THE USEFULNESS OF HYDRAZINE DERIVATIVES FOR MASS SPECTROMETRIC ANALYSIS OF CARBOHYDRATES

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Over the last years, extensive studies have evaluated glycans from different biological samples and validated the importance of glycosylation as one of the most important post-translational modifications of proteins. Although a number of new methods for carbohydrate analysis have been published and there has been significant progress in their identification, the development of new approaches to study these biomolecules and understand their role in living systems are still vivid challenges that intrigue glycobiologists. In the last decade, the success in analyses of oligosaccharides has been driven mainly by the development of innovative, highly sensitive mass spectrometry techniques. For enhanced mass spectrometry detection, carbohydrate molecules are often derivatized. Besides, the type of labeling can influence the fragmentation pattern and make the structural analysis less complicated. In this regard, in 2003 we introduced the low scale, simple non-ductive tagging of glycans employing phenylhydrazine (PHN) as the derivatizing reagent. PHN-labeled glycans showed increased detection and as reported previously they can be analyzed by HPLC, ESI, or MALDI immediately after derivatization. Under tandem mass spectrometry conditions, PHN-derivatives produced useful data for the structural elucidation of oligosaccharides. This approach of analysis has helped to reveal new isomeric structures for glycans of known/unknown composition and has been successfully applied for the profiling of N-glycans obtained from serum samples and cancer cells. The efficacy of this labeling has also been evaluated for different substituted hydrazine reagents. This review summarizes all types of reducing-end labeling based on hydrazone-linkage that have been used for mass spectrometric analyses of oligosaccharides. This review is also aimed at correcting some past misconceptions or interpretations reported in the literature. © 2013 Wiley Periodicals, Inc. Mass Spec Rev 32:366–385, 2013

Keywords: derivatization; glycans; hydrazines; hydrazides; hydrazone; mass spectrometry; oligosaccharides

I. INTRODUCTION

For the identification of carbohydrate molecules, different strategies have been used. Among them, mass spectrometry (MS) provides many advantages over traditional analytical methods, such as a low sample consumption and high sensitiv-
can influence MS/MS fragmentation patterns and significantly simplify the structural analysis of glycans. Each derivatization procedure has its advantages and the final result depends on the complexity of the sample and the type of targeted carbohydrates. Besides widely used permethylation (Ciucanu & Kerek, 1984; Dell et al., 1994; Ciucanu & Costello, 2003; Kang et al., 2005; Yu, Wu, & Khoo, 2006; Alvarez-Manilla et al., 2007; Ciucanu & Caprita, 2007; Alley et al., 2010), reactions with various types of aromatic substituted amines under mild acidic conditions are other popular methods used for derivatization of saccharides (Hase, Ibuki, & Ikenaka, 1984; Bigge et al., 1995; Okafo et al., 1996; Anumula & Dhume, 1998). This type of labeling among others was thoroughly reviewed, for example, by Lamari, Kuhn, and Karamanos (2003) and recently updated, for example, by Ruhaak et al. (2010), Harvey (2011), and Yamada and Kakehi (2011). The reaction with aromatic amines is usually followed by the reduction to stabilize a Schiff base by providing stable open ring structures at the former reducing terminus. This step requires clean-up step procedure to remove a large excess of a reducing agent (e.g., commonly used sodium cyanoborohydride, 2-picoline-borane) which can lead to a significant loss of the sample (Pabst et al., 2009). Omitting the reducing step can prevent a sample loss caused during the purification step, however on the other hand Schiff bases are not very stable and the amount of non-derivatized saccharides is usually high (Harvey, 2000b). This problem can be avoided by derivatization with substituted hydrazine reagents which allow coupling to reducing terminus of oligosaccharides under non-reductive conditions (Scheme 1). The reactions can be carried out under neutral or acidic conditions, at room temperature or increased, depending on the substituent linked at the nitrogen in the hydrazine molecule. This labeling offers an advantage over other procedures; since no salts are used or produced in the reaction, the hydrazine products can be after derivatization immediately injected into HPLC or analyzed by MS.

II. REDUCING END LABELING BASED ON HYDRAZONE LINKAGE

The phenylhydrazine chemistry had its beginning in 1875 when famous German scientist Emil Fischer was able to prepare this reagent and later used it to explore the chemistry of glucose and related monosaccharide derivatives (Helferich, 1953). The reaction of phenylhydrazine and other substituted hydrazine compounds (e.g., dansylhydrazine, 2,4-dinitrophenylhydrazine, N,N-diphenylhydrazine, p-hydrazinobenzenesulfonic acid) have been employed extensively for identification of monosaccharides by different analytical approaches including nuclear magnetic resonance (NMR), high performance liquid chromatography (HPLC) or capillary zone electrophoresis (CEF) (Avigad, 1977; Takeda, 1979; Karamanos, Tsegendis, & Antonopoulos, 1987; Lin & Wu, 1987; Muramoto, Goto, & Kamiya, 1987; Zhang, Cao, & Hearn, 1991; Perez & Colón, 1996; Miksik, Gabriel, & Deyl, 1997; Wang & Chen, 2001). However, it is not long ago since the reagents with a hydrazine function have become utilized for MS detection of carbohydrates (Table 1, Scheme 2).

A. Derivatization with Arylhydrazines

Derivatization with phenylhydrazine (PHN) is perhaps the simplest labeling procedure and was introduced for detection of oligosaccharides by ESI- and MALDI-MS in 2003 (Lattova & Perreault, 2003a). It was reported that phenylhydrazide derivatives of small saccharides provided better HPLC separation on a reversed-phase column with improved sensitivities in comparison with their native analogs. Along this study, it was shown that despite using the same concentrations of each saccharide, their PHN derivatives did not produce peaks of the same heights; arabinose, galactose, and N-acetyl-galactosamine produced smaller peaks than their gluco-epimers (Fig. 1). The NMR study of several PHN-sugars led to the conclusion that their ratios of cyclic to acyclic forms depended on the solvent used and on the sugar configuration (Takeda, 1979). Therefore, the difference in the UV response of individual di- and monosaccharides under HPLC and MS conditions could be explained by different concentration effects of acyclic forms in solution (Lattova & Perreault, 2003a). Moreover, discrepancies in the extent of electron delocalization around PHN groups, depending on a saccharide structure, could amplify observed differences in the absorption patterns. Lactose and glucose (1) have three secondary hydroxyl groups at carbons 2, 3, and 4 in the gauche arrangement in their zigzag conformations (Scheme 3). The same can apply to N-acetyl-glucosamine (2), where a hydroxyl is replaced by an N-acetyl function group. These groups could take part in a PHN delocalization through hydrogen bridges. The lower UV absorbivities observed for arabinose, galactose (3), and N-acetyl-galactosamine (4) could result only from two such groups in cis-position and therefore with less bridging taking place.

The suitability of PHN-labeling for MS analysis of larger oligosaccharides was first demonstrated on N-glycans detached from ovalbumin (Lattova & Perreault, 2003b). PHN-glycans showed enhanced sensitivity under both ESI- and MALDI-MS conditions and provided again better retention and separation.
TABLE 1. List of substituted hydrazine reagents used for the mass spectrometric detection of carbohydrates

<table>
<thead>
<tr>
<th>Abbreviations*</th>
<th>Reaction conditions</th>
<th>Oligosaccharides originated</th>
<th>MS</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-BPH</td>
<td>water/methanol/acetic acid 80°C</td>
<td>Bacterial gonococcal strains, Haemophilus ducreyi</td>
<td>ESI-MS</td>
<td>Mondrell et al., 1991</td>
</tr>
<tr>
<td>PHN</td>
<td>water/methanol or acetonitrile 25-70°C</td>
<td>Mono, di, and trisaccharide standards, standard glycans – NG2, NG2F, NA2F, A1F, A2F, glycans detached from albumin, IgG, transferrin, serum samples, herceptin, MCF-7, CEM cells and MKN45 cancer cells</td>
<td>HPLC-ESI ESI-MS, MS/MS MALDI-MS, MS/MS</td>
<td>Lattova et al., 2003-2011</td>
</tr>
<tr>
<td>Girard's reagent T</td>
<td>methanol/acetic acid 75°C</td>
<td>Standard glycans – Man3-9, glycans detached from albumin, Gle, sorbitol, biose, triose and tetrose analyzed in beer</td>
<td>ESI-MS, MS/MS MALDI-MS</td>
<td>Naven &amp; Harvey, 1996</td>
</tr>
<tr>
<td>Tripropyl reagent GP</td>
<td>methanol/acetic acid 56-75°C</td>
<td>Standard glycans – NA2, A2F, glycans cleaved from human plasma</td>
<td>ESI-MS, MS/MS</td>
<td>Bereman et al., 2010</td>
</tr>
<tr>
<td>Phenyl-GP GPN GPN2 GPN3 GPN4 Phenyl-GPN Phenyl-2-GPN</td>
<td>methanol/acetic acid 60°C</td>
<td>Standard glycans – GN2, LacNAc, Man2-5, NA2, A2F</td>
<td>LC-ESI</td>
<td>Leiteux et al., 1999</td>
</tr>
<tr>
<td>BNAN</td>
<td>methanol/water/acetic acid (90°C)</td>
<td>Mono, di, and trisaccharide standards, standard glycans – Man5-8, NG2, NA2, A1F, glycans detached from albumin, glycans released from KHL</td>
<td>LC-ESI, MS/MS MALDI-MS, MS/MS</td>
<td>Leiteux et al., 1999 Kapkova 2009 Wuhrer et al. 2010-2011</td>
</tr>
<tr>
<td>BACH</td>
<td>methanol/water/acetic acid (60°C) methanol/water (90°C)</td>
<td>Mono, di, and trisaccharide standards, standard glycans – Man5-8, NG2, NA2, A1F, glycans detached from albumin, glycans released from KHL</td>
<td>LC-ESI, MS/MS MALDI-MS, MS/MS</td>
<td>Leiteux et al., 1999 Kapkova 2009 Wuhrer et al. 2010-2011</td>
</tr>
<tr>
<td>AMCA</td>
<td>methanol/acetic acid 80°C</td>
<td>Lacto-N-fucopentaose, -oligosaccharides, mannosylglycosylpeptide</td>
<td>LC-ESI, CID, UVPD</td>
<td>Ko &amp; Broadbent, 2011</td>
</tr>
</tbody>
</table>

*Structures of all listed hydrazine derivatives are in Scheme 2.

on reversed-phase HPLC columns than their native analogs. The level of separation depended on the mobile phase and flow rate (Fig. 2a). Glycans with molecular weights higher than 1,000 Da ionized mainly as [M + 2H]^{2+} and [M + 3H]^{3+} ions in ESI-MS, depending on the gradient and instrumentation conditions (Fig. 2b). The total profile of derivatized ovalbumin N-glycans found by ESI was in good agreement with results obtained by MALDI-MS.

In both reports discussed above, it was demonstrated that derivatization can be completed after less than 1 hr of heating an aqueous saccharide solution under neutral conditions, that is, without the presence of acid or any additional reagent. The works following this study demonstrated that this labeling reaction can be completed even at a room temperature with a shorter time and no purification after derivatization is required. Since the phenylhydrazine is an oily liquid, a small amount of an organic solvent (e.g., acetonitrile, methanol) is suggested to add to the final reaction mixture (~1–5%). For maximum conversion of the carbonyl to the hydrazone form, frequent mixing of the reaction mixture is recommended. This labeling showed to be appropriate for the characterization of all types of oligosaccharides with a free reducing end. Acidic oligosaccharides (with sialic/uronic acids) can be successfully labeled without apparent cleavage during reaction.

Suzuki et al. (2009) applied PHN derivatization for the post-column labeling of sugars separated by HPLC in the HILIC mode and their results supported the usefulness of the reaction in the proteomic research. It can be noted that lowering pH facilitates the derivatization, however stronger acidic conditions especially in combination with higher temperatures should be avoided, to prevent a peeling reaction or a loss of labile sialic residue. Beside that a large excess of reagent under such conditions can lead to the formation of by-products called osazones.

General characteristic that the hydrazone formation does not require the presence of additional reagents and therefore the purification steps to remove salts or excess of reagent can thus be avoided, make this reaction suitable for direct on-target derivatization for MALDI-MS (Lattova & Perreault, 2003a,b; Lattova et al., 2006). Usually after the PNGase treatment of glycoprotein or glycopeptides, free glycans are not detectable because of ionization suppression by stronger [M + H]^{+} ions of peptides. Therefore, in a case of the glycopeptide analysis, glycans composition is only derived from the calculated mass differences between glycopeptides and deglycosylated peptide. However, hydrazone-tagging (in-tube or on-target) enables visualization of glycans peaks (Lattova et al., 2006). Labeled oligosaccharides and deglycosylated peptides can be thus analyzed in one step; the glycan profile and site occupancy of peptides can be monitored and characterized from one fraction or even one single spot (e.g., Fig. 3 and Table 2). This significantly simplifies the analysis and the total time from the beginning of derivatization to the end of MS analysis can be less than 1 hr. Besides that, neutral and sialylated glycans can be analyzed simultaneously. This approach, that is, the detection of glycans next to peptides after deglycosylation from the same spot was presented on the
analysis of glycopeptides isolated from human IgG. Tandem mass spectra provided sufficient data for the structural composition of glycans attached at Asn 297 in a known sequence of the IgG molecule—EEQY

Moreover, two other glycosylated peptides in IgG were identified—EEQF

and EEQFNSTFYR (Fig. 3). A new glycosylated amino acid sequence—ANQTVYR was found in the IgG1 isolated from myeloma plasma (Lattová et al., 2006).

SCHEME 2. Structures and abbreviation of hydrazine derivatives used for mass spectrometric analyses of carbohydrates listed in Table 1.
Shortly after that an additional step—on-target enzymatic deglycosylation was combined with procedure described above. In this approach, the glycopeptides spotted on the target with a suitable matrix (e.g., 2-aza-2-thiothymine/phenylhydrazine hydrochloride) are first analyzed by MS. Then, this spot can be treated with PNGaseF (~5 min) and then the released oligosaccharides can be immediately labeled with a PHN solution (Lattová et al., 2007). It makes this method important in case when no more material is available. This simple approach was demonstrated on the analysis of glycosylated peptides isolated from standard glycoprotein samples (ovalbumin, fetuin, IgG, and transferrin) and successfully applied to, for example, in the identification of glycoconjugates isolated from breast MCF-7 cancer and leukemic CEM cells after treatment with monoclonal antibody (Lattová et al., 2011).

B. Derivatization with Hydrazides

Hydrazine compounds with at least one acyl substituent covalently linked to nitrogen are usually called hydrazide compounds (Scheme 1). The first hydrazine of this type applied for MS study of glycans was carboxymethyltrimethylammonium chloride hydrazide, marketed as Girard’s T reagent (Table 1). Naven and Harvey (1996) reported a simple derivatization strategy by employing this reagent to introduce a cationic site and improve MS detection limit of oligosaccharides released from glycoproteins by hydrazinolysis. No clean-up stage was required and the products were obtained in a high yield. The authors reported that this derivatization helped to eliminate problems associated with the presence of reducing terminal N-acetylamino groups, often introduced when oligosaccharides were obtained by chemical cleavage with hydrazine (NH₂NH₂) from glycoprotein. Girard’s T reagent was later used by other research group for the analysis of small saccharides (Gouw et al., 2002). The authors referred that observed gain in sensitivity of derivatized sugars did not demonstrate a great advantage for the analysis of complex samples in which oligosaccharide analyte was as a minor component.
However, with more concentrated samples, the identification of small saccharides as their quaternized derivatives was facilitated by the absence of matrix derived signals in the MALDI spectra.

It was reported lately that three methyl groups in Girard’s T reagent are not sufficient to overcome the hydrophilicity imparted by the permanent charge and that Girard’s T-derivative ions were even less abundant than ions of native sugars (Bereman, Comins, & Muddiman, 2010). It was demonstrated that a house-synthesized variant of Girard’s T reagent with tripropyl groups instead, afforded approximately a 5- to 12-fold increase in electrospray response compared with the native saccharides (Table 1). Although charged tripropyl hydrazide provided much significant increase in the sensitivity then its analog with phenyl group, the same research group chose to follow a comparative study with Phenyl-GP (Walker et al., 2010). Interestingly, it was stated that the incorporation of a permanent charge generally decreases the signal intensity and hampers glycan analysis. A comparative study of neutral hydrazide derivatives versus their corresponding counterparts with permanent charges was demonstrated on evaluation of saccharides coupled with phenyl-phenylacetic hydrazide (Phenyl-GPN), phenylacetic hydrazide (GPN) against those with permanently charged Phenyl-Girard’s P (Phenyl-GP) and Girard’s P (GP) reagents (Table 1). Walker et al. (2010) reported that saccharides coupled with neutral Phenyl-GPN and GPN provided better increase in electrospray response than their corresponding charged hydrazide derivatives—Phenyl-GP and GP. This study supported the hypothesis that the positive charge actually affects the molecule to be more solvated in the electrospray droplet, thus less competitive for the excess surface charge and producing a decreased ESI response in comparison with neutral hydrazide analogs. Shortly after that the same research group examined other house-synthesized analogs of neutral hydrazides (GPN2-4; Table 1). They confirmed that ESI-MS signal for tagged oligosaccharide standards was observed significantly more abundant and the detection of sialylated glycans was enhanced (Walker et al., 2011a). Among these derivatives, Phenyl-2-GPN was selected for labeling glycans from complex mixtures and examined on the analysis of N-linked glycans obtained from human plasma. The detailed investigation led to modify conditions to minimize the peeling reaction or loss of labile sugar residues (NeuAc). It was found that 56°C in 25% acetic acid in...
methanol for 3 hr provided more than 95% conversion to the derivatized glycans with minimized peeling. Recently, the Phenyl-2-GPN reagent was synthesized with incorporation of $^{13}$C$_6$ stable isotopes and then applied for labeling glycans (Walker et al., 2011b). This study demonstrated the capability of this stable-isotope derivative to be used for the quantification of N-glycans (Fig. 4).

Over the years a number of hydrazide-containing reagents have been devised and many of them constitute versatile and effective probes for labeling oxidized saccharides in functional studies (Wilchek & Bayer, 1987; Bayer, Ben-Hur, & Wilchek, 1988). Among them, biotinylated derivatives have emerged as an additional class of promising probes that enable immobilization of desired glycans on streptavidin matrices, exploiting carbohydrate–protein interactions (Gitlin, Bayer, & Wilchek, 1987; Shao, Chen, & Wold, 1990; Shao & Chin, 1992; Shinohara et al., 1995; Leteux et al., 1999). These types of oligosaccharide derivatives have been mostly analyzed by MS as part of the preparation procedure, that is, verifying the purity of these derivatives aimed for another studies. Among these reports, the UV absorbing and fluorescent biotinyl-L-3-(2-naphthyl)-alanine hydrazide (BNAH) and 6-(biotinyl)-amino-caproyl-hydrazide (BACH) prepared as non-reduced derivative coupled via hydrazone linkages to oligosaccharides were compared with 2-amino-6-amidobiotinyl-pyridine (BAP) derivative obtained by classical reductive amination (Table 1). The purification of these derivatives was carried out by RP-HPLC, analyzed by LC-MS and their identities were established from

![Figure 3](image-url)
positive-ion spectra $\text{MH}^+$ and/or $\text{MNa}^+$ ions (Leteux et al., 1998). Although BACH lacks an aromatic group, which makes this biotinylated derivative a weak UV-absorbent, it was selected as an alternative tag for MS detection of saccharides (Kapková, 2009). The signal intensities of BACH-oligosaccharides were generally lower in comparison with corresponding reducing AB-derivatives. However, BACH derivatives performed better than their native analogs and still bear the advantage of their usefulness in functional studies. Wuhrer et al. (2010) combined BACH-labeled glycans with a microtitration plate binding assay, in which case the need for glycan immobilization and carbohydrate-binding proteins (CPBs) detection is avoided. Instead, the presented glycan–CPB binding assay employs a microtitration plate format that relies on immobilized (adsorbed) CPBs that are screened for the binding of glycans in solution. Glycans were isolated directly from a natural source and labeled with BACH at the reducing end a 1:1 stoichiometry. In the next step, labeled glycans were fractionated by HPLC. Fractions containing glycan ligands can be then characterized by tandem mass spectrometry (MS/MS) in conjunction with exoglycosidase treatment and rechromatography. The authors reported that this approach allowed them to separate efficiently the glycans carrying the target epitope from non-relevant BACH-glycans. The method was exemplified by analyzing keyhole limpet hemocyanin (KLH) N-glycans for their reactivity with anti-KLH antibodies as well as cross-reactive antibodies directed against the human parasite Schistosoma mansoni.

### III. STRUCTURAL ANALYSIS OF HYDRAZONE LABELED SACCHARIDES

The published results, reviewed in previous section, were mostly aimed at demonstrating the suitability of hydrazine derivatives for MS carbohydrate detection in regard of a simple preparation and gained sensitivity. A few articles also demonstrated the influence of the hydrazone linkage on MS fragmentation behavior of oligosaccharides and the usefulness of these derivatives in the structural analysis. Perhaps, the first detailed report with this purpose was presented in a study of N-glycans released from ovalbumin and labeled with PHN (Lattova, Perreault, & Krokhin, 2004). It was demonstrated that PHN labeling influences the fragmentation behavior of glycans under MALDI-PSD and MS/MS conditions. Neutral PHN-oligosaccharides produced $[\text{M} + \text{Na}]^+$ parent ions which fragmented in a predictable manner. The most abundant fragment ions at higher $m/z$ values corresponded to B and C types of glycosidic cleavages associated with the loss of labeled GlcNAc, followed by the cleavage of one more GlcNAc residue consistent with total loss of the chitobiose core. Less abundant cross-ring cleavage ions were observed in all MS/MS spectra, and were useful for the determination of linkage positions. On a large number of PHN labeled glycans, it was observed that monosaccharide residues attached to the 3-antenna of the core mannose were cleaved preferentially (B/Y$_3$ ions) and after that residues from 6-linked mannose followed. B/Y$_3$ ions produced by cleavages from the 3-antenna typically underwent to the characteristic loss of water, the same phenomenon as observed in the fragmentation patterns of some native glycans (Harvey, 2000b). The presence of a bisecting moiety was signaled by the loss of 221 Da. Moreover, the extent of the loss of GlcNAc with mass 221 Da seemed to increase with the number of residues attached to the 6-linked mannose. When no substituent was attached on the 6-linked mannose, the Man residue itself was lost instead of the bisecting GlcNAc moiety, producing ions at $m/z$ 388 (Scheme 4).

### TABLE 2. Key symbols and abbreviations used for depiction of glycans structures

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Monosaccharide residue</th>
<th>Abbreviation</th>
<th>Residual mass (monoisotopic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>○</td>
<td>Mannose</td>
<td>Man</td>
<td>162.053</td>
</tr>
<tr>
<td>●</td>
<td>Galactose</td>
<td>Gal</td>
<td>162.053</td>
</tr>
<tr>
<td>■</td>
<td>N-Acetyl-glucosamine</td>
<td>GlcNAc</td>
<td>203.079</td>
</tr>
<tr>
<td>▲</td>
<td>Fucose</td>
<td>Fuc</td>
<td>146.078</td>
</tr>
<tr>
<td>◊</td>
<td>N-Acetyl-neuraminic acid</td>
<td>NeuAc</td>
<td>291.095</td>
</tr>
<tr>
<td>◦</td>
<td>N-Glycolyl-neuraminic acid</td>
<td>NeuGc</td>
<td>307.090</td>
</tr>
</tbody>
</table>
isomeric structures. Structure the non-fractionated mixture was supportive for three possible oligosaccharides, the fragmentation pattern recorded from the high-resolution mass spectrum of the \(^{13}C_6\)-labeled P2GPN. The \(^{13}C_6\) was incorporated at 95%, while the \(^{13}C_0\) and \(^{13}C_5\) side products account for 1 and 5%, respectfully; \((\text{b})\) a control study in which one plasma sample was tagged with the “heavy” reagent and a blank vial was tagged with the “light.” The samples were mixed together, and a negligible cross-reaction was observed, as the 1% of “light” sample was observed from the incomplete incorporation of the \(^{13}C_6\). Reprinted with permission from Walker et al. (2011b). Copyright 2011, American Chemical Society.

\textbf{FIGURE 4.} Characterization of the heavy P2GPN reagent: \((\text{a})\) the high resolving power mass spectrum of the \(^{13}C_6\)-labeled P2GPN. The \(^{13}C_6\) was incorporated at 95%, while the \(^{13}C_0\) and \(^{13}C_5\) side products account for 1 and 5%, respectfully; \((\text{b})\) a control study in which one plasma sample was tagged with the “heavy” reagent and a blank vial was tagged with the “light.” The samples were mixed together, and a negligible cross-reaction was observed, as the 1% of “light” sample was observed from the incomplete incorporation of the \(^{13}C_6\). Reprinted with permission from Walker et al. (2011b). Copyright 2011, American Chemical Society.

Besides high-mannose, hybrid, and complex glycans, two sialylated structures were identified in ovalbumin (Scheme 5). Parent ions were detected as \([M + H + 2Na]^+\) adducts (Lattova, Perreault, & Krokhin, 2004). All neutral fragment ions were monosodiated and the cleavages with acidic residue produced \([M + H + 2Na]^+\) ions. The MS/MS spectrum for parent ions at \(m/z\) 2716.9 indicated glycans with composition of NeuAcGalGlcNAc\(_3\)Man\(_2\)GlcNAc\(_2\)PHN (Scheme 5a). The fragmentation pattern corresponded to two isomers, one with Gal substitution on the 4-linked GlcNAc (3-antenna) and the other with Gal on the 6-antenna instead. Abundant ions at \(m/z\) 2403.9 corresponded to an \(Y_6\)-cleavage of the NeuAc residue. The isomer with Gal on the 3-arm was consistent with the loss of a bisecting GlcNAc detected at \(m/z\) 938.3. This type of the cleavage for the isomer with Gal on the 6-arm was observed at \(m/z\) 1100.5. Sialylated glycans similar to those discussed, however with additional hexose residue, were detected as \([M + H + 2Na]^+\) adducts at \(m/z\) 2879.1 and corresponded to composition NeuAcGal\(_2\)GlcNAc\(_3\)Man\(_2\)GlcNAc\(_2\)PHN (Scheme 5b).

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residues from the 3-antenna were observed in CID spectra of sialylated glycans, along with the ions indicating the loss of bisecting GlcNAc.

Whether the hydrazone linkage in a labeling group has an impact on behavior of saccharides in regard to ionization, sensitivity or fragmentation, a comparative study involving two other reducing-end labeling methods was presented (Lattova et al., 2005). Both these methods, the widely used aminobenzamide (AB) under reduced conditions (Bigge et al., 1995) and derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP) yielding bis-PMP products (Honda et al., 1989; Shen & Perreault, 1998) produce open ring structures at the former reducing ends (Scheme 6a,b). Small oligosaccharides as lactose, N-acetylactosamine, fucosyllactose, and sialyllactose were studied under ESI- and MALDI-MS conditions and large glycans were characterized by MALDI-MS and MS/MS. It was shown that the intensities of MS signals depended on both, the type of reducing end label and the nature of saccharide investigated. For example, PMP-sialyllactose in ESI/MALDI produced a mixture of \([M + Na]^+\), \([M - H + 2Na]^+\) ions in the positive mode and \([M - 2H + Na]^+\) ions in the negative mode. The AB and PHN derivatives provided abundant protonated ions in ESI, and by MALDI produced abundant monosodiated ions. MS sensitivities depended again on the type of labeling and the type of carbohydrate. The influence of the labeling group on the fragmentation pattern was clearly observed even in a case of small saccharides. For instance, AB and PMP sialyllactose

![Diagram](chart.png)
**Scheme 5.** Structures of sialylated N-glycans identified in hen-ovalbumin (Grade V). The type of cleavage ions (nomenclature Domon & Costello, 1988) were based on MS/MS fragmentation pattern recorded with qQTOF mass spectrometer (Manitoba Sciex). Key symbols are in Table 2.

**Scheme 6.** General structures of oligosaccharides labeled at reducing terminus with: (a) aminobenzamide (AB); (b) pyrazolone (PMP); and (c) phenylhydrazine (PHN). Reprinted with permission from Lattová et al. (2005). Copyright 2005. American Society for Mass Spectrometry, Elsevier, Inc.
produced only Y-type ions under ESI or MALDI-MS/MS conditions (Fig. 6c–e). In the ESI-MS/MS spectrum of PHN-sialyllactose, abundant ions corresponded to B, Z cleavages (Fig. 6b) and in its MALDI-MS/MS spectrum, the abundant ions were consistent with Y-type glycosidic cleavages accompanied by B, C, and cross-ring fragment ions.

In the MALDI-MS spectra of large N-glycans acquired immediately after derivatization, it was possible to detect only PHN-oligosaccharides. After purification, spectra of all three types of derivatives showed high signal-to-noise ratios with the best sensitivity observed in the spectra of AB reduced glycans. $[M+Na]^+$ ions were the dominant peaks in all spectra and their fragmentation patterns were influenced again by the type of the labeling and type of oligosaccharide (Lattova et al., 2005). In CID-spectra of AB-glycans, higher $m/z$ ions corresponded to B and Y cleavages and the loss of bisecting GlcNAc was detected only as a weak signal or was not observed at all. Fragmentation patterns observed for hybrid/complex PHN and PMP glycans were more comparable; fragment ions at higher $m/z$ values corresponded to B and C-glycosidic cleavages. For PHN glycans, the abundance of ions resulting from the loss of bisecting GlcNAc depended on the number of residues linked to the 6-positioned mannose. Cross-ring cleavages in the spectra of PHN and PMP derivatives were observed in abundances higher than for AB derivatized oligosaccharides. For example, the fragmentation patterns recorded for derivatized galactosylated complex glycans of composition Gal$_3$GlcNAc$_6$Man$_3$GlcNAc$_2$ (MW$_{native}$ 2290.8) are shown in Figure 7. In the spectra of PHN (Fig. 7a) and PMP (Fig. 7c) derivatized glycans, higher $m/z$ ions corresponded to B, C and cross ring cleavages—$3.5A_3$, $0.2A_3$, and $0.2A_6$. The spectrum of AB derivatives contained dominant Y-type ions (Fig. 7b). The presence of bisecting GlcNAc in both isomers was apparent with PHN derivatives, where the loss of 221 U from B$_3$/Y$_3$ ions appeared at $m/z$ 1100.4 and 938.3 (Fig. 7a). These cleavages were present in the spectra of the other derivatives, but observed as low abundant ions (Fig. 7b,c).

High-mannose glycans produced some cleavages different relative to complex and hybrid glycans. The most abundant ions for AB derivatives were consistent with Y-type cleavages, whereas the PHN and PMP derivatives produced the most abundant ion again corresponding to B, C glycosidic cleavages. Diagnostically useful B$_3$/Y$_3$ ions resulting from the loss of chitobiose core and the 3-antenna ($m/z$ 671.3) were observed for AB and PHN glycans with high intensities. However, this signal was observed as a very weak peak in the spectra of PMP derivatives.

Recently, Ko and Brodbelt (2011) compared the fragmentation patterns of hydrazide-conjugated and reductively

![FIGURE 6. ESI-MS/MS spectra of $[M+H]^+$ ions of sialyllactose detected in the native form (a); derivatized with PHN (b); AB-unreduced (c); AB-reduced (d); and PMP (e). Reprinted with permission from Lattova et al. (2005). Copyright 2005, American Society for Mass Spectrometry, Elsevier, Inc.](image-url)
aminated oligosaccharides under CID (collisionally induced dissociation) and UVPD (ultraviolet photodissociation) in a quadrupole ion trap. The hydrazide conjugation procedure proved again to be an easy reaction (Table 1). The resulting UVPD spectra of AMCA (7-amino-4-methyl-coumarin-3-acetic acid hydrazide) derivatized oligosaccharides produced different cross-ring cleavage ions relative to reductively aminated oligosaccharides. Hydrazide derivatives generally resulted mainly in $0.2\Lambda$ and $2.4\Lambda$ type ions, whereas $0.1\Lambda$ and $0.2\Lambda$ type cross-ring cleavages were dominant in the spectra of saccharides derivatized under reductive conditions. On a series of small oligosaccharide standards ($\text{lacto-}N\text{-fucopentaoses, lacto-}N\text{-difucohexaoses, and mannopentaose}$) it was demonstrated that the differences in the fragment ions produced by UVPD versus CID were observed more dramatically for the reductively aminated derivatives, than for hydrazide coupled saccharides. However, integrating more derivatization procedures and more activation methods can provide additional complementary information for more comprehensive mapping of oligosaccharide structures.

IV. APPLICATION OF HYDRAZINE DERIVATIVES IN PROTEOMIC STUDIES

Mass spectrometry as one of the most sensitive technique is a leading tool in proteomic studies, and its enormous capability has already made positive contributions in the search for markers of diseases (Duchesne et al., 2006; Schulz & Aebi, 2009; Balog et al., 2010; Hua et al., 2011; Seward et al., 2011; Yates, 2011). Glycosylation is a highly conserved post-translational modification of biologically active proteins (Rademacher & Dwek, 1989; Dwek, 1996; Aebi & Hennet, 2001; Hart & Copeland, 2010) and by means of mass
spectrum, a lot of valuable information has been gathered. The published reports on using hydrazine derivatives for labeling of carbohydrates, which were reviewed in the previous sections, have targeted mostly saccharides from commercial sources as a part of the method development. A few articles have been published to demonstrate also the applicability and suitability of these derivatives for MS analysis of glycans obtained from real biological samples.

One first proteomic report mentioning MS detection of oligosaccharides through labeling based on hydrazone linkage was a study of meningococcal lipopolysaccharides (Mandrell et al., 1991). Dephotosphorylated oligosaccharides were derivatized with 4-buty1 phenylhydrazine (4-BPH) under acidic and heating conditions and analyzed in the negative ion mode (Table 1). The same derivatization procedure was used in the identification of surface lipooligosaccharides isolated from gram-negative human mucosal pathogen Haemophilus ducreyi (Melaugh et al., 1992). The reaction mixture followed HPLC fractionation on C18-column and purified 4-BPH-oligosaccharides were analyzed by liquid secondary ion MS. The mass spectral data combined with a compositional and methylation analyses provided evidence for oligosaccharides attached on lipid A. These oligosaccharides were terminated with 3-deoxy-D-manno-octulosonic acid (KDO) in which phosphate was at C-4. A similar approach was reported for the analysis of outer membrane lipooligosaccharides (LOS) from Haemophilus influenzae type b strain A2 (Phillips et al., 1993). The majority of these structures contained a variable number of hexoses, three L-glycero-D-manno-heptoses, and one KDO residue attached to a diphotosphorylated 0-deacetylated Lipid A moiety. Additional phosphate and phosphoethanolamine groups were shown to be also present on the oligosaccharide structures.

The first extensive MS study of oligosaccharides obtained from biological material and analyzed through hydrazone linkage was demonstrated on N-glycans obtained from total serum of nude mice with implanted head and neck tumors (Lattová et al., 2008). Serum samples of cancerous and healthy mice, after digestions (trypsin/chymotrypsin/PNGaseF), were fractionated by HPLC. The analysis of glycans fractions was achieved directly on-target after non-reductive derivatization with PHN. Neutral and sialylated oligosaccharides were detected from the same spot. The major differences between serum samples were observed mainly in the level of high-mannose glycans and neutral/sialylated hybrid oligosaccharides without GlcNAc linked on the 6-positioned mannose. These glycans were distinctly more abundant in the sera from cancerous mice. In this study, the presence of PHN-labeling on the reducing-terminus showed a benefit also in the identification of unknown glycan structures. A few glycans with unusual residues were detected in mouse serum. Among them, some atypical N-glycans had a residue with an extra 60 Da. No information was obtained by MS/MS relative to the position of this unknown moiety for the parent precursors of the native glycans (e.g., Fig. 8a). After labeling with PHN, producing peak with shift mass 90 Da (m/z 2016.7), the mass increment of 60 Da showed to be part of the non-reducing GlcNAc (Fig. 8b). The derivatization excluded the possibility that this uncommon residue may be linked to the chitobiase core; MS/MS data provided evidence for its presence on the non-reducing terminus and indicated the presence of an N-acetyl-octosamine residue (residual mass 263 Da) in two isomeric glycan structures.

A similar approach, PHN tagging in combination with MS, was applied in the examination of N-glycans obtained from the PNGase treated serum samples of woodchucks with liver cancer (Lattová et al., 2009). Moreover, the direct comparison of glycosylation profiles of both animal models, that is, mouse and woodchuck was an excellent marker to confirm differences in their profiles (Fig. 9). It helped to validate the existence of new N-glycan structures found in mouse serum (e.g., shown in Fig. 8) and to eliminate the possibility of their presence as side products formed during a sample preparation. The comparison of N-glycan pools obtained from mouse and woodchuck serum samples showed discrepancies in their glycosylation profiles and suggested differences in the protein glycosylation between these two animal models. The dominant N-glycan type obtained from both samples corresponded to a biantennary sialylated structure with the sialic residues on both antennae. NeuGc type residues were found in glycans from mouse serum (Fig. 9a). In woodchuck serum, glycans with NeuAc residues were dominant and MS/MS analysis of PHN-glycans also supported the presence of minor N-glycans bearing both types of sialic acids—NeuAc and NeuGc (Fig. 9b). While three and higher sialylated oligosaccharides were observed extensively in the samples of mice sera, these
glycans were observed only sporadically and at low abundances in pools obtained from serum of woodchucks analyzed under the same conditions. Some minor glycans of the same m/z values were detected in the samples from both types of animals. However, the MS/MS fragmentation pathways evidently indicated the existence of different glycans types for the same parent ions. For example, in mouse serum, the fragmentation pattern of parent ion with m/z 2206 corresponded to only a complex biantennary fucosylated N-glycan with a NeuGc residue on one of the antennae (Fig. 9a; m/z 2206). In serum obtained from woodchucks, the precursor ions with the same m/z value provided fragment ions for additional N-glycan—hybrid structure with NeuAc (Fig. 9b; m/z 2206). In the previous work presented by Block et al. (2005), the increase of biantennary sialylated glycan with 1,6-linked fucose was observed in the serum of diseased woodchucks with the value of serum gamma glutamyl transferase (GGT) higher than 100 IU/L. However, in the serum of woodchuck diagnosed with HCC and GGT values under 100 IU/L, only high-mannose and hybrid glycans showed to be elevated when compared with oligosaccharide pools obtained from healthy animals (Lattova et al., 2009). As mentioned above, these glycans were also observed increased in sera from cancerous mice (Lattova et al., 2008). These facts suggest that high-mannose and hybrid structures could be good indicators for early cancer detection. The relationship between increase of high-mannose glycans and cancer, analyzed through PHN labeling of N-glycans obtained from serum samples (Lattová et al., 2008, 2009) can be supported by the similar finding, when N-glycans were obtained from sera of human and mice with breast cancer (de Leoz et al., 2011).

In 2010, PHN derivatization was employed in a study of N-glycans isolated from cancer cells and compared with profiles of the same models after treatment with antibody (Lattová et al., 2010). The investigation of N-glycans indicated changes in the glycosylation during treatment with humanized monoclonal antibody (Herceptin). Detailed MS analyses of glycans cleaved from human breast MCF-7 carcinoma and T-lymphoblastoid CEM cells showed a dominant occurrence of high-mannose glycans accompanied with minor peaks corresponding to bi-, tri-, and tetraantennary higher sialylated N-glycans and most of them were core fucosylated (Fig. 10a). After treatment, most of these glycans were suppressed and instead complex biantennary fucosylated oligosaccharides with the same compositions as found in IgG1 were detected (Fig. 10b,c). A number of additional significant oligosaccharide peaks were observed only in cells treated with Herceptin without presence of Lipoplex. These glycans corresponded to complex non-fucosylated triantennary galactosylated structures (marked with arrows in Fig. 10c). Although sialylated oligosaccharides were generally present at lower abundances in all samples examined, the alterations were notable at this level too. Fucosylated tri- and tetraantennary sialylated oligosaccharides present in the original leukemic and breast cancer cells were suppressed after treatment and the most abundant acidic glycan corresponded to a non-fucosylated triantennary structure with NeuAