Chapter 4

Liquid Chromatography – Mass Spectrometry of Carbohydrates Derivatized with Biotinamidocaproyl Hydrazide

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Additional information is available at the end of the chapter

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1. Introduction

Glycans are involved in a number of cell processes. The glycocomponents of a protein encompass structural and modulatory functions, trigger receptor/ligand binding and many other processes which include mechanisms of interest to immunology, hematology, neurobiology and others (Marino *et al.*, 2010; Raman *et al.*, 2005). Because they are associated with both the physiological and the pathological processes, there is constant interest paid to analysis of protein glycosylation and its changes (North *et al.*, 2009; Leymarie & Zaia, 2012).

In order to get a full picture about the structure and function of a carbohydrate, combination of different analytical methods, detection and derivatization techniques is usually applied (Bindila & Peter-Katalinic, 2009; Geyer & Geyer, 2006; Harvey, 2011). Derivatization of sugars for the purpose of their structural or functional analysis bears various advantages (Rosenfeld, 2011). Most of the labels used for the derivatization of carbohydrates possess a chromophor or a flurophor, enabling a sensitive detection of these analytes by means of spectroscopic methods. Apart from an increase in detection sensitivity, chromatographic properties of derivatized sugars may improve in comparison with their underivatized counterparts (Melmer *et al.*, 2011; Pabst *et al.*, 2009). Furthermore, mass spectrometric signal intensity showed to profit from the derivatization step, as an effective ionization was achieved through derivatization. Moreover, depending on the type of the carbohydrate considered, mass spectrometric fragmentation showed to be influenced by the kind of label introduced, and provided additional information about the structure of sugars (Lattová *et al.*, 2005).

There are many possible labelling reactions for the generation of respective derivatives. The most applied derivatization method is the reductive amination via Schiff-base in the presence of the sodium cyanoborohydride as reductant. The greatest success and a broad area of usage

over the last decades experienced the arylamine tag such as 2-aminobenzamide (2-AB) and is still extensively used in mass spectrometric studies of glycans (Bigge *et al.*, 1995). Other common tags from this group are 2-aminobenzoic acid (2-AA), 2-aminopyridine (2-AP) and 2-aminoacridone (2-AMAC). Another labelling approach which has been widely used is the formation of hydrazones, where the hydrazine label of the tag reacts with the aldehyde group of the sugar. The mentioned approaches and other derivatizations which have shown their use in analytics of glycans were reviewed recently (Harvey, 2011; Ruhaak *et al.*, 2010).

Generally, selection criteria for a certain derivatization approach depend on many different factors, including the nature of the sample, the compatibility of the label with the analytical protocol being applied and very importantly, the objective of the analysis. Here, very often, not only the structure of a glycan is a matter of a question but also its functionality, which is an essential feature, that often wants to be examined. Consequently, a derivatization method, which would allow for both the structural and functional studies of sugars can be of interest in the characterization of the glycan species. Such a possibility is given e.g. by biotinylated reagents (Grün *et al.*, 2006; Hsu *et al.*, 2006; Leteux *et al.*, 1998; Ridley *et al.*, 1997; Rothenberg *et al.*, 1993; Shinohara *et al.*, 1996; Toomre & Varki, 1994).

In our recent study, biotinamidocaproyl hydrazide (BACH) was presented as a label for carbohydrates analyzed by mass spectrometry (Kapková, 2009). This bifunctional label combines two features: the sugar reactivity and the bioaffinity. In this way, beside the structural characterization of carbohydrates, it enables also for performing interaction studies with carbohydrate-binding proteins. The derivatization was performed under non-reducing conditions via hydrazone formation and showed an increase in the ion abundance of small sugars and *N*-linked glycans.

Here, chip-based electrospray tandem mass spectrometric structural analysis of different glycan-derivatives generated via BACH-labeling using triple quadrupole instrument was performed. The methodology has been applied to glycans released from glycoproteins (ovalbumin, ribonuclease B) including different types of *N*-glycans e.g. high-mannose, complex and hybride glycans. Further, an analysis of small BACH-labeled carbohydrates was conducted as these represent important building unit of the large glycan entities. Reversed liquid chromatography and hydrophilic interaction chromatography (HILIC) coupled with ion-trap mass spectrometry (LC-ESI-IT-MS) of BACH-derivatized monosaccharides were carried out. Advantageous properties of HILIC in terms of separation of non-reducing trisaccharides and BACH-derivatized isomeric disaccharides are presented. Here, derivatization helped to obtain a favorable separation profile or a higher signal intensity. The isomers were compared in their fragmentation pattern, and a distinction of their linkage or anomeric configuration was sought.

2. Experimental

2.1. Materials

Hen ovalbumin, ribonuclease B from bovine pancreas, melezitose, raffinose and biotinamidocaproyl hydrazide were purchased from Sigma-Aldrich (Taufkirchen,

Germany). The BACH-label was used without further purification. Monosaccharides (glucose, galactose, mannose, fucose, xylose, *N*-acetylglucosamine) and disaccharides (maltose, cellobiose) were kindly provided by the Division of Chemicals (Department of Pharmacy, University Würzburg). The standard *N*-glycans MAN5, MAN6, NA2, NGA2 as well as PNGase F and the GlycoClean™ S Cartridges were from Europa Bioproducts Ltd. (Cambridge, UK). The water was from a Milli-Q system.

2.2. Enzymatic digestion of glycoproteins

About 500µg of the protein were dissolved in water (40µl) and reaction buffer (10µl) and denatured by boiling in a water-bath (100°C, 10min). After cooling, the samples were incubated with PNGase F (2µl) at 37°C for 18 hours. By adding 200µl of cold ethanol and keeping in ice for 2h, the deglycosylated protein was precipitated and centrifuged down to a pellet. The supernatant, containing the glycans, was dried in vacuum and then the residue was derivatized.

2.3. Derivatization with BACH

The released glycans from glycoproteins were mixed with 100µl of a 5mmol solution of BACH in 30% acetonitrile and subsequently evaporated. For the derivatization of standard *N*-glycans (0.5µg) the same protocol was followed. According to the evaporation, the mixture was dissolved in 30µl of methanol/water (95/5), and incubated at 90°C for 1h. Then the BACH conjugates were further purified using GlycoClean™ S cartridges according to the accompanying protocol.

2.4. Derivatization with 2-Aminobenzamid (2-AB)

50µl of the AB-derivatization solution, containing 2-AB (5mg), $NaBH_3CN$ (7.5mg), DMSO (500µl) and acetic acid (200µl), were added to the glycans or small sugars. After the incubation at 65°C for 2.5h, the AB-conjugates were purified with GlycoClean™ S cartridges according to the manufacturer's protocol.

2.5. LC/ESI-MS

2.5.1. Nano LC/ESI-MS of N-glycans

The mass spectra were measured on the triple quadrupole mass spectrometer 6460 (Agilent Technologies, Waldbronn, Germany) coupled with an Agilent 1200 liquid chromatography nanoflow system with a ChipCube interface. The HPLC-Chip used for the separation contained a 0.075 × 43mm porous graphitized carbon column and an integrated 40nl enrichment column. This column was filled with identical media, e.g. the same phase was used for both the separation and trapping. Solvents for the capillary and the nanoflow pump were A: 0.1% formic acid in water and B: 0.1% formic acid in methanol. The chromatographic conditions were as follows: capillary pump flow rate (1µl/min), nanoflow

pump (0.4μl/min for standard glycans and 0.5μl/min for released glycans); 4μl of the samples were loaded. Gradient was set from 30% to 90%B in 50min. MS conditions were: positive ionization, drying gas flow 4l/min at a drying temperature of 325°C, capillary voltage 1800V, and a scan range from 300 to 2200 m/z.

The MS/MS experiments were performed based on the collision-induced dissociations (CID). In order to selectively monitor the glycans in the triple quadrupole instrument, precursor ion scanning was performed. Characteristic fragment ions used in this mode were the oxonium ions of hexose (HexNAc) at m/z 163 and of *N*-acetylhexosamine (HexNAc) at m/z 204 and the larger oxonium ion at m/z 366 (HexHexNAc). In this approach, quadrupole 1 works as a scanner across the range of interest in order to determine the mass of injected glycans, the second quadrupole acts as a collision cell and the third quadrupole serves to analyze the fragment ions generated in the collision cell. Upon the detection of precursor ions which are losing a specific fragment ion of interest (oxonium ion) the precursor ions of glycans were analyzed in the product ion scan mode which gave the fragment spectrum of the glycan.

2.5.2. LC/ESI-MS of mono-, di- and trisaccharides

Analyses of small sugars were performed on the LC-MSD ion-trap instrument (Agilent) operated with Agilent HPLC 1100 or infusion syringe pump. The separation was conducted on the C18 reversed-phase and HILIC-phase (Kinetex™, Phenomenex, 100 × 2.1 mm, 100Å, 2.6 μm). The chromatographic conditions of C18 were: A: 100mM ammonium acetate (pH 5.8), B: MeOH (0.1% formic acid), flow: 0.3 mL/min, gradient: 0 min, 11% B; 17 min, 11% B; 30 min, 50% B. The chromatographic conditions of HILIC: A: 100mM ammonium acetate (pH 5.8), B: acetonitril (0.1% formic acid), flow: 0.3 mL/min, gradient: 0 min, 99% B; 12 min, 99% B, 16min, 92% B; 30min, 60% B.

3. Results and discussion

3.1. Derivatization with BACH

Labeling reagent biotinamidocaproyl hydrazide (BACH) is a bifunctional label which possesses a hydrazide group for coupling with the reducing end of sugars and a biotin group in order to interact with a solid support which possesses affinity to biotin. This chemical process may be run under reducing or non-reducing conditions depending on the purpose of the analysis. Because the reaction with hydrazines gives much more stable products than the easily reversible Schiff-base generated by the reaction of an amine with an aldehyde, carbohydrate-hydrazone bond formed *via* hydrazide chemistry does not necessarily require a reducing agent (Hermannson, 2008).

In this way, the resultant product is a glycosylhydrazide (Fig. 1), which preserves the pyranose ring closed, i.e. it preserves its near-native conformation. This is a favorable feature in many functional studies where the closed pyranose ring is essential, in order to be recognized by a carbohydrate-binding protein. Differences in binding activity of certain

sugars have been shown (Grün *et al.*, 2006), when reacting with lectins or other carbohydrate binding proteins. Hence, whether a biological interaction is affected by the reduction or not, depends on the type of the sugar and on the kind of the carbohydrate binding protein.

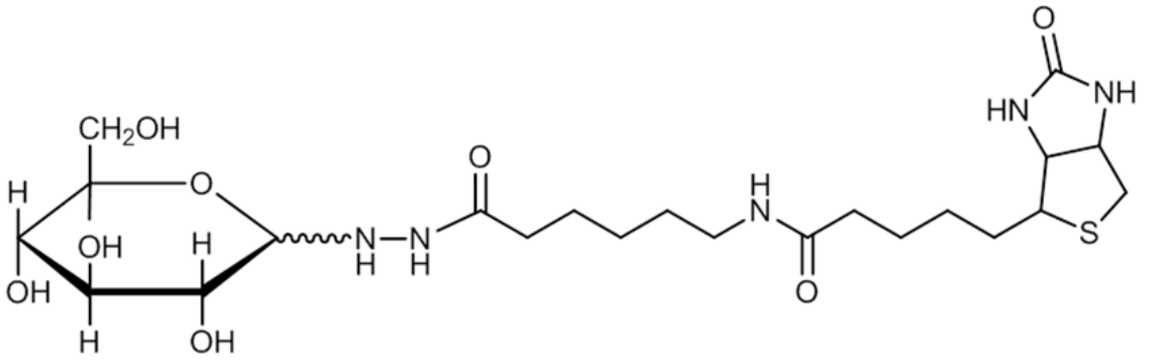


Figure 1. Scheme of glycosylhydrazide formation of carbohydrate with biotinamidocaproyl hydrazide

3.2. Structural characterization of BACH-derivatized *N*-linked glycans

In general, the structures of *N*-linked glycans fall within three main types, namely, high-mannose, complex and hybrid. These all share a common chitobiose core and differ in branching patterns mainly at the non-reducing end.

Here, different standard asparagine-linked glycans and glycans released from model glycoproteins such as ovalbumin and ribonuclease B (RNAse B) were analyzed by means of nano-LC-chip/electrospray mass spectrometry in order to cover the main types of *N*-linked glycans. The *N*-linked glycans were released by treating the glycoproteins with peptide-*N*-deglycanase F (PNGaseF). Released glycans were labeled with the biotinamidocaproyl hydrazide and analyzed by nanoLC- triple quadrupole mass spectrometry using the precursor ion scan mode. The oxonium ions chosen for the scanning mode were m/z 163 (Hex+H), m/z 325 (Hex-Hex+H) and m/z 204.1 (GlcNAc+H). Fragmentation was carried out at three different collision energies (15V, 25V and 50V). The best fragmentation spectra resulted from the application of the collision energy of 15V and this was used throughout. Tandem mass spectra of the respective glycans were recorded in the product ion scan mode by the collision induced dissociation.

RNase B has one glycosylation site occupied by five high-mannose glycans differing in one mannose residue. Because of the high-mannose nature of these glycans, precursor ion scanning with the oxonium ion of hexose (m/z 163) was performed and the resultant mass spectra showed high abundance.

Fig. 2 shows the mass spectrum of the BACH-derivatized high-mannose glycans present in RNaseB and features the characteristic spacing pattern of its glycoforms $[(Man)_nGlcNAc)_2$ with n = 5,6,7,8,9]. The signals were registered as doubly charged hydrogenated species. Hence, the spectrum exhibits the differences of 81 Da, which account for half of the mass of one mannose unit. The most abundant glycoforms were MAN6 and MAN5. This proportionality in pattern was observed also in studies on high-mannose glycopeptides (Alley *et al.*, 2009).

In Fig. 3 the tandem mass spectrum of the doubly charged BACH-labeled MAN6 glycan is shown, which is dominated by the glycosidic cleavages, which correspond with losses of mannose residues. The prominent signals were the doubly charged signals related to consecutive losses of 81Da directly from the molecular ion $[M+2H]^{2+}$. A series of weaker singly charged ions corresponded to losses of mannose residues (162Da) from the chitobiose cleavage between the two GlcNAc-moieties (B_4-ion). In this way, the collision-induced dissociation of BACH-derivatized ribonuclease B glycans provided fast and informative spectra in order to characterize the identity of the analyzed glycans. Fragments have been assigned using the systematic nomenclature for carbohydrate fragmentation proposed by Domon and Costello (Domon & Costello, 1988).

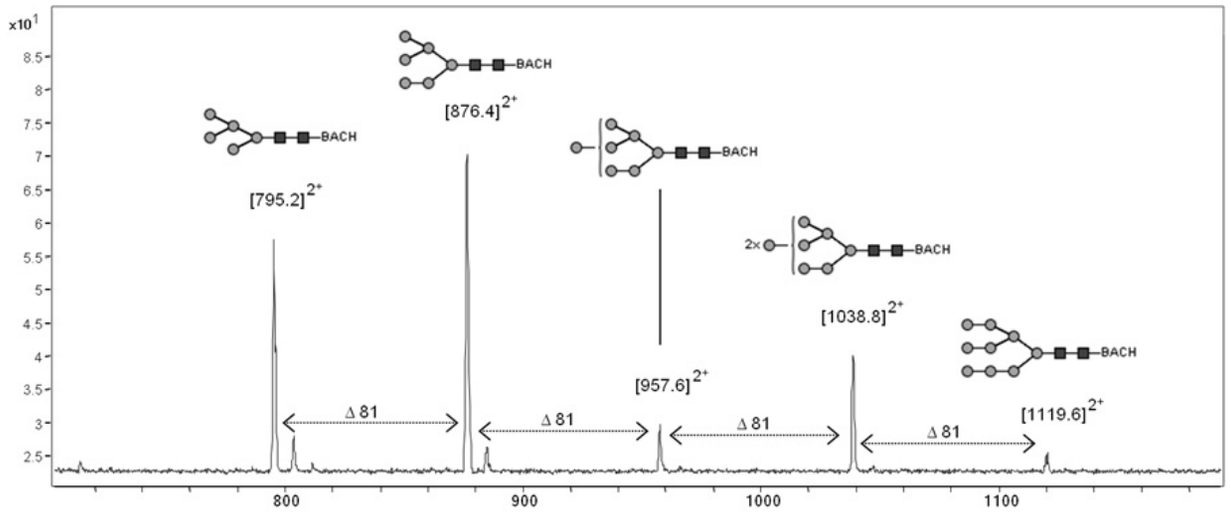


Figure 2. Precursor ion scan mass spectrum of BACH-biotinylated high-mannose glycans from ribonuclease B for oxonium ion of hexose at m/z 163. Doubly charged ions show the differences of 81Da.

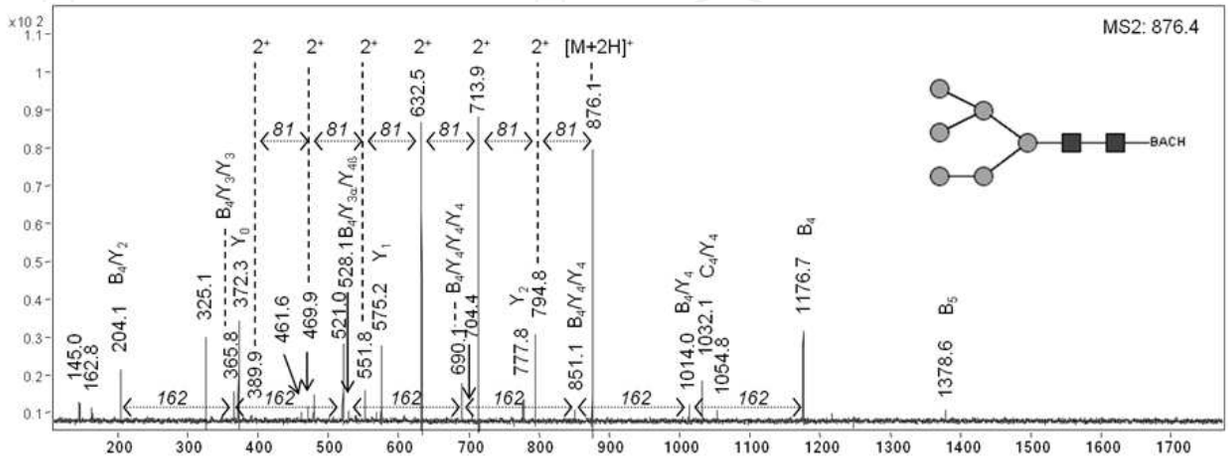


Figure 3. Product ion mass spectrum of the high-mannose glycan $(GlcNAc)_2(Man)_6$ prepared by BACH-derivatization. Spectrum was derived by fragmenting the $[M+2H]^{2+}$ glycan precursor ion of the m/z 876.4.

Ovalbumin, a major glycoprotein in hen egg white, contains one glycosylation site with a series of asparagine-linked oligosaccharides of mannose- and hybrid-type; some rare complex-type oligosaccharides are also known. Due to its structural diversity, this protein is an optimal source of a library of *N*-linked glycans and a good model glycoprotein. The mixture of glycans released from ovalbumin was analyzed by precursor ion scanning in the triple quadrupole. The oxonium ion which has been scanned for was the protonated *N*-acetylglucosamine (GlcNAc) at m/z 204. The mass spectrum of the identified glycans can be seen in the Fig. 4. Found oligosaccharides cover the glycan forms typically present in this glycoprotein (Fig. 4, Table 1). Under the conditions described in the experimental part, the glycan ions appeared as doubly or triply charged ions. Doubly charged ions were fragmented by product ion scanning and their structure was elucidated. Elucidation of the fragment spectra of triply charged ions was a cumbersome task, as the spectra contained ions with different charge states. The fragment ion with m/z 204, corresponding to the oxonium ion $[HexNAc+H]^+$, was frequently observed in the MS/MS spectra of ovalbumin glycans. Generally, this type of ion is highly diagnostic of the presence of glycans that terminate in one or more *N*-acetylglucosamine residues.

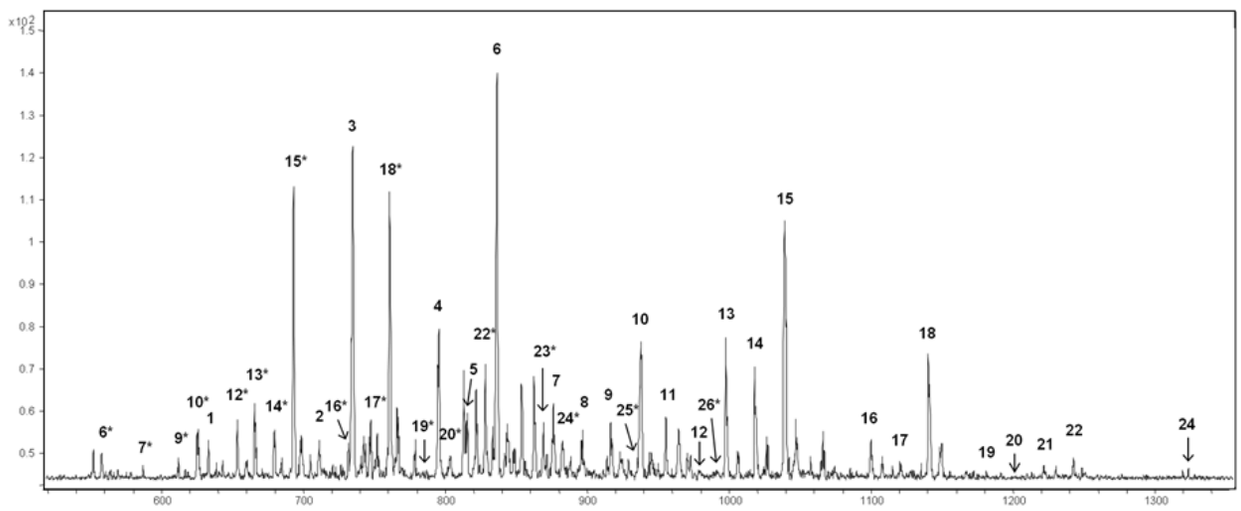
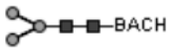
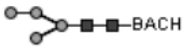
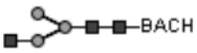
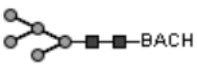
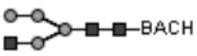
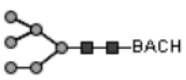
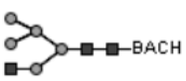
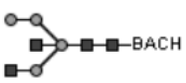
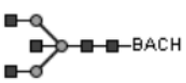
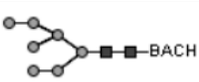
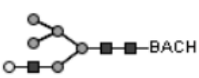
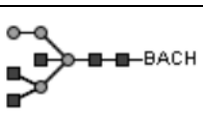
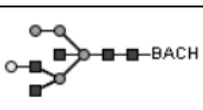


Figure 4. Precursor ion scan for oxonium ion of *N*-acetylglucosamine at m/z 204 of BACH-biotinylated glycans from ovalbumin. Doubly and triply (*) charged ions are depicted. For the structure see the Table 1.

Compd.[1]	Comp.[2]	Observed *m/z* BACH-glycans, $[M+2H]^{2+}/[M+3H]^{3+}$ *	Structure
1	H_3N_2	632.9	
2	H_4N_2	714.0	
3	H_3N_3	734.5	
4	H_5N_2	795.4	
5	H_4N_3	815.4	
6	H_3N_4	836.4/557.4*	
7	H_6N_2	876.1/584.5*	
8	H_5N_3	896.7	
9	H_4N_4	916.5/611.9*	
10	H_3N_5	937.8/625.6*	
11	H_7N_2	956.6	
12	H_6N_3	978.0/652.3*	
13	H_5N_4	997.6/665.4*	
14	H_4N_5	1018.0/679.8*	
15	H_3N_6	1039.1/692.8*	
16	H_5N_5	1099.8/733.6*	

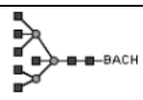
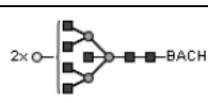
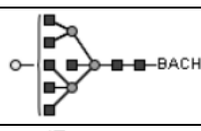
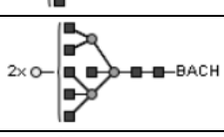
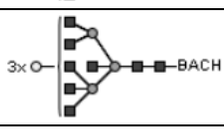
Compd.[1]	Comp.[2]	Observed *m/z* BACH-glycans, $[M+2H]^{2+}/[M+3H]^{3+}$ *	Structure
17	H_4N_6	1120.0/747.3*	
18	H_3N_7	1140.0/760.5*	
19	H_6N_5	1181.0/786.8*	
20	H_5N_6	1201.9/801.8*	
21	H_4N_7	1221.9	
22	H_3N_8	1242.5/828.2*	
23	H_5N_7	869.2*	
24	H_4N_8	1323.5/882.6*	
25	H_5N_8	936.8*	
26	H_6N_8	990.9*	

Table 1. Composition and m/z of the doubly and triply charged (*) glycans released by PNGase from the hen ovalbumin observed by precursor ion scanning experiment (m/z 204). For the spectrum see the Fig. 4. [1]Compound number, [2]Composition of *N*-glycans (H = hexose, N = GlcNAc)

Several diagnostic ions were present in the ESI-MS/MS spectra of complex and hybride glycans from ovalbumin. Fig. 5 shows the tandem mass spectrum of one of the complex glycans of ovalbumin. The MS/MS fragmentation of this BACH-derivatized glycan was characterized by sequential losses of sugar residues from its non-reducing end. Arrows with continuous lines indicate differences in the number of mannose, and the dashed lines in the number of *N*-acetylglucosamines.

The Fig. 6 represents the product ion spectrum of the galactosylated standard glycan NA2. Several diagnostic ions were present in the MS/MS spectrum. The loss of the protonated fragment with m/z 366 was the preferred route in the breaking up of this galactosylated species. The oxonium ion is diagnostic of the loss of a fragment containing a hexose residue attached to *N*-acetylhexosamine. The sequencial losses of 365 (i.a. Hex-HexNAc) from the

precursor ion (m/z 1630 and m/z 1264) show that this glycan accommodates two galactosyl-*N*-acetylglucosamine residues. The appearance of the fragment at m/z 1264 distinguishes this molecule from the core-bisected species.

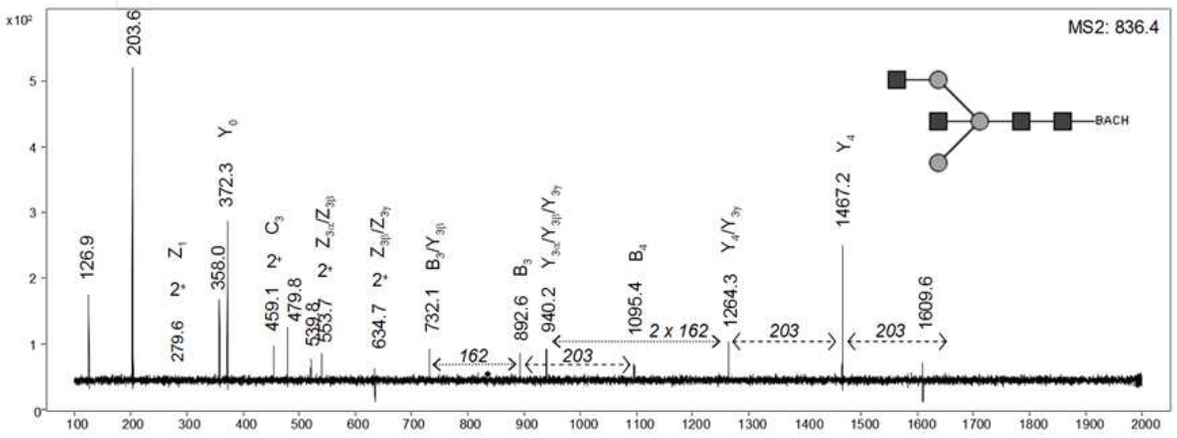


Figure 5. Product ion scan of BACH-derivatized ovalbumin glycan $GlcNAc_4Man_3$ at m/z 836.4.

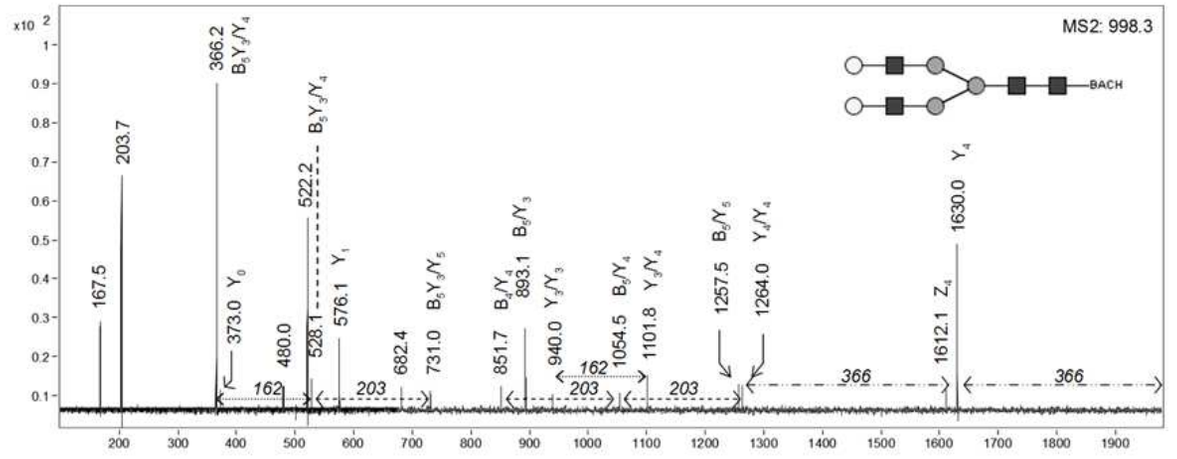


Figure 6. Product ion mass spectrum of the galactosylated NA2 glycan derivatized with BACH. The fragmentation was performed by collision-induced dissociation of the $[M+2H]^{2+}$-ion at the m/z 998.3

When compared with literature of frequently used 2-AB derivatization, tandem mass spectra of BACH-derivatives showed similar fragmentation as 2-AB-derivatives encompassing dominant glycosidic cleavages from reducing and non-reducing end (Harvey, 2000; Morelle & Michalski, 2004; Wuhrer *et al.*, 2004). These simple spectra make the MS/MS elucidation a straightforward task. Moreover, one should keep in mind that with BACH-derivatives not only the structure of the glycans could be explored, but also the function (Kapková, 2009). The bioaffinity of the BACH-label makes these derivatives suitable for the experiments exploring the interaction capabilities of a glycan (Wuhrer *et al.*, 2010).

Usually, the complete sugar analysis encompasses the characterization of the composition, sequence, branching and linkage of the monosaccharide residues constituting the oligosaccharides. The fragments arising from glycosidic cleavages are useful for the elucidation of the sugar composition, sequence and branching pattern of the glycan. Using

this methodology alone, the type of linkage between the various sugar residues was not obtained. However, combining the product ion scanning with other methods e.g. enzymatic exoglycosidase digestion studies would provide some of this information.

3.3. LC-MS of BACH-derivatized monosaccharides

Mixture of carbohydrates (*N*-acetylglucosamine (GlcNAc), galactose (Gal), mannose (Man), Fucose (Fuc) and Xylose (Xyl)) which are typically present in mammalian proteins was analyzed by ESI-MS in the ion trap. Comparative examinations of pre-column labeled BACH-monosaccharides were performed by means of reversed phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) as these methods shown to be highly complementary in resolution of mixtures of carbohydrates (Melmer *et al.*, 2011; Schlichtherle-Cerny *et al.*, 2003; Strege *et al.*, 2000). HILIC is based on the hydrophilic character of the analyte which accounts for the interaction of the molecule with the stationary phase (Wuhrer *et al.*, 2009). In our study, diol-modified silica phase was used. Chemically bonded diol groups were shown in the past to give similar functions as an amino-bonded silica. Moreover, they possess better stability than amino-sorbent and provide a good separation of structurally related polar molecules, e.g. sugars (Brons & Olieman, 1983; Churms, 1996; Ikegami *et al.*, 2008).

The BACH-derivatized monosaccharides, non-labeled and 2-AB-labeled counterparts (as 2-AB-labeling is one of the most widely used methods) were compared in terms of their chromatographic behavior and signal intensity of the mass spectrometric measurement.

Under the conditions used, underivatized sugars did not provide sufficient retention and separation on either of the phases (C18 and HILIC) and eluted almost at the same time (t_R = 1 min, C18; t_R = 2-5 min, HILIC) with a poor sensitivity (data not shown), especially for glucose, xylose and fucose (Intens. = 0.5 × 10^6). In order to seek an enhanced detection and better separation, derivatization of sugars was performed. After the derivatization with BACH or 2-AB, mass spectrometric signal intensity significantly enhanced about an order of a magnitude (BACH-derivatives Ints. = 0.5-4 ×10^7, 2-AB-derivatives 0.5-3 ×10^7). The extent of the intensity enhancement depended on the kind of the sugar analyzed (Fig. 7).

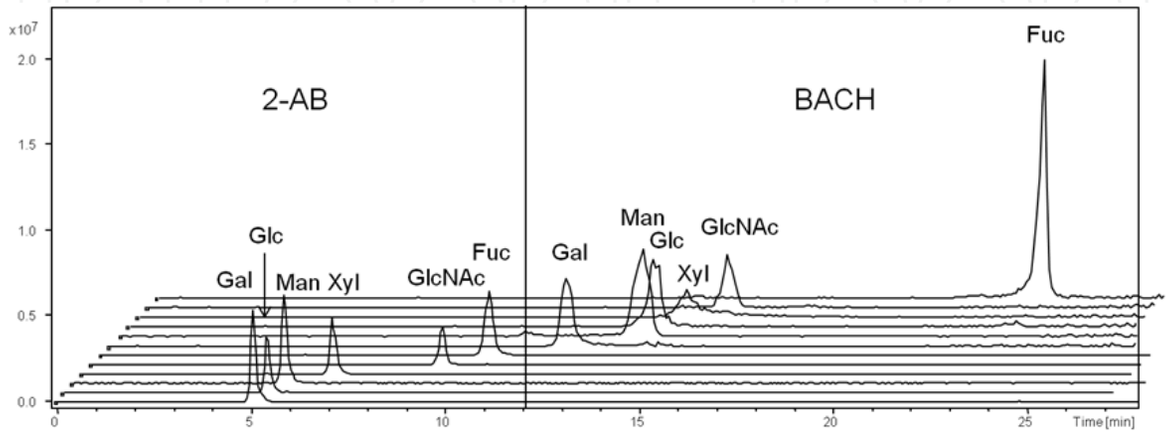


Figure 7. Total ion chromatogram (TIC) of RPLC-MS of 2-AB and BACH-derivatized monosaccharides

The BACH-derivatives interacted with the C18-phase ~8-15 min longer than the respective 2-AB-derivatives. The elution order of the sugars on the C-18-phase remained similar except for the triplet of the sugars galactose, glucose and mannose (Gal-2-AB, Glc-2AB, Man-2AB and Gal-BACH, Man-BACH, Glc-BACH). Galactose and mannose separation was achieved with both labels under the named conditions on the RP (Fig. 7), whereas the BACH-derivatives provided a clear baseline resolution.

Glucose is rarely present in glycoproteins. It can however originate from hydrolysis of contaminating polysaccharides from the environment. This problem was often encountered in reversed phase chromatography of monosaccharides (Toomre & Varki, 1994; Kwon & Kim, 1993) where glucose (at least partially) overlaps with the peak of galactose. Therefore, glucose contamination in carbohydrate samples should be addressed and examined.

In our experiments on C18 and HILIC-phase, 2-AB-derivatized glucose and galactose eluted very close to each other (Fig. 7 and Fig. 8, respectively). Through BACH-derivatization, on the other hand, glucose and galactose could have been separated on C18 (Fig. 7). However, the separation of glucose from mannose was still insufficient with BACH-labeling.

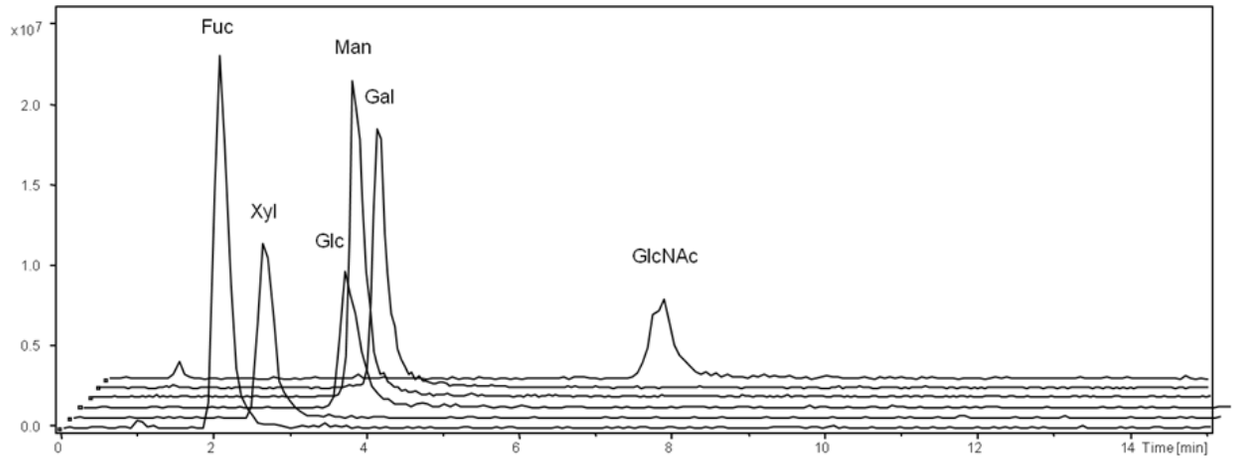


Figure 8. Hydrophilic interaction LC/MS of 2-AB-derivatized monosaccharides

A complete separation of glucose from galactose and mannose was reached only after the application of BACH-sugars to the HILIC-phase as shown in Fig. 9. However, the monosaccharides galactose and mannose could have been separated from each other only by means of RP chromatography (Fig.7). In this regard, the two phases (RP and HILIC) provided complementary features and their interchange may help to solve some analytical obstacles.

The BACH-label was used in the original form without any purification after its purchase. The presence of an isomeric byproduct was evident when the chromatography of the native label was performed. Two peaks (t_R: 16.8 min and 24 min, C18; 2 min and 6 min, HILIC) with the same m/z at 372 were present when chromatographically examined (data not shown). An example of this fact can be seen later in Fig.11 with the disaccharides, where the unreacted BACH shows two peaks. Due to this fact, we obtained after derivatization of saccharides a main product with a byproduct of the same m/z (Fig. 9). When necessary, the

byproduct can be easily purified away with the excess BACH-label. Detailed structure of this byproduct is under investigation. Our further measurements concentrated only on the main product. As the BACH-tag eluted on the reversed phase between 16- 17 min with other sugars such as Man and Glc, a simultaneous clean-up of this label with the method on the C18 would not be feasible. However, when applied to HILIC-phase, the BACH-reagent eluted earlier than all the sugars and could be separated away when desired. As 2-Aminobenzamid label eluted before the mixture of monosaccharides on both stationary phases RP (6 min.) and HILIC (1.5 min.), a clean-up of the 2AB-label by this methodology would be very straightforward by both chromatographic methods.

The peaks of the investigated monosaccharides on the HILIC phase show broadening or splitting (Fig. 9). This is due to mutarotation of the sugars and consequently the presence of its anomers. The broadening is more evident with sugars, which mutarotate fast e.g. glucose and mannose. This phenomenon was observed with small reducing sugars on the HILIC-phase already in the past (Churms, 1996; Moriyasu *et al.*, 1984).

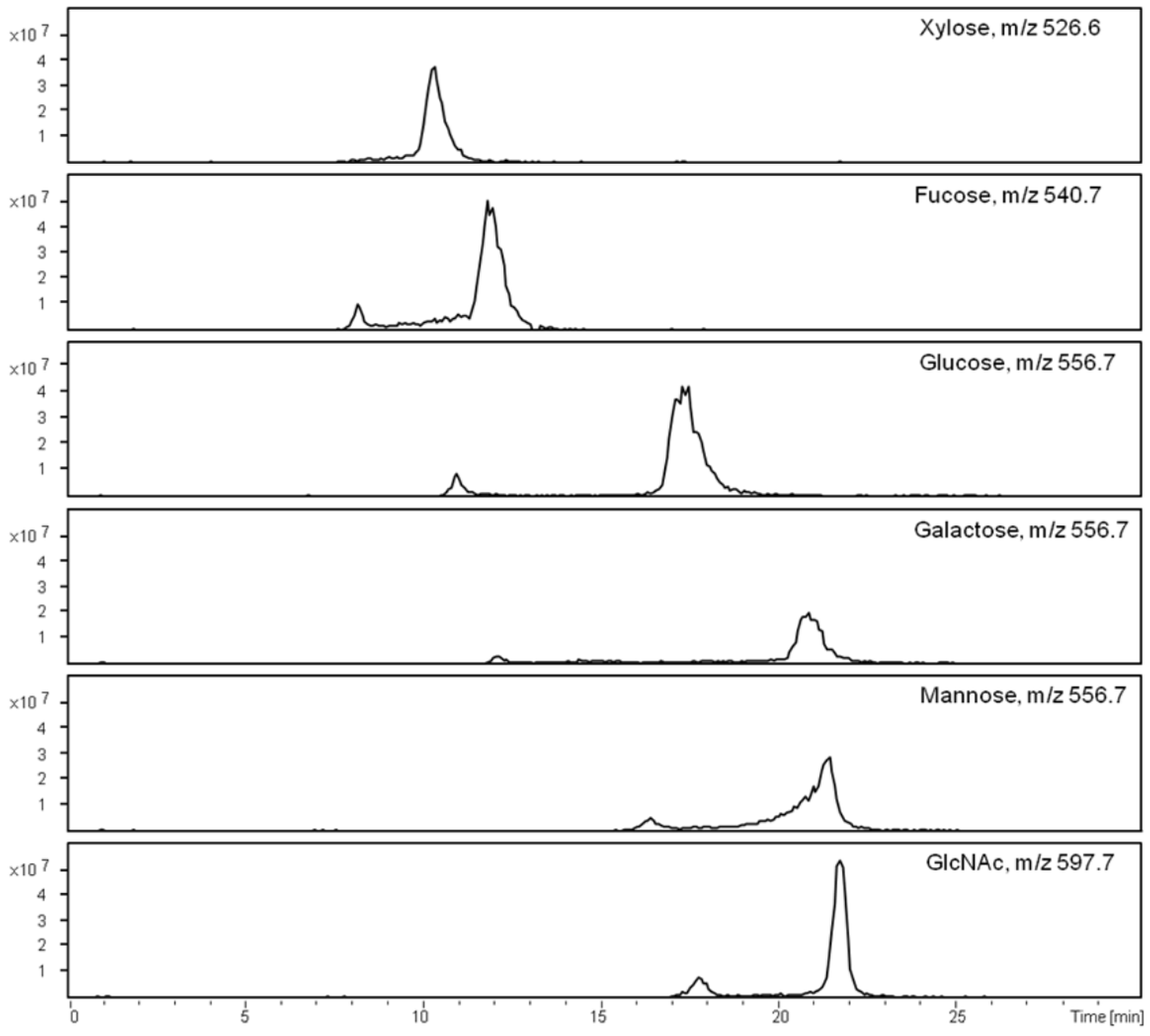


Figure 9. Extracted ion chromatograms (EIC) of BACH-derivatized monosaccharides separated by hydrophilic interaction liquid chromatography.

If the mutarotation should be suppressed, chromatography on the reversed phase can be followed and usage of a solvent with a higher content of organic phase might be preferable. A full separation of such isomeric structures (anomers) was achieved hitherto by means of chiral HPLC (Lopes & Gaspar, 2008), ion-exchange chromatography or amino-columns (Schumacher & Kroh, 1995; Nishikawa *et al.*, 1996; Moriyasu *et al.*, 1984). More recently, separation of metal adducts of anomeric methyl glycoside isomers was achieved by ion mobility mass spectrometry (Dwivedi *et al.*, 2007).

3.3.1. MS/MS of BACH-derivatized monosaccharides

The BACH-derivatized monosaccharides were subjected to tandem mass spectrometry. Analysis by ESI-ion trap showed that sodium adducts were the most abundant ions. Less abundant $[M+H]^+$ ions were detected in positive ion mode as well. Negative ion mode MS yielded $[M-H]^-$ ions of derivatized monosaccharides. All three types of ions were subjected to MS/MS in order to examine their fragmentation (Fig. 10).

MS/MS of sodium adducts $[M+Na]^+$ of neutral monosaccharides provided X-ring-cleavage in position 0-2 (m/z 436, 10% rel. intensity (RI)) and cleavage between two nitrogen atoms of the hydrazide group with m/z 379 (100% RI). With the sodiated ion of BACH-derivatized aminosugar *N*-acetylglucosamine, both of the cleavages were abundantly present in the spectrum ($^{0,2}X$ at m/z 477, 100% RI; m/z 379, ~60% RI) and were accompanied by minor water losses from the molecular ion.

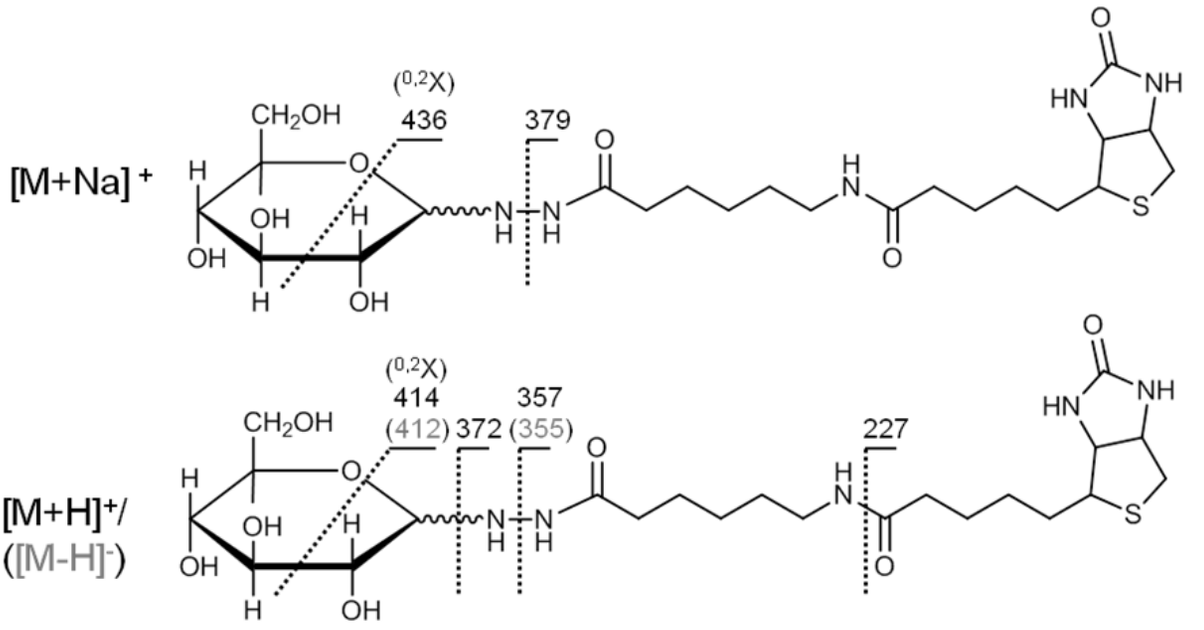


Figure 10. Scheme of fragmentation observed in MS/MS of BACH-labeled monosaccharides with different types of ions. Nominal masses are given.

The protonated ion species of the BACH-derivatized monosaccharides showed under the MS-fragmentation multiple water losses from the molecular ion (100%), ^{0,2}X-fragment at m/z 414 (~20%) and some abundant cleavages associated with the BACH label: m/z 372 (100%) – detachment of the whole label, m/z 357 (~23%) – loss of an ammonia group and m/z 227 (~43%) which presents the acylium ion of the biotin component (Fig. 10).

The deprotonated form of the molecular ion $[M-H]^-$ of BACH monosaccharides showed similarity with the fragmentation spectrum of the $[M+Na]^+$- ion and yielded weak ^{0,2}X-ring cleavage (m/z 412, ~7%) and the abundant cleavage of the hydrazine label (m/z 355, 100%). In the case of *N*-acetylglucosamine, on the other hand, the ^{0,2}X-fragment was the only prominent peak in the MS/MS spectrum with 100% of rel. intensity. Hence, variation in abundances of fragment-ions of the aminosugar GlcNAc in comparison with fragments of neutral sugars was observed when performing CID of $[M+Na]^+$ and $[M-H]^-$ ions.

3.4. Hydrophilic interaction liquid chromatography of BACH-derivatized disaccharides

A common obstacle in the mass spectrometry of saccharides is the characterization of the stereochemistry of the glycosidic bond (Zhu *et al.*, 2009; Morelle & Michalski, 2005). Isomers usually have very similar MS^2- or MS^3-fragmentation and are difficult to differentiate without a previous separation. Therefore, the MS discrimination of isomers is often aided by one or more orthogonal pre-mass separation techniques.

The formation of closed-ring derivatives (glycosylhydrazides) through non-reducing derivatization has demonstrated some advantages in the chromatographic separation and in the analysis of isomers (Li & Her, 1998, 1993). Also, differentiation of anomeric configuration has been observed in some cases following derivatization (Xue *et al.*, 2004; Ashline *et al.*, 2005). Clowers et al. achieved this with reduced form of isobaric di- and trisaccharides by ion mobility mass spectrometry (Clowers *et al.*, 2005). These research groups exploring isomeric sugars with the focus on their MS fragmentation, observed differences in the negative-ion electrospray mass spectra of structurally related compounds. The presence/absence and abundance of certain fragment ions allowed for an assembly of empirical criteria for assigning either a linkage position or the anomeric configuration of a disaccharide. For example, diagnostic ions (m/z 221, 263, 281) which were characteristic for 1-4-linkage were observed by negative ion mode mass spectrometry (Mulroney *et al.*, 1995; Garozzo *et al.*, 1991). With glucose-containing disaccharides, dissociation of the m/z 221 generated product ions that allowed for the differentiation of anomeric configuration (Fang & Bendiak, 2007; Fang *et al.*, 2007).

Here we sought to examine whether the BACH-derivatized isomeric disaccharides would be separable on HILIC and would in MS/MS dissociate to yield product ions from which stereochemical information might be obtained about the glycosidic bond. For this purpose, maltose (α-D-Glc-(1-4)-D-Glc) and cellobiose (β-D-Glc-(1-4)-D-Glc), sugars which vary solely in their anomeric configuration were derivatized by non-reducing BACH-labeling according to the protocol described in the experimental part.

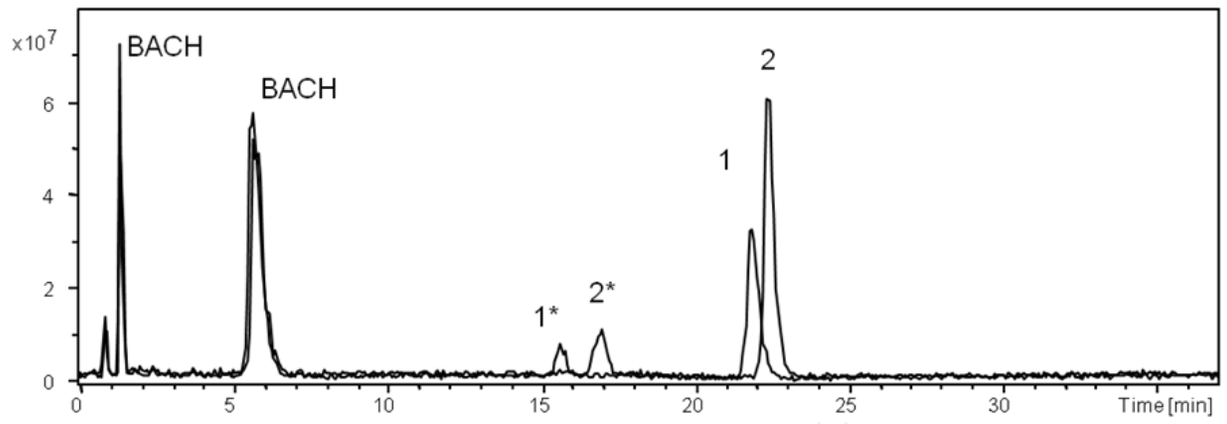


Figure 11. TIC showing separation of BACH-labeled isomeric disaccharides by means of hydrophilic interaction liquid chromatography. Peak assignements: 1 = BACH-maltose, 2 = BACH-cellobiose, * isomeric byproduct upon derivatization when BACH-label used in original unpurified form, BACH = excess of labeling reagent

With underivatized maltose and cellobiose, HPLC-separation and signal intensity were very poor (data not shown). Through BACH-derivatization, enhancement of signal intensity of two orders of magnitude (from $2x10^5$ to $6x10^7$) in comparison with native counterparts was achieved and at least a partial separation on the HILIC-phase was reached (Fig. 11). Even if they were not completely resolved, this demonstrated the feasibility of determining the presence of isomeric hits. For MS/MS investigation, the sugars were subjected separately to mass spectrometry, in order to obtain discrete fragmentation data originating only from one of the isomers. MS/MS spectra of underivatized maltose and cellobiose were without any significant difference in fragmentation pattern. Thus, no assignment could have been done with the native sugars.

In the study of BACH-labeled maltose and cellobiose, different types of molecular ions were detected: $[M+Na]^+$ (m/z 718), $[M+H]^+$ (m/z 696) and $[M-H]^-$ (m/z 694). These were subsequently subjected to tandem mass spectrometry in order to examine their fragmentation profile. Each of the precursor ions showed different fragmentation:

In positive ion mode, same fragmentation patterns for maltose and cellobiose were observed (data not shown). MS/MS of the sodium adduct contained the $^{0,2}X$-ring cleavage (m/z 436) from the reducing end of the derivatized disaccharide and the N-N-cleavage of the hydrazide group of the BACH-label (m/z 355). Fragmentation spectra of the $[M+H]^+$-ion were similar to $[M+Na]^+$. They provided week $^{0,2}X$-fragment (m/z 414) and the hydrazide group cleavage (m/z 355). Additionally to these, glycosidic cleavages (Y_0 and Y_1), which confirmed the sequence and hexose composition of the saccharide, appeared. With Y_1-ion, up to four water losses were observed. Hence, in positive ion mode, when observing either $[M+Na]^+$ or $[M+H]^+$, no difference in the fragmentation of these isomers was observed.

CID of the $[M-H]^-$ion, on the contrary (Fig. 12), yielded cleavages (m/z 412 and m/z 355) and also fragments between m/z 179 and m/z 355, having higher masses than monosaccharide and encompassing the glycosidic bond of the sugar.

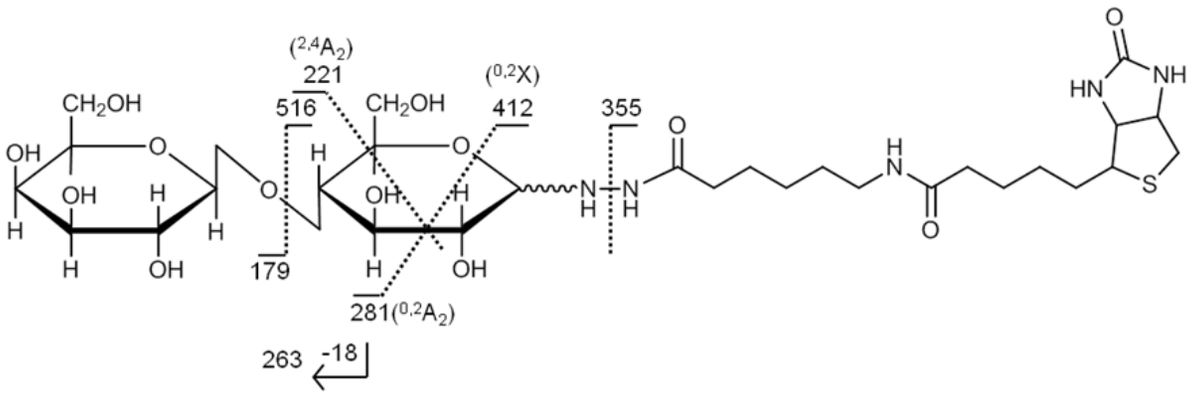


Figure 12. Fragmentation of $[M-H]^-$-ion observed for BACH-labeled isomeric disaccharides maltose and cellobiose in negative ion mode

Negative MS/MS fragmentation spectra of BACH-labeled maltose and cellobiose were similar in terms of presence of diagnostic ions. However, some variation in the relative abundance of the product ions was visible. Ions observed were: m/z 179, 221, 263, 281 (Fig. 12), where m/z 179 represents the non-reducing monosaccharide containing the glycosidic oxygen; m/z 221 ($^{2,4}A_2$) comprises intact non-reducing sugar glycosidically linked to a glycoaldehyde molecule and m/z 281 corresponds to loss of one –CHOH backbone-unit and also one -CH with the BACH-label. This ion eliminated a molecule of water yielding an m/z 263 ion. This has been shown to be characteristic for 1,4-bonded underivatized glucose dimers (Dallinga & Heerma, 1991a).

In the spectrum of α-linked maltose (Fig. 13a), intensity of the m/z 221 was slightly higher than m/z 263. In the fragmentation spectra of beta-linked cellobiose, on the contrary, the ion m/z 221 appeared only in traces. With cellobiose, the intensity of m/z 263 was always higher than m/z 221. Further, the ion at m/z 281 was more abundant in the spectrum of maltose, whereas almost absent in the MS/MS of cellobiose. In order to confirm the reproducibility of the fragmentation we performed the measurements 15 times in total (5 rounds of measurements for each disaccharide on three different days) indicating the presence and intensity of respective fragments to be reproducible and constant. These observations are in accordance with the fragments reported for 4-linked disaccharides and their proportionalities correspond with dissociation studies reported for the differentiation of this type of isomeric disaccharides. In the study of Li and Her (Li & Her, 1993), where relative abundance of fragment ions of 1-4 linked disaccharides was compared, the intensity of the m/z 221 was higher than m/z 263 in alpha-linked disaccharides and smaller than m/z 263 ion in beta-linked disaccharides. As reported (Fang & Bendiak, 2007; Fang *et al.*, 2007), the cross-ring cleavage m/z 221 of the disaccharides also showed to be useful in the discrimination of anomeric configuration. Under conditions applied here, further dissociation of m/z 221 (MS^3) of the BACH- labeled maltose or cellobiose provided no additional differential information. Noneless, through the combination of BACH-labeling and tandem mass spectrometry, distinctive structural information was obtained at the MS^2-stage of fragmentation and disaccharides with the same linkage but different anomeric configurations (maltose: 1-4-α-linkage, cellobiose: 1-4-β-linkage) could have been distinguished.

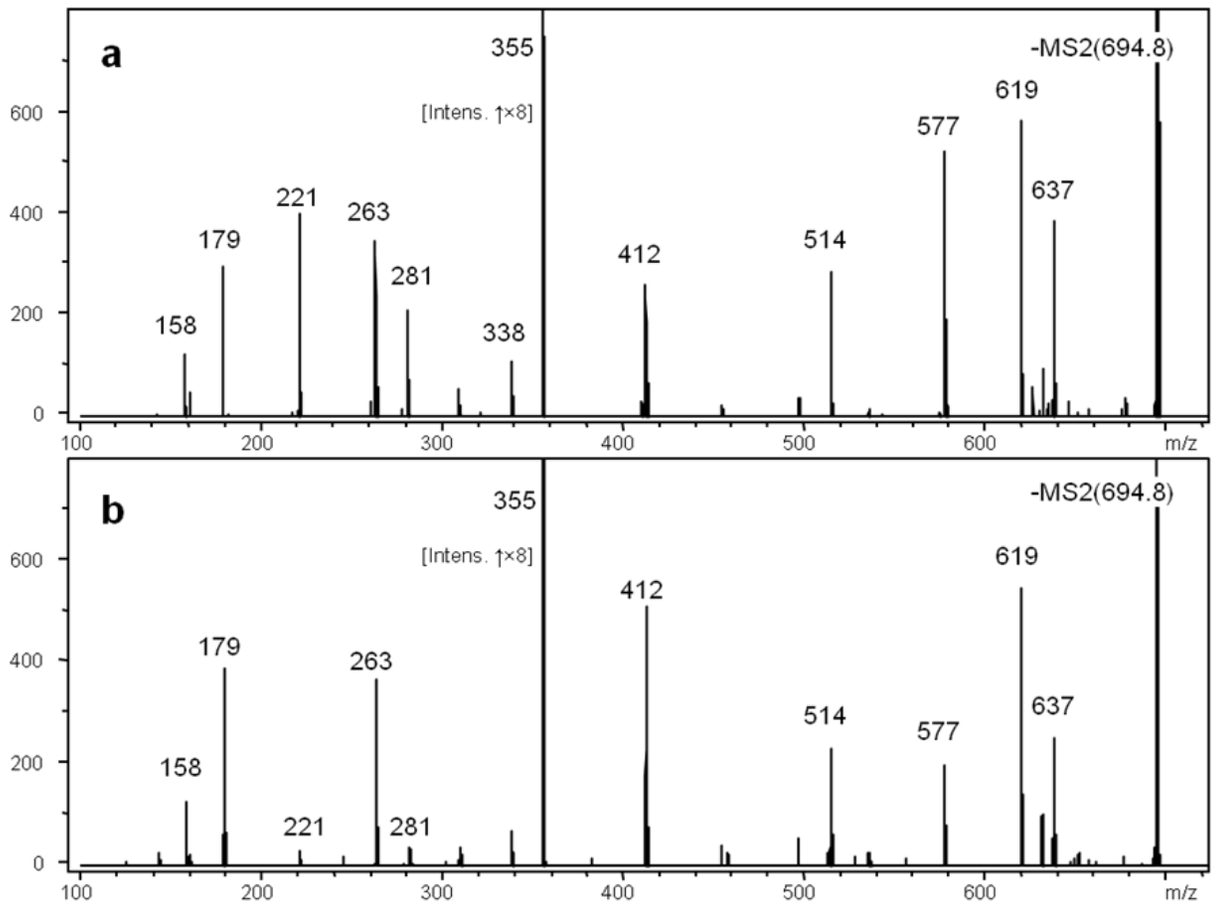


Figure 13. MS/MS of BACH-derivatized isomeric disaccharides. a) Maltose-BACH b) Cellobiose-BACH

3.5. Hydrophilic interaction liquid chromatography of isomeric non-reducing trisaccharides

In order to further examine the ability of the current analytical system to distinguish between the sugar isomers, we have analyzed two trisaccharides melezitose [α-D-Glc-(1→3)-β-D-Fru-(2→1)-α-D-Glc] and raffinose [α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru] which differ in sequence, linkage and anomeric configuration. Both of them were examined by LC-MS/MS in negative ion mode as this one showed to be the preferential mode in order to observe some distinctive fragmentation of related compounds (Section 3.4.). Figure 14a shows the total ion current of melezitose and raffinose. The chromatography of these sugars on the HILIC-phase provided a full baseline separation.

The fragmentation mass spectra of these trisaccharides were obtained from the deprotonated form of the molecular ion $[M-H]^-$ at m/z 503 (Figure 14b-e). Melezitose displayed a dominant fragment at m/z 323 which derives from cleavage of one of the two possible glycosidic bonds (Fig. 14b). This route was confirmed through the MS^3 experiment, where the dissociation of m/z 341 generated no m/z 323 but solely the ions with m/z 179 and m/z 161 (Fig. 14d). This indicates that m/z 323 occurs directly through B-fragmentation and not via formation of m/z 341 and a subsequent water loss. The peak at m/z 341 was found at

MS^2-stage of both trisaccharides and is due to the elimination of a site residue from the $[M-H]^-$-molecular ion. Interestingly, in positive-ion MS-studies on $[M+H]^+$-ions of non-reducing oligosaccharides containing fructose (including melezitose and raffinose), the formation of m/z 323 was proposed to preferably occur via the water loss from m/z 341 (Dallinga & Heerma, 1991; Perez-Victoria *et al.*, 2008).

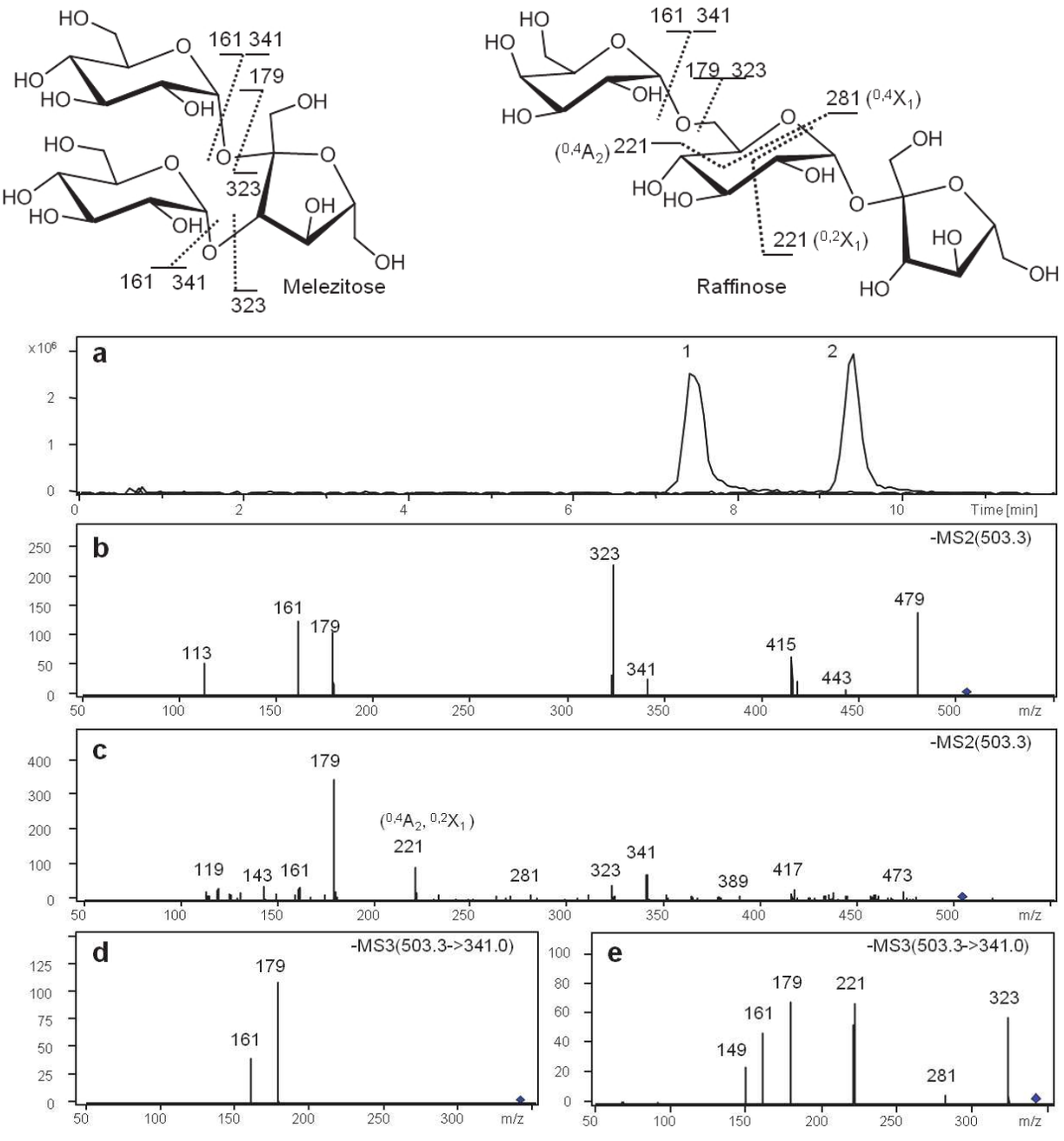


Figure 14. a) Separation of the mixture of two isomeric trisaccharides melezitose (1) and raffinose (2), demonstrating the value of coupling HILIC with ion-trap mass spectrometry performed to MS^3. b) MS^2 of melezitose c) MS^2 of raffinose d) MS^3 of melezitose e) MS^3 of raffinose

Raffinose fragmented to form preferably m/z 179, m/z 221 and glycosidic fragment at m/z 341 (Fig. 14c). Other ions (m/z 323 and 161) were present as well. At this stage of fragmentation (MS^2), the formation of cross-ring cleavage (m/z 221) involving either the 0-2 or 0-4 bonds of the center residue appeared. This ion has not been seen in the spectrum of melezitose (compare to Fig. 14b). The same fact was reported in the studies of fragmentation of trisaccharides by negative FAB (Dallinga & Heerma, 1991b) and negative ion mobility mass spectrometry (Liu & Clemmer, 1997): raffinose yielded m/z 221 in the MS^2-spectrum, whereas melezitose showed only the product ion at m/z 323 without presence of m/z 221. The presence of m/z 221 was also evident in MS^3 of raffinose (Fig. 14e). The different fragmentation routes observed may be attributed to the difference in the steric character of these isomers: Melezitose has a branched character and raffinose represents a linear molecule.

Finally, the differences in the chromatography on the HILIC-phase and in the fragmentation pattern (presence and rel. intensity of the product ions in the MS^2 and MS^3 stage) of the melezitose and raffinose indicated the occurrence of distinct isomeric structures.

This distinguished fragmentation would not have been possible to obtain without the previous pre-mass spectrometric separation on the HILIC-phase. Considering mass spectrometric techniques, such differential identification of these isomers out of their mixture was achieved up to now, only by means of ion-mobility mass spectrometry (Zhu *et al.*, 2009; Liu & Clemmer, 1997). To our best knowledge, this is the first report on separation and identification of such isomers by coupling of hydrophilic interaction chromatography and mass spectrometry.

4. Conclusion

Different types of *N*-glycans (high-mannose, complex and hybrid) have been derivatized with biotinamidocaproyl hydrazide and analyzed by chip-based LC/ESI triple-quadrupole mass spectrometry. This approach allowed for rapid mass spectrometric selection of glycans out of glycan mixtures on the base of the scanning for a common feature ion (oxonium ion). The glycans observed upon MS were primarily in the doubly charged state. For each type of glycan, product ion spectra with the characteristic B- and Y-ions were observed which allowed for elucidation of the structure and segregation of the glycans into the respective class. This methodology provided fast structural information about the nature of the glycan pool present in a glycoprotein and may be used in the profiling of *N*-linked glycans. Beyond structural mass spectrometric studies, BACH-derivatized glycans can potentially be used also in functional studies with carbohydrate binding proteins, as recently reported.

HILIC and reversed phase liquid chromatography of BACH- and 2-AB-labeled monosaccharides enabled the investigation of different analytical objectives and showed some orthogonal features in terms of the separation of monosaccharides frequently present in glycoproteins. The HILIC-system was not sufficient for separation of anomers of BACH-derivatized monosaccharides. It was, however, capable to partially resolve the

BACH-derivatized isomeric disaccharides and to fully separate non-reducing isomeric trisaccharides.

In terms of the enhancement of mass spectrometric signal of mono- and disaccharides, BACH-labeling provided higher intense ions than 2-AB-derivatization. Analysis of the BACH-derivatives in combination with the mass spectrometry can be pursued, when investigation of isomers is desired. Unlike positive ESI-MS, negative ion MS of BACH-derivatized isomeric disaccharides provided ring-cleavage ions which were helpful in the differentiation of the anomeric configuration of the glycosidic bond. Further, separation of structurally related non-derivatized trisaccharides was achieved on HILIC and their differentiation on the basis of multiple stage mass spectrometry was possible. This differentiation would not have been possible without the suitable pre-mass spectrometric separation. In this way, coupling of the HILIC-separation with MS^n-fragmentation might be a valuable approach on the way to the distinction of certain types of isomeric structures.

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5. References

Alley, W. R., Jr., Mechref, Y. & Novotny, M. V. (2009). Use of activated graphitized carbon chips for liquid chromatography/mass spectrometric and tandem mass spectrometric analysis of tryptic glycopeptides. *Rapid Communications in Mass Spectrometry,* Vol.23, No.4, pp. 495-505, ISSN 0951-14198

Ashline, D., Singh, S., Hanneman, A. & Reinhold, V. (2005). Congruent strategies for carbohydrate sequencing. 1. Mining structural details by MSn. *Analytical Chemistry,* Vol.77, No.19, pp. 6250-6262, ISSN 0003-2700

Bigge, J. C., Patel, T. P., Bruce, J. A., Goulding, P. N., Charles, S. M. & Parekh, R. B. (1995). Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. *Analytical Biochemistry,* Vol.230, No.2, pp. 229-238, ISSN 0003-2697

Bindila, L. & Peter-Katalinic, J. (2009). Chip-mass spectrometry for glycomic studies. *Mass Spectrometry Reviews,* Vol.28, No.2, pp. 223-253, ISSN 1098-2787

Brons, C. & Olieman, C. (1983). Study of the high-performance liquid-chromatographic separation of reducing sugars, applied to the determination of lactose in milk. *Journal of Chromatography,* Vol.259, No.1, pp. 79-86, ISSN 0021-9673

Churms, S. C. (1996). Recent progress in carbohydrate separation by high-performance liquid chromatography based on hydrophilic interaction. *Journal of Chromatography A,* Vol.720, No.1-2, pp. 75-91, ISSN 0021-9673

Clowers, B. H., Dwivedi, P., Steiner, W. E., Hill, H. H. & Bendiak, B. (2005). Separation of sodiated isobaric disaccharides and trisaccharides using electrospray ionization-atmospheric pressure ion mobility-time of flight mass spectrometry. *Journal of the American Society for Mass Spectrometry,* Vol.16, No.5, pp. 660-669, ISSN 1044-0305

Dallinga, J. W. & Heerma, W. (1991a). Positive-Ion Fast-Atom-Bombardment Mass-Spectrometry of Some Small Oligosaccharides. *Biological Mass Spectrometry,* Vol.20, No.3, pp. 99-108, ISSN 1052-9306

Dallinga, J. W. & Heerma, W. (1991b). Reaction-Mechanism and Fragment Ion Structure Determination of Deprotonated Small Oligosaccharides, Studied by Negative-Ion Fast-Atom-Bombardment (Tandem) Mass-Spectrometry. *Biological Mass Spectrometry,* Vol.20, No.4, pp. 215-231, ISSN 1052-9306

Domon, B. & Costello, C. E. (1988). A Systematic Nomenclature for Carbohydrate Fragmentations in Fab-Ms Ms Spectra of Glycoconjugates. *Glycoconjugate Journal,* Vol.5, No.4, pp. 397-409, ISSN 0282-0080

Dwivedi, P., Bendiak, B., Clowers, B. H. & Hill, H. H. (2007). Rapid resolution of carbohydrate isomers by electrospray ionization ambient pressure ion mobility spectrometry-time-of-flight mass spectrometry (ESI-APIMS-TOFMS). *Journal of the American Society for Mass Spectrometry,* Vol.18, No.7, pp. 1163-1175, ISSN 1044-0305

Fang, T. T. & Bendiak, B. (2007). The stereochemical dependence of unimolecular dissociation of monosaccharide-glycolaldehyde anions in the gas phase: A basis for assignment of the stereochemistry and anomeric configuration of monosaccharides in oligosaccharides by mass spectrometry via a key discriminatory product ion of disaccharide fragmentation, m/z 221. *Journal of the American Chemical Society,* Vol.129, No.31, pp. 9721-9736, ISSN 0002-7863

Fang, T. T., Zirrolli, J. & Bendiak, B. (2007). Differentiation of the anomeric configuration and ring form of glucosyl-glycolaldehyde anions in the gas phase by mass spectrometry: isomeric discrimination between m/z 221 anions derived from disaccharides and chemical synthesis of m/z 221 standards. *Carbohydrate Research,* Vol.342, No.2, pp. 217-235, ISSN 0008-6215

Garozzo, D., Impallomeni, G., Spina, E., Green, B. N. & Hutton, T. (1991). Linkage analysis in disaccharides by electrospray mass-spectrometry. *Carbohydrate Research,* Vol.221, pp. 253-257, ISSN 0008-6215

Geyer, H. & Geyer, R. (2006). Strategies for analysis of glycoprotein glycosylation. *Biochimica Biophysica Acta,* Vol.1764, No. 12, pp. 1853-1869, ISSN 0006-3002

Grün, C. H., van Vliet, S. J., Schiphorst, W. E., Bank, C. M., Meyer, S., van Die, I. & van Kooyk, Y. (2006). One-step biotinylation procedure for carbohydrates to study carbohydrate-protein interactions. *Analytical Biochemistry,* Vol.354, No.1, pp. 54-63, ISSN 0003-2697

Harvey, D. J. (2000). Electrospray mass spectrometry and collision-induced fragmentation of 2-aminobenzamide-labelled neutral N-linked glycans. *Analyst,* Vol.125, No.4, pp. 609-617, ISSN 0003-2654

Harvey, D. J. (2011). Derivatization of carbohydrates for analysis by chromatography; electrophoresis and mass spectrometry. *Journal of Chromatography B,* Vol.879, No.17-18, pp. 1196-1225, ISSN 1570-0232

Hermannson, G. T. (2008). *Bioconjugate Techniques,* Academic Press, New York

Hsu, J., Chang, S. J. & Franz, A. H. (2006). MALDI-TOF and ESI-MS analysis of oligosaccharides labeled with a new multifunctional oligosacharide tag. *Journal of the American Society for Mass Spectrometry,* Vol.17, pp. 194-204, ISSN 1044-0305

Ikegami, T., Tomomatsu, K., Takubo, H., Horie, K. & Tanaka, N. (2008). Separation efficiencies in hydrophilic interaction chromatography. *Journal of Chromatography A,* Vol.1184, No.1-2, pp. 474-503, ISSN 0021-9673

Kapková, P. (2009). Mass spectrometric analysis of carbohydrates labeled with a biotinylated tag. *Rapid Communications in Mass Spectrometry,* Vol.23, No.17, pp. 2775-2784, ISSN 0951-4198

Kwon, H. & Kim, J. (1993). Determination of monosaccharides in glycoproteins by reverse-phase high-performance liquid chromatography. *Analytical Biochemistry,* Vol.215, pp. 243-252, ISSN 0003-2697

Lattová, E., Snovida, S., Perreault, H. & Krokhin, O. (2005). Influence of the labeling group on ionization and fragmentation of carbohydrates in mass spectrometry. *Journal of the American Society for Mass Spectrometry,* Vol.16, No.5, pp. 683-696, ISSN 1044-0305

Leteux, C., Childs, R. A., Chai, W., Stoll, M. S., Kogelberg, H. & Feizi, T. (1998). Biotinyl-l-3-(2-naphthyl)-alanine hydrazide derivatives of N-glycans: versatile solid-phase probes for carbohydrate-recognition studies. *Glycobiology,* Vol.8, No.3, pp. 227-236, ISSN 0959-6658

Leymarie, N. & Zaia, J. (2012). Effective use of mass spectrometry for glycan and glycopeptide structural analysis. *Analytical Chemistry,* Vol.84, pp. 3040-3048, ISSN 0003-2700

Li, D. T. & Her, G. R. (1993). Linkage Analysis of Chromophore-Labeled Disaccharides and Linear Oligosaccharides by Negative-Ion Fast-Atom-Bombardment Ionization and Collisional-Induced Dissociation with B/E Scanning. *Analytical Biochemistry,* Vol.211, No.2, pp. 250-257, ISSN 0003-2697

Li, D. T. & Her, G. R. (1998). Structural analysis of chromophore-labeled disaccharides and oligosaccharides by electrospray ionization mass spectrometry and high-performance liquid chromatography electrospray ionization mass spectrometry. *Journal of Mass Spectrometry,* Vol.33, No.7, pp. 644-652, ISSN 1076-5174

Liu, Y. S. & Clemmer, D. E. (1997). Characterizing oligosaccharides using injected-ion mobility mass spectrometry. *Analytical Chemistry,* Vol.69, No.13, pp. 2504-2509, ISSN 0003-2700

Lopes, J. F. & Gaspar, E. M. S. M. (2008). Simultaneous chromatographic separation of enantiomers, anomers and structural isomers of some biologically relevant monosaccharides. *Journal of Chromatography A,* Vol.1188, pp. 34-42, ISSN 0021-9673

Marino, K., Bones, J., Kattla, J. J. & Rudd, P. M. (2010). A systematic approach to protein glycosylation analysis: A path through the maze. *Nature Chemical Biology,* Vol.6, pp. 713-723, ISSN 1552-4450

Melmer, M., Stangler, T., Premstaller, A. & Lindner, W. (2011). Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis. *Journal of Chromatography A,* Vol.1218, pp. 118-123, ISSN 0021-9673

Morelle, W. & Michalski, J. C. (2004). Sequencing of oligosaccharides derivatized with benzlyamine using electrospray ionization-quadrupole time of flight-tandem mass spectrometry. *Electrophoresis,* Vol.25, No.14, pp. 2144-2155, ISSN 0173-0835

Morelle, W. & Michalski, J. C. (2005). Glycomics and mass spectrometry. *Current Pharmaceutical Design,* Vol.11, No.20, pp. 2615-2645, ISSN 1381-6128

Moriyasu, M., Kato, A., Okada, M. & Hashimoto, Y. (1984). HPLC separation of sugar anomers in a very low temperatur region. *Analytical Letters,* Vol.17, pp. 689-699, ISSN 0003-2719

Mulroney, B., Traeger, J. C. & Stone, B. A. (1995). Determination of Both Linkage Position and Anomeric Configuration in Underivatized Glucopyranosyl Disaccharides by Electrospray Mass-Spectrometry. *Journal of Mass Spectrometry,* Vol.30, No.9, pp. 1277-1283, ISSN 1076-5174

Nishikawa, T., Suzuki, S., Kubo, H. & Ohtani, H. (1996). On-column isomerization of sugars during high-performance liquid chromatography: analysis of the elution profile. *Journal of Chromatography A,* Vol.720, pp. 167-172, ISSN 0021-9673

North, S. J., Hitchen, P. G., Haslam, S. M. & Dell, A. (2009). Mass spectrometry in the analysis of N-linked and O-linked glycans. *Current Opinion in Structural Biology,* Vol.19, pp. 498-506, ISSN 0959-440X

Pabst, M., Kolarich, D., Poltl, G., Dalik, T., Lubec, G., Hofinger, A. & Altmann, F. (2009). Comparison of fluorescent labels for oligosaccharides and introduction of a new postlabeling purification method. *Analytical Biochemistry,* Vol.384, No.2, pp. 263-273, ISSN 0003-2697

Perez-Victoria, I., Zafra, A. & Morales, J. C. (2008). Positive-ion ESI mass spectrometry of regioisomeric nonreducing oligosaccharide fatty acid monoesters: In-source fragmentation of sodium adducts. *Journal of Mass Spectrometry,* Vol.43, No.5, pp. 633-638, ISSN 1076-5174

Raman, R., Raguram, S., Venkataraman, G., Paulson, J. C. & Sasisekharan, R. (2005). Glycomics: an integrated systems approach to structure-function relationships of glycans. *Nature Methods,* Vol.2, pp. 817-824, ISSN 1548-7091

Ridley, B. L., Spiro, M. D., Glushka, J., Albersheim, P., Darvill, A. & Mohnen, D. (1997). A method for biotin labeling of biologically active oligogalacturonides using a chemically

stable hydrazide linkage. *Analytical Biochemistry,* Vol.249. No.1, pp. 10-19, ISSN 0003-2697

Rosenfeld, J. (2011). Enhancement of analysis by analytical derivatization. *Journal of Chromatography B,* Vol.879, pp. 1157-1158, ISSN 1570-0232

Rothenberg, B. E., Hayes, B. K., Toomre, D., Manzi, A. E. & Varki, A. (1993). Biotinylated diaminopyridine: an approach to tagging oligosaccharides and exploring their biology. *Proceedings of the National Academy of Sciences USA,* Vol.90, No.24, pp. 11939-11943, ISSN 1091-6490

Ruhaak, L. R., Zauner, G., Huhn, C., Bruggink, C., Deelder, A. & Wuhrer, M. (2010). Glycan labeling strategies and their use in identification and quantification. *Analytical and Bioanalytical Chemistry,* Vol.397, pp. 3457-3481, ISSN1618-2650

Schlichtherle-Cerny, H., Affolter, M. & Cerny, C. (2003). Hydrophilic interaction liquid chromatography coupled to electrospray mass spectrometry of small polar compounds in food analysis. *Analytical Chemistry,* Vol.75, pp. 2349-2354, ISSN 0003-2700

Schumacher, D. & Kroh, L. W. (1995). A rapid method for separation of anomeric saccharides using cyclodextrin bonded phase and for investigation of mutarotation. *Food Chemistry,* Vol.54, pp. 353-356, ISSN 0308-8146

Shinohara, Y., Sota, H., Gotoh, M., Hasebe, M., Tosu, M., Nakao, J., Hasegawa, Y. & Shiga, M. (1996). Bifunctional labeling reagent for oligosaccharides to incorporate both chromophore and biotin groups. *Analytical Chemistry,* Vol.68, No.15, pp. 2573-2579, ISSN 0003-2700

Strege, M. A., Stevenson, S. & Lawrence, S. M. (2000). Mixed mode anion-cation exchange/hydrophilic interaction liquid chromatography-electrospray mass spectrometry as an alternative to reversed phase for small molecule drug discovery. *Analytical Chemistry,* Vol.72, No.19, pp. 4629-4633, ISSN 0003-2700

Toomre, D. K. & Varki, A. (1994). Advances in the use of biotinylated diaminopyridine (BAP) as a versatile fluorescent tag for oligosaccharides. *Glycobiology,* Vol.4, No.5, pp. 653-663, ISSN 0959-6658

Wuhrer, M., de Boer, A. R. & Deelder, A. M. (2009). Structural glycomics using hydrophilic interaction chromatography (HILIC) with mass spectrometry. *Mass Spectrometry Reviews,* Vol.28, No.2, pp. 192-206, ISSN 1098-2787

Wuhrer, M., Koeleman, C. A. M., Hokke, C. H. & Deelder, A. M. (2004). Nano-scale liquid chromatography-mass spectrometry of 2-aminobenzamide-labeled oligosaccharides at low femtomole sensitivity. *International Journal of Mass Spectrometry,* Vol.232, No.1, pp. 51-57, ISSN 1387-3806

Wuhrer, M., van Remoortere, A., Balog, C. I. A., Deelder, A. M. & Hokke, C. H. (2010). Ligand identification of carbohydrate-binding proteins employing a biotinylated glycan binding assay and tandem mass spectrometry. *Analytical Biochemistry,* Vol. 406, No.2, pp. 132-140, ISSN 0003-2697

Xue, J., Song, L. G., Khaja, S. D., Locke, R. D., West, C. M., Laine, R. A. & Matta, K. L. (2004). Determination of linkage position and anomeric configuration in Hex-Fuc disaccharides

using electrospray ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, Vol.18, No.17, pp. 1947-1955, ISSN 0951-4198

Zhu, M. L., Bendiak, B., Clowers, B. & Hill, H. H. (2009). Ion mobility-mass spectrometry analysis of isomeric carbohydrate precursor ions. *Analytical and Bioanalytical Chemistry*, Vol.394, No.7, pp. 1853-1867, ISSN 1618-2650

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