glycoproteins for therapeutic purposes, but nevertheless special care has to be taken that the glycan moieties of the produced proteins are highly similar to those of the natural counterparts in order to keep their function. At the moment many of the pharmaceutically relevant glycoproteins, where a correct glycan structure is essential for their function, are produced in mammalian cell cultures because of their similarity to the human system; however, these are expensive and sensitive systems with some risks of virus transfer and so non-mammalian sources are under closer investigation. Therefore the investigation of glycosylation capacities combined with the analysis of substrate specificity and the order of involved enzymes is a growing field in this area.

Bacteria do not N-glycosylate in the same way as eukaryotes and yeasts often produce very large oligomannosidic glycans but lack the ability to synthesise complex ones. The glycosylation potential of insect cells is, due to their advantage in terms of costs as well as of biosafety, of pharmaceutical interest.

Baculovirus expression systems as well as non-lytic systems are already used for the expression of recombinant proteins with small complex glycans, but they seem to lack the ability to produce negatively charged complex N-glycans in an appreciable amount, if at all [7, 8]. Currently the engineering of transgenic insect cell lines is under investigation in order to express mammalian glycosyltransferases and enzymes synthesising the appropriate activated sugar molecules but up to now these systems are not in use for large scale production [9, 10]. Furthermore other non-mammalian features, such as α1,3-fucosylation of the inner GlcNAc-residue of N-glycans, may occur on recombinant glycoproteins derived from insect cell culture. This structural feature is also typical for plants and has been shown to be highly immunogenic [1, 11]. For this reason also plant cells have to be modified before they can be used as production system for pharmaceutical relevant glycoproteins. Another deficit of plant glycans, the production of too small antennae lacking terminal GlcNAc-residues, seems to be solved by the co-expression of a human N-acetylglucosaminyltransferase III which prevents the degradation of the antennae [12].

So, up to now, no optimal system has been found which allows the production of recombinant glycoproteins including all types of N-glycosylation in large quantities and constantly high quality. This is the reason why other non-mammalian sources are under investigation for their glycosylation abilities. The current knowledge on gastropod N-glycosylation is described here.

Biosynthesis of N-glycans

N-Glycosylation starts in the Endoplasmatic Reticulum by formation of a lipid linked precursor, containing two GlcNAc residues, nine mannoses and three glucoses, which is transferred *en bloc* onto the growing polypeptide chain. The processing of the N-glycans from $Glc_3Man_9GlcNAc_2$ to complex structures is based on a strict order of the action of glycosidases and glycosyltransferases [13]. The individual glycosylation pattern produced by a cell is based on an interplay between substrates and enzymes, as well as their current availability and location within this cell. These factors are responsible for the creation of an individual glycosylation pattern in each specific cell, yielding species-, organ- and tissue-specific glycans.

While the first steps of the biosynthetic pathway of N-glycans seem to be similar in nearly all kinds of organisms, only very small modifications have been found so far there, the final modification steps are highly heterogeneous and lead to the wide range of various glycan structures.

The key enzyme for the formation of complex glycans is the GlcNAc-transferase I, which forms the $GlcNAcMan_5GlcNAc_2$-structure [14].

After previous action of GlcNAc-transferase I, the glycan is a suitable substrate for a number of further modifying enzymes such as core α1, 6-fucosyltransferase, core α1,3-fucosyltransferase, β1,2-xylosyltransferase, β1,2-GlcNAc-transferase II and β1,3 - or β1,4- galactosyltransferase which build up larger and more complex glycan structures (Fig. 1). Most of the known modifying glycosyltransferases depend on the previous action of GlcNAc-transferase I, only few exceptions have been found up to now [15]. In some tissues the glycans are also further modified by additional non-sugar structural features, such as methylation, sulphation, phosphorylation or others to create even more variations.

Analysis of the N-glycosylation potential

The glycosylation pathway of a specific cell or organism is usually elucidated on one hand by the analysis of the glycan structures and on the other hand by the determination of the enzymes which are responsible for the formation of these glycans.

First the glycan structures are released from the protein-backbone by specific glycopeptidases followed by labelling with a fluorescent dye. Routine analysis is carried out by two-dimensional HPLC-technique (separation by size and hydrophobicity) and mass spectrometry with or without fragmentation. Sometimes also digestion with specific exoglycosidases is used to get further information on type and linkage of terminal sugar residues [16, 17]. However, even though this approach sounds quite simple, the strategy and the purification protocols have to be optimised for each kind of tissue separately, due to the highly complex sample matrices. With permanently evolving methods a continuous increase in detection sensitivity can be ensured [18].

Mammalian enzymes involved in the biosynthesis of glycans show a relatively high genetic similarity throughout different species and therefore it is often possible to identify further enzymes by homology search. Even though invertebrates and plants contain several enzymes with quite similar substrate specificity, the genetic homology of these enzymes with the mammalian ones is usually not high enough for determination by homology search.

N-glycans of molluscs

Hemocyanin, the oxygen-carrier of arthropods and molluscs, was the first target of investigation in the elucidation of the gastropod proteome. There, the occurrence of N-glycans in molluscs was detected [19, 20] and soon a novel modification of glycans was identified: the methylation of hexoses (mannoses and galactoses) [21]. This kind of modification does not occur in mammals. Up to now it has been found

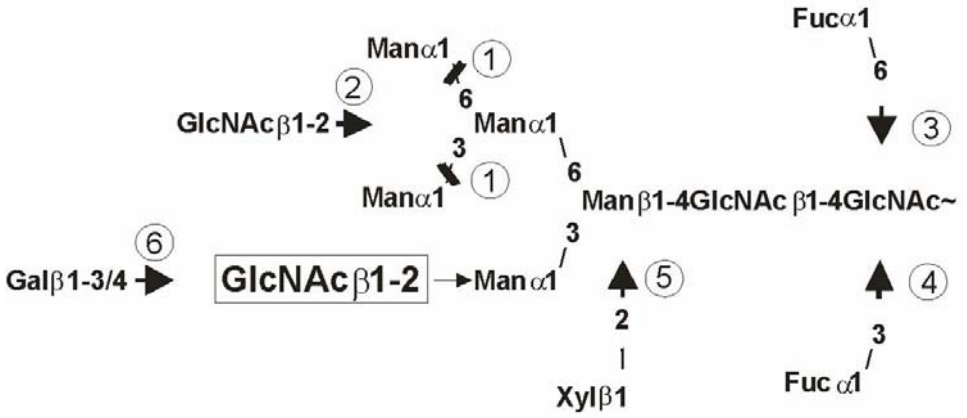


Fig. 1. Possible modifications of a glycan after the action of GlcNAc-transferase I. (1) Mannosidase II; (2) GlcNAc-transferase II; (3) Core α1,6-fucosyl-transferase; (4) Core α1,3-fucosyltransferase; (5) β1,2-xylosyltransferase; (6) β1,3- or β1,4-galactosyltranferase..

in the kingdom of animals only in nematodes (2-O-methylated terminal fucose as well as 4-O-methylated galactose) [22] and in molluscs (see later in detail). All other studies on gastropods at that time were dealing with the analysis of the biochemical parameters of exoglycosidases or the binding specificities of lectins derived from snails.

Since the nineteen-eighties the increase of advanced technical opportunities facilitated a more accurate and precise investigation of glycans. With this impact the detailed analysis of gastropod structures started again with the hemocyanin of some selected snails. It was shown that *Helix pomatia* hemocyanin includes complex structures containing a common core with a fucose α1,6-linked to the reducing GlcNAc and a xylose β1,2-linked to the β-mannose residue. One or both α-mannose residues may be substituted by GalNAcβ1,4GlcNAcβ1,2-elements which contain two to four β1,3- or β1,6-linked galactoses with or without 3- or 4-O-methylgroups (Fig. 2) [23, 24]. A recent study shows that especially the xylose and terminal 3-O-methylated galactoses seem to be responsible for the cross-reactivity of *Helix pomatia* glycoproteins [25].

Lymnaea stagnalis hemocyanin contains low and high molecular mass biantennary oligosaccharides. They lack the α1,6-linked fucose at the inner GlcNAc-residue but some antennae are terminated by an α1,2-linked fucose. The basic element of the antennae is a Galβ1,3GalNAcβ1,4GlcNAc unit [26, 27].

The glycans of the *Unio elongatulus* gp273, which is the ligand for sperm-egg interaction in this mollusc bivalve, are of the oligomannosidic type ($Glc_1Man_9GlcNAc_2$ and $Man_9GlcNAc_2$) [28].

Megathura crenulata (keyhole limpet) hemocyanin glycans carry a novel type of modification with galactose directly linked in β1,6-linkage to the α1,3- or the α1,6-linked mannose residues or even the β-linked mannose of the N-glycan core [29]. Some of the glycans are decorated at the α1,3-antenna of the trimannosyl core with the Fucα1, 3GalNAcβ1, 4[Fucα1,3] GlcNAc motif, which also occurs in schistosomal glycoconjugates mediating cross reactivities between these two organisms [30]. For the first time an elongation by up to two galactose residues of the fucose α1,6-linked to the inner core N-acetylglucosamine was found [31].

The two N-glycans of the functional unit RvH1-a of *Rapana venosa* hemocyanin are biantennary non-fucosylated oligosaccharides with terminal 3-O-methylated β1,3-linked galactose residues. One of them carries a sulphate group on the α1,6-linked core mannose and a 3-O-methylated GlcNAc residue linked β1,2 to the β-mannose of the core [32]. Furthermore, an unusually branched fucose residue substituted by a hexosamine and a hexouronic acid has been found here [33]. In *Rapana thomasiana* a similar fucose residue is elongated by 3-O-methylgalactose and N-acetylgalactosamine [34].

In *Biomphalaria glabrata* a core structure terminated by two 3-O-methylated mannose residues linked to the major soluble protein of the organic shell matrix was initially identified [35].

Later, several carbohydrate determinants such as terminal Fucα1,3GalNAc units or β1,2-linked xylose which are common to this intermediate host snail, as well as to the corresponding parasite, *Schistosoma mansoni*, were found (Fig. 3) [36, 37].

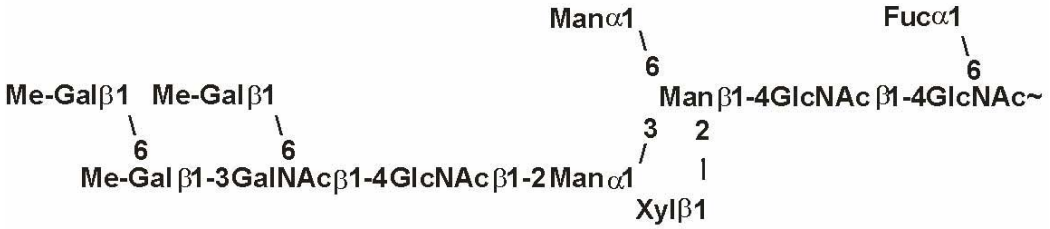


Fig. 2. The most complex N-glycan-chain of *Helix pomatia* [24].

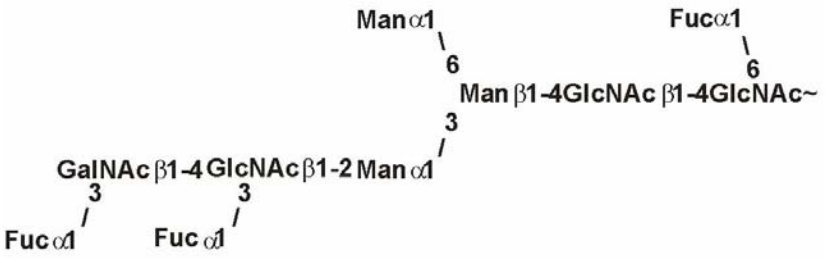


Fig. 3. One example of a N-glycan of *Biomphalaria glabrata* hemolymph [36].

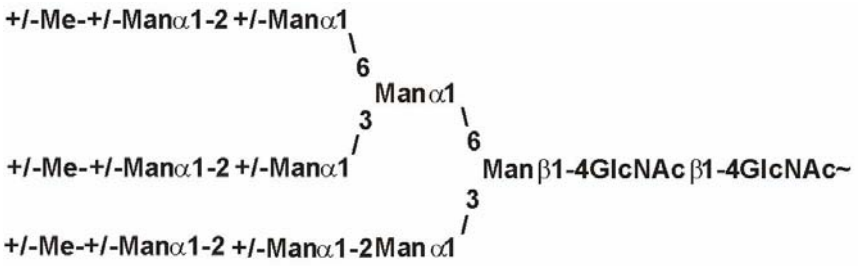


Fig. 4. High mannosidic N-glycans with three to nine mannose residues and up to three methyl groups [39, 40].

In the course of our own previous projects we analysed the N-glycosylation patterns of whole tissue extracts derived from *Arion lusitanicus*, *Limax maximus*, *Cepaea hortensis*, *Planorbarius corneus*, *Arianta arbustorum* and *Achatina fulica*.

There we found glycans with all structural features mentioned above and some more. The snails contain a broad spectrum of elements which are already known from different organisms [38-40]. Besides the common high mannosidic structures ($Man_3GlcNAc_2$ to $Man_9GlcNAc_2$) the same structures with up to three additional methyl groups occur (Fig. 4). Xylosylation at the β-mannose and core fucosylation in α1,6-linkage are frequent modifications, but also core α1,3 fucosylation, terminal α1,3 fucosylation and terminal α1,2 fucosylation were found. The larger, complex glycans are terminated by galactose residues with or without a methyl group. A study on *Achatina fulica* eggs and young snails of different ages (up to 120 days) showed that at these young ages structures with several terminal GlcNAc-residues are dominant [41]. These changes of the N-glycan pattern during development may be due to different expression levels of various glycosyltransferases and glycosidases.

Snail enzymes involved in glycosylation

The information given on enzymes which are involved in glycan biosynthesis is restricted in most cases to enzyme specificity *in vitro* and some biochemical parameters. *Lymnaea stagnalis* has been shown to express the key enzyme for the formation of complex N-glycans, GlcNAc-transferase I, the prerequisite for the action of further modifying glycosyltransferases [42]. This water snail also expresses GlcNAc-transferase II and xylosyltransferase [42], a β1,4-GalNAc-transferase, which shows high homology to mammalian β1,4-galactosyltransferase [43], a β1,3-galactosyltransferase and an α1,2-fucosyl-transferase, both located in the connective tissue [44, 45]. Another β1,3-galactosyltransferase purified from the albumen gland is not involved in N-glycan biosynthesis but in the formation of Galβ1,3Galβ1,4Glc units. This enzyme may be essential for the production of galactogen, the main polysaccharide of *Lymnaea stagnalis* [46].

Hybridization experiments using the cDNA of bovine β1,4-galactosyltransferase as probe, revealed a β1,4-GlcNAc-transferase which requires a different nucleotide sugar but is similar

to the mammalian galactosyltransferase in acceptor specificity and definitely not involved in the biosynthesis of the chitobiose core of N-glycans [47, 48]. This enzyme was the first glycosyltransferase described where an exon duplication seems to have taken place during evolution (exons 6, 7 and 8 show extremely high sequence similarity) [49].

Furthermore an α1,3-fucosyltransferase catalysing the transfer of fucose from GDP-fucose to a Galβ1,4GlcNAc acceptor forming the Lewis^x -unit has been found in the connective tissue of *Lymnea stagnalis* [45] and an α1,3-fucosyltransferase catalysing the transfer of fucose from GDP-fucose to the asparagine-linked GlcNAc has been found in the albumin and prostate glands of the same snail [50]. However, neither Lewis^x-containing structures nor core α1,3-fucosylated structures have been detected in this snail so far. Another enzyme of which the *in vivo* products are still unknown, is the β1,4-glucosyltranferase present in the prostate gland of *Lymnaea stagnalis*. It catalyses the transfer of glucose from UDP-glucose to terminal GlcNAc-residues which are linked β1,6 to a galactose or a GalNAc residue in O-glycans, or, in N-glycans to a terminal GlcNAc which is β1,2-linked to mannose [51]. In *Helix pomatia* an α1,2-L-galactosyltransferase which seems to be involved in the elongation of the storage polysaccharides of the snail was found [52]. Besides its galactose transferring ability, this galactosyltransferase catalyses the transfer of a fucose from GDP-fucose into α1,2-linkage to a Galβ1,3Gal-O-Me substrate *in vitro* as well; nothing is known about this ability *in vivo*. Other galactosyltransferases with β1,6-specificity building linear chains of D-galactoses or creating branching points in galactans were identified in *Biomphalaria glabrata, Helix pomatia* and *Arianta arbustorum* [53, 54].

Besides the glycosyltransferases some glycosidases (glucosidase I and II, mannosidase I and II) are involved in the biosynthetic pathway of N-glycans too. So far none of these specific exoglycosidases have been determined. Those glycosidases which have been found in snails seem to be more likely part of the degradation system for galactans, mannans and cellulose [55, 56]. However, these enzymes may be of economical interest and some are checked for a use in food industry [57].

From *Pomacaea canaliculata* three exoglycosidases have been characterised. The α-mannosidase has a broad specificity cleaving α1,2-, α1,3- and α1,6-mannosidic linkages [58]. The fucosidase and the xylosidase are able to release the corresponding sugar residues from plant-derived oligosaccharides, α1,3-linked fucose and β1,2-linked xylose respectively, but have not been tested with other glycans [59]. For some exoglycosidases also genetic information is available. An endo-β-mannosidase from *Haliotis discus hannai* has been isolated and cloned and two cellulases from *Pomacaea canaliculata* were investigated in detail for their genomic organization and their expression in snails of different ages [60, 61].

The genetic information on snails in general is marginal. There are some data mainly on enzymes involved in the oxidative phosphorylation process which are known to be extremely conserved. So far there are no data on glycosyltransferases. Because of its medical relevance as an intermediate host a genome initiative on *Biomphalaria glabrata* is ongoing which supplies at least some genetic information on this snail (http://biology.unm.edu/biomphalaria-genome/).

FUNCTION

Due to their diversity, glycans provide the most perfect instruments for each single cell to assign its communication and other recognition processes. These processes are well investigated in mammals and somehow in plants but until now not in gastropods. However, there is no doubt that glycans are involved in these processes in snails too.

For gastropods only medical relevant recognition events are under investigation. They can be divided into two groups: one, where the interaction of the intermediate host gastropod with a parasite is analysed. It has been known for about twenty years that snails and parasites share some epitopes which cause cross-reactivity *in vitro* and antibody production *in vivo* [62]. This reaction

can be used on one hand for the diagnosis and on the other hand for the design of drugs against the parasites [63-68].

And the second one, where gastropod glycoproteins are studied for their use in cancer therapy. Some gastropod lectins bind specifically to certain types of cancer cells allowing diagnoses and prognoses [69]. Also natural or modified gastropod glycoproteins are utilised against cancer cells by stimulating the human immune response [70-72].

CONCLUSION

The analysis of gastropod glycosylation is an emerging field in glycobiology, but we are still at the very beginning. A number of structure analyses have already been performed and several completely new glycan modifications have been detected. However, only few enzymes which are involved in the glycosylation process have been isolated and characterised so far and none have been cloned. Lacking these genetic data, not much can be said about the evolution of the enzymes involved in glycosylation. From the specificity data of the enzymes and the structural analysis of the glycans no distinctive differences between land and water snails or snails with and without shell can be made out yet.

Currently the main driving force for this field of research is the interest in new model organisms with a broad glycosylation spectrum to understand the biosynthesis of all different kinds of glycans. This new information could than be applied to medicine for the establishment of new diagnosis and therapy tools as a consequential reward.

ACKNOWLEDGEMENTS

Our work was funded by the Austrian Fonds zur Förderung der wissenschaftlichen Forschung projects P13928-BIO and P20393-B11.

ABBREVIATIONS

Fuc, fucose; Gal, galactose; GalNAc, N-Acetylgalactosamine; Glc, glucose; GlcNAc, N-Acetylglucosamine; Man, mannose; Xyl, xylose

REFERENCES

1. Tretter, V., Altmann, F., Kubelka, V., März, L., and Becker, W. M. 1993, Fucose α1,3-linked to the core region of glycoprotein N-glycans creates an important epitope for IgE from honeybee venom allergic individuals. Int. Arch. Allergy Immunol., 102, 259-266.
2. Wilson, I. B. H., Harthill, J. E., Mullin, N. P., Ashford, D. A., and Altmann, F. 1998, Core α1,3-fucose is a key part of the epitope recognized by antibodies reacting against plant N-linked oligosaccharides and is present in a wide variety of plant extracts. Glycobiology, 8, 651-661.
3. Altmann, F. 2007, The role of protein glycosylation in allergy. Int. Arch. Allergy Immunol., 142, 99-115.
4. Elbein, A. D. 1987, Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. Annu. Rev. Biochem., 56, 497-534.
5. Rademacher, T. W., Parekh, R. B., and Dwek, R. A. 1988, Glycobiology. Annu. Rev. Biochem., 57, 785-838.
6. Aeed, P. A., and Elhammer, Å. P. 1994, Glycosylation of recombinant prorenin in insect cells: the insect cell line Sf9 does not express the mannose 6-phosphate recognition signal. Biochemistry, 33, 8793-8797.
7. Altmann, F., Staudacher, E., Wilson, I. B. H., and März, L. 1999, Insect cells as hosts for the expression of recombinant glycoproteins. Glycoconjugate J., 16, 109-123.
8. Choi, O., Tomiya, N., Kim, J. H., Slavicek, J. M., Betenbaugh, M. J., and Lee, Y. C. 2003, N-glycan structures of human transferrin produced by *Lymantria dispar* (gypsy moth) cells using the LdMNPV expression system. Glycobiology, 13, 539-548.
9. Hollister, J., Conradt, H., and Jarvis, D. L. 2003, Evidence for a sialic acid salvaging pathway in lepidopteran insect cells. Glycobiology, 13, 487-495.
10. Aumiller, J. J., Hollister, J., and Jarvis, D. L. 2003, A transgenic insect cell line engineered to produce CMP-sialic acid and sialylated glycoproteins. Glycobiology, 13, 497-507.

11. Van Ree, R., Cabanes-Machetau, M., Akkerdaas, J., Milazzo, J. P., Loutelier-Bourhis, C., Rayon, C., Villalba, M., Koppelman, S., Aalberse, R., Rodriguez, R., Faye, L., and Lerouge, P. 2000, J. Biol. Chem. 275, 11451-11458.
12 Rouwendal, G. J. A, Wuhrer, M., Florack, D. E. A., Koeleman, C. A. M., Deeelder, A. M., Bakker, H., Stoopen, G. M., Van Die, I., Helsper, J. P. F. G., Hokke, C. H., and Bosch, D. 2007, Efficient introduction of a bisecting GlcNAc residue in tobacco N-glycans by expression of the gene encoding human N-acetylglucosaminyltransferase III. Glycobiology, 17, 334-344.
13. Kornfeld, R., and Kornfeld, S. 1985, Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem., 54, 631-664.
14. Longmore G. D., and Schachter H. 1982, Product-identification and substrate-specificity studies of the GDP-L-fucose:2-acetamido-2-deoxy-β-D-glucoside (FUC goes to Asn-linked GlcNAc) 6-α-L-fucosyltransferase in a Golgi-rich fraction from porcine liver. Carbohydr Res., 100, 365-392.
15. Paschinger, K., Staudacher, E., Stemmer, U., Fabini, G., and Wilson, I. B. 2005, Fucosyltransferase substrate specificity and the order of fucosylation in invertebrates. Glycobiology, 15, 463-474.
16. Geyer, H., and Geyer, R. 2006, Strategies for analysis of glycoprotein glycosylation. Biochim Biophys Acta., 1764, 1853-1869.
17. Domann, P. J.**,** Pardos-Pardos, A. C.**,** Fernandes, D. L.**,** Spencer, D. I.**,** Radcliffe, C. M.**,** Royle, L.**,** Dwek, R. A., and Rudd, P. M. 2007, Separation-based glycoprofiling approaches using fluorescent labels. Proteomics, 7 (Suppl. 1), 70-76.
18. North, S. J., Hitchen, P. G., Haslam, S. M., and Dell, A. 2009, Mass spectrometry in the analysis of N-linked and O-linked glycans. Curr. Opin. Struct. Biol., 19, 1-9.
19. Dijk, J., Brouwer, M., Coert, A., and Gruber, M. 1970, Structure and function of hemocyanins. VII. The smallest subunit of alpha- and beta-hemocyanin of *Helix pomatia*: size, composition, N- and C-terminal amino acids. Biochim. Biophys. Acta, 221, 467-479.
20. Waxman, L. 1975, The structure of arthropod and mollusc hemocyanins. J. Biol. Chem., 250, 3796-3806.
21. Hall, R. L., Wood, E. J., Kamerling, J. P., Gerwig, G. J., and Vliegenthart, J. F. G. 1977, 3-O-Methyl sugars as constituents of glycoproteins. Biochem. J., 165, 173-176.
22. Khoo, K. H., Maizels, R. M., Page, A. P., Taylor, G. W., Rendell, N. B., and Dell, A. 1991, Characterization of nematode glycoproteins: the major O-glycans of Toxocara excretory-secretory antigens are O-methylated trisaccharides. Glycobiology, 1, 163-171.
23. van Kuik, J. A., van Halbeek, H., Kamerling, J. P., and Vliegenthart, J. F. G. 1985, Primary structure of the low molecular weight-carbohydrate chains of *Helix pomatia* α-hemocyanin. Xylose as a constituent of N-linked oligosaccharides in an animal glycoprotein. J. Biol. Chem., 260, 13984-13988.
24. Lommerse, J. P. M., Thomas-Oates, J. E., Gielens, C., Préaux, G., Kamerling, J. P., and Vliegenthart, J. F. G. 1997, Primary structure of 21 novel monoantennary and diantennary N-linked carbohydrate chains from α_D-hemocyanina of *Helix pomatia*. Eur. J. Biochem., 249, 195-222.
25. Siddiqui, N. I., Yigzaw, Y., Préaux, G., and Gielens, C. 2009, Involvement of glycans in the immunological cross-reaction between α-macroglobulin and hemocyanin of the gastropod *Helix pomatia*. Biochimie, 91, 508-516.
26. Van Kuik, J. A., Sijbesma, R. P., Kamerling, J. P., Vliegenthart, J. F. G., and Wood, E. J. 1986, Primary structure of a low-molecular-mass N-linked oligosaccharide from hemocyanin of *Lymnaea stagnalis*. 3-O-methyl-D-mannose as a constituent of the xylose-containing core structure in an animal glycoprotein. Eur. J. Biochem., 160, 621-625.
27. Van Kuik, J. A., Sijbesma, R. P., Kamerling, J. P., Vliegenthart, J. F. G., and Wood, E. J. 1987, Primary structure determination of seven novel N-linked carbohydrate chains derived from hemocyanin of *Lymnaea stagnalis.* 3-O-methyl-D-galactose and N-acetyl-D-galactosamine as constituents of xylose-containing N-linked oligosaccharides in an animal glycoprotein. Eur. J. Biochem., 169, 399-411.

28. Di Patrizi, L., Capone, A., Focarelli, R., Rosati, F., Gallego, R. G., Gerwig, G. J., and Vliegenthart, J. F. G. 2001, Structural characterization of the N-glycans gp273, the ligand for sperm-egg interaction in the mollusc bivalve of the *Unio elongatulus*, Glycoconjugate J., 18, 511-518.
29. Kurokawa, T., Wuhrer, M., Lochnit, G., Geyer, H., Markl, J., and Geyer, R. 2002, Hemocyanin from the keyhole limpet *Megathura crenulata* (KLH) carries a novel type of N-glycans with Gal(β1-6)Man-motifs. Eur. J. Biochem., 269, 5459-5473.
30. Geyer, H., Wuhrer, M. Resemann, A., and Geyer, R. 2005, Identification and characterization of keyhole limpet hemocyanin N-glycans mediating cross-reactivity with Schistosoma mansoni. J. Biol. Chem., 280, 40731-40748.
31. Wuhrer, M., Robijn, M. L. M., Koeleman, CAM, Balog, C. I. A., Geyer, R, Deelder, A. M., and Hokke, C. H. 2004, A novel Gal(β1,4)Gal(β1,4)Fuc(α1,6)-core modification attached to the proximal N-acetyl-glucosamine of keyhole limpet haemocyanin (KLH) N-glycans. Biochem. J., 378, 625-632.
32. Dolashka-Angelova, P., Beck, A, Dolashiki, A., Beltramini, M., Stevanovic, S., Salvato, B., and Voelter, W. 2003, Characterization of the carbohydrate moieties of the functional unit RvH_1-a of *Rapana venosa* haemocyanin using HPLC/electrospray ionization MS and glycosidase digestion. Biochem. J., 374, 185-192.
33. Sandra, K., Dolashka-Angelova, P., Devereese, B., and Van Beeumen, J. 2007, New insights in Rapana venosa hemocyanin N-glycosylation resulting from on-line mass spectrometric analyses. Glycobiology, 17, 141-156.
34. Gielens, C., Idakieva, K., van den Bergh, V., Siddiqui, N. I., Parvanova, K., and Compernolle, F. 2005, Mass spectral evidence for N-glycans with branching on fucose in a molluscan hemocyanin. *Biochem*. Biophys. Res. Commun., 331, 562-570.
35. Marxen, J. C., Nimtz, M., Becker, W., and Mann, K. 2003, The major soluble 19.6 kDa protein of the organic shell matrix of the freshwater snail *Biomphalaria glabrata* is an N-glycosylated dermatopontin. Biochim. Biophys. Acta, 1650, 92-98.
36. Lehr, T., Geyer, H., Maass, K., Doenhoff, M. J., and Geyer, R. 2007, Structural characterization of N-glycans from teh freshwater snail biomphalaria glabrata cross-reacting with *Schistosoma mansoni* glycoconjugates. Glycobiology, 17, 82-103.
37. Lehr, T., Beuerlein, K., Doenhoff, M. J., Grevelding, C. G., and Geyer, R. 2008, Localization of carbohydrate determinants common to *Biomphalaria glabrata* as well as to sporocysts and miracidia of *Schistosoma mansoni*. Parasitology, 135, 931-942.
38. Bürgmayr, S., Grabher-Meier, H., and Staudacher, E. 2001, Sialic acids in gastropods. FEBS Lett., 508, 95-98.
39. Gutternigg, M., Ahrer, K., Grabher-Meier, H., Bürgmayr, S., and Staudacher, E. 2004, Neutral N-glycans of the gastropod *Arion lusitanicus.* Eur. J. Biochem., 271, 1348-1356.
40. Gutternigg, M., Bürgmayr, S., Pöltl, G., Rudolf, J., and Staudacher, E. 2007, Neutral N-glycan patterns of the gastropods *Limax maximus*, *Cepaea hortensis*, *Planorbarius corneus*, *Arianta arbustorum* and *Achatina fulica.* Glycoconjugate J., 24, 475-489.
41. Park, Y., Zhang, Z., Laremore, T. N., Li, B., Sim, J-S, Im, A., Ahn, M. Y., Kim, Y. S., and Linhardt, R. J. 2008, Variation of acharan sulfat ad monosaccharide composition and analysis of neutral N-glycans in African giant snail (*Achatina fulica*) Glycoconjugate J., 25, 863-877.
42. Mulder, H., Dideberg, F., Schachter, H., Spronk, B. A., De Jong-Brink, M., Kamerling, J. P., and Vliegenthart, J. F. G. 1995, In the biosynthesis of N-glycans in connective tissue of the snail *Lymnaea stagnalis* of incorporation GlcNAc by β2GlcNAc-transferase I is an essential prerequisite for the action of βGlcNAc-transferase II and β2Xyl-transferase. Eur. J. Biochem., 232, 272-283.

43. Mulder, H., Spronk, B. A., Schachter, H., Neeleman, A. P., Van den Eijneden, D. H., De Jong-Brink, M., Kamerling, J. P., and Vliegenthart, J. F. G. 1995, Identification of a novel UDP-GalNAc:GlcNAcβ-R β1-4 N-acetylgalactosaminyltransferase from the albumen gland and connective tissue of the snail *Lymnaea stagnalis*. Eur. J. Biochem., 227, 175-185.
44. Mulder, H., Schachter, H., De Jong-Brink, M., Van der Ven, J. G. M., Kamerling, J. P., and Vliegenthart, J. F. G. 1991, Identification of a novel UDP-Gal:GalNAc β1-4GlcNAc-R β1-3-galactosyltransferase in the connective tissue of the snail *Lymnaea stagnalis*. Eur. J. Biochem., 201, 459-465.
45. Mulder, H., Schachter, H., Thomas, J. R., Halkes, K. M., Kamerling, J. P., and Vliegenthart, J. F.G. 1996, Identification of a GDP-Fuc:Gal β1-3GalNAc-R (Fuc to Gal) α1-2fucosyltransferase and a GDP-Fuc:Gal β1-4GlcNAc (Fuc to GlcNAc) α1-3 fucosyltransferase in connective tissue of the snail *Lymnaea stagnalis*. Glycoconjugate J., 13, 107-113.
46. Joziasse, D. H., Damen, H. C. M., de Jong-Brink, M., Edzes, H. T., and Van den Eijnden, D. H. 1987, Identification of a UDP-Gal:β-galactoside β1,3-galactosyl-transferase in the albumen gland of the snail *Lymnaea stagnalis*. FEBS Lett., 221, 139-144.
47. Bakker, H., Agterberg, M., Van Tetering, A., Koeleman, C. A. M., Van den Eijnden, D. H., and Van Die, I. 1994, A *Lymnaea stagnalis* gene, with sequence similarity to that of mammalian β1-4-galactosyl-transferases, encodes a novel UDP-GlcNAc:GlcNAc βR β1-4-N-acetylglucosaminyltransferase. J. Biol. Chem., 269, 30326-30333.
48. Bakker, H., Schoenmakers, P. S., Koeleman, C. A. M., Joziasse, D. H., Van Die, I., and Van den Eijnden, D. H. 1997, The substrate specificity of the snail *Lymnea stagnalis* UDP-GlcNAc:GlcNAcβ-Rβ4-N-acetylglucosaminyltransferase reveals a novel variant pathway of complex-type oligosaccharide synthesis. Glycobiology, 7, 539-548.
49. Bakker, H., Van Tetering, A., Agterberg, M., Smith, A. B., Van den Eijnden, D. H., and Van Die, I. 1997, Deletion of two exons from the *Lymneaea stagnalis* β1,4-N-acetylglucosaminyltranferase gene elevates the kinetic efficiency of the encoded enzyme for both UDP-sugar donor and acceptor substrates. J. Biol. Chem., 272, 18580-18585.
50. Van Tetering, A., Schiphorst, WECM, Van den Eijnden, D. H., and Van Die, I. 1999, Characterization of a core α1-3-fucosyltransferase from the snail *Lymnaea stagnalis* that is involved in the synthesis of complex-type N-glycans FEBS Lett., 461, 311-314.
51. Van Die, I., Cummings, R. D., Van Tetering, A., Hokke, C. H., Koeleman, CAM, and Van den Eijnden, D. H. 2000, Identification of a novel UDP-Glc:GlcNAcβ1,4-glucosyltranferase in *Lymnaea stagnalis* that may be involved in the synthesis of complex-type oligo-saccharide chains. Glycobiology, 10, 263-271.
52. Lüttge, H., Heidelberg, T. Stangier, K., Thiem, J., and Bretting, H. 1997, The specificity of an α(1-2)-L-galactosyltransferase from albumen glands of the snail *Helix pomatia*. Carbohydr. Res., 297, 281-288.
53. Stangier, K., Lüttge, H., Thiem, J. E., and Bretting, H. 1995 Biosynthesis of the storage polysaccharide from the snail Biomphalaria glabrata, identification and specificity of a branching β1,6-galactosyltransferase. J. Comp. Physiol. B, 165, 278-290.
54. Bretting, H., Messer, M., Bornaghi, L., Kroger, L., Mitschnick, P., and Thiem, J. 2000, Galactan biosynthesis in snails: a comparative study of β1,6-galactosyltransferases from *Helix pomatia* and *Biomphalaria glabrata*. J. Comp. Physiol., 170, 601-613.
55. Bretting, H., Jacobs, G., Müller, S., and Breitfeld, O. 1991, The galactan-degrading enzymes in the snail *Biomphalaria glabrata*. Comp. Biochem. Physiol. B, 99, 629-636.

56. Yamaura, I., and Tatsumoto, T. 1993, Purification and some properties of endo1,4-β-D-mannanase from a mud snail, *Pomacea insularius* (de Ordigny) Biosci. Biotech. Biochem., 57, 1316-1319.
57. Tramice, A. Andreotti, G., and Trincone, A. 2008, Direct enzymatic glucosylation of naringin in grapefruit juice by α-D-glucosidase from the marine mollusc *Aplysia fasciata*. Biotechnol. J., 3, 545-554.
58. Hirata, K., Aso, Y., and Ishiguro, M. 1998 Properties of α-mannosidase partially purified from the apple snail *Pomacea canaliculata*. Biosci. Biotechnol. Biochem., 62, 2242-2245.
59. Hirata, K., Nakahara, Y., Kimura, Y., and Funatsu, G. 1996, Purification and some properties of a β-xylosidase and an α-fucosidase from apple snails (*Pomacea canaliculata*). *Biosci. Biotechnol.* Biochem., 60, 249-254.
60. Ootsuka, S., Saga, N., Suzuki, K., Inoue, A., and Ojima, T 2006, Isolation and cloning of an endo-β1,4-mannosidase from Pacific abalone *Haliotis discus hanna*i. J. Biotechnol., 125, 269-280.
61. Imjongjirak, C., Amparyup, P., and Sittipraneed, S. 2008, Cloning, genomic organization and expression of two glycosyl hydrolase family 10 (GHF10) genes from golden apple snail (*Pomacea canaliculata*). DNA Seq., 19, 224-236.
62. Dissous, C., Grzych, J. M., and Capron, A. 1986, *Schistosoma mansoni* share a protective oligosaccharide epitope with freshwater and marine snails. Nature, 323, 443-445.
63. Mansour, M. M., Ali, P. O., Farid, Z., Simpson, A. J., and Woody, J. W. 1989, Serological differentiation of acute and chronic schistosomasis mansoni by antibody responses to keyhole limpet hemocyanin. *Am*. J. Trop. Med. Hyg., 4, 338-344.
64. Alves-Brito, C. F., Simpson, A. J., Bahia-Oliveira , L. M., Rabello, A. L., Rocha, R. S., Lambertucci, J. R., Gazzinelli, G., Katz, N., and Correa-Oliveira, R. 1992, Analysis of anti-keyhole limpet haemocyanin antibody in Brazilians support its use for the diagnosis of acute schistosomiasis mansoni. Trans. R. Soc. Trop. Med. Hyg., 86, 53-56.
65. Xue, C. G., Taylor, M. G., Bicklie, Q. D., Savioli, L., and Renganathan, E. A. 1993, Diagnosis of *Schistosoma haematobium* infection: evaluation of ELISA using keyhole limpet haemocyanin or soluble egg antigen in comparison with detection of eggs or haematuria. Trans. R. Soc. Trop. Med. Hyg., 87, 654-658.
66. Li, Y., Rabello, A. L., Simpson, A. J., and Katz, N. 1994, The serological differentiation of acute and chronic Schistosoma japonicum infection by ELISA using keyhole limpet haemocyanin as antigen. Trans. R. Soc. Trop. Med. Hyg., 88, 249-251.
67. Verweij, J. J., Polderman, A. M., Visser, L. G., and Deelder, A. M. 1995, Measurement of antibody response to keyhole limpet haemocyanin was not adequate for early diagnosis of schistosomiasis in a group of Dutch visitors to Mali. Trans. R. Soc. Trop. Med. Hyg., 89, 48-50.
68. El-Ansary, A., and Al-Daihan, S. 2006, Important aspects of Biomphalaria snail-schistosome interactions as targets for antischistosome drug. Med. Sci. Monit., 12, RA282-292.
69. Fenion, S., Ellis, I. O., Bell, J., Todd, J. H., Elston, C. W., and Blamey, R. W. 1987, *Helix pomatia* an *Ulex europeus* lectin binding in human breast carcinoma. J. Pathol., 152, 169-176.
70. Linn, J., Black, P., Derksen, K., Rubben, H., and Thuroff, J. W. 2000, Keyhole limpet haemocanain in experimental bladder cancer: literature review and own results. Eur. Urol., 37 (Suppl.)3, 34-40.
71. McFadden, D. W., Riggs, D. R., Jackson, B. J., and Vona-Davis, L. 2003, Keyhole limpet hemocyanin, a novel immune stimulant with promising anticancer activity in Barrett´s esophageal adenocarcinoma. Am. J. Surg., 186, 552-555.
72. Krug, L. M., Ragupathi, G., Hood C., Kris, M. G., Miller, V. A., Allen, J. R., Keding, S. J., Danishefsky, S. J., Gomez, J., Tyson, L., Pizzo, B., Baez, V., and Livingston, P. O. 2004, Vaccination of patients with small-lung cancer with synthetic fucosyl GM-I conjugated to keyhole limpet hemocyanin. Clin. Cancer Res., 10, 6094-6100.

10 Table of abbreviations

(k)Da	(kilo) Dalton
2D	2-dimensional
AA	anthranilic acid; 2-aminobenzoic acid
AB	2-aminobenzamide
Ac	acetyl
All	allose
Alt	altrose
AMAC	2-aminoacridone
ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid
APTS	8-aminopyrene-1,3,6-trisulfonic acid
Ara	arabinose
Asn	asparagine
ATT	6-aza-2-thiotymine
CE	capillary electrophoresis
DEAE	diethylaminoethyl
DHA	2,5-dihydroxyacetophenone
DHB	2,5-dihydroxybenzoic acid
DNA	Deoxyribonucleic acid
EI	electron impact
ER	endoplasmatic reticulum
ESI	electrospray ionization
FACE	fluorophore assisted carbohydrate electrophoresis
Fuc	fucose
GAG	glycosaminoglycan
GalN	galactosamine
GalNAc	N-acetylgalactosamine
GalUA	galacturonic acid
GC	gas chromatography
GlcN	glucosamine
GlcNAc	N-acetylglucosamine
GlcUA	glucuronic acid
GPI	glycosylphosphatidylinositol
Gul	gulose
Hex	hexose
HexNAc	N-acetylhexosamine
HIC	hydrophobic interaction chromatography
HILIC	hydrophilic interaction chromatography
HPAEC-PAD	High-pH anion-exchange chromatography with pulsed amperometric detection
HPLC	high performance liquid chromatography
Hyl	hydroxylysine
Hyp	hydroxyproline
Ido	idose

IM	isomaltose
IT	ion trap
LC	liquid chromatography
LU	light unit
m/z	mass-to-charge ratio
MALDI	matrix-assisted laser desorption/ionization
ManN	mannosamine
ManNAc	N-acetylmannosamine
mAU	milli absorbance unit
Me	methyl
MS	mass spectrometry
MWCO	molecular weight cut-off
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NMR	nuclear magnetic resonance
NMWL	nominal molecular weight limit
NP	normal phase
PA	2-aminopyridine
PAGE	polyacrylamide gel electrophoresis
PGC	porous graphitized carbon
PMAA	partially methylated alditol acetate
PMP	1-phenyl-3-methyl-5-pyrazolone
Q	quadrupole
R_g	radius of gyration
Rib	ribose
RNA	ribonucleic acid
RP	reversed phase
SDS	sodium dodecyl sulfate
Ser	serine
Sia	sialic acid
SPE	solid phase extraction
Tal	talose
Thr	threonine
TOF	time-of-flight
Tyr	tyrosine
UDP	uracil-diphosphate
UV	ultraviolet
Xyl	xylose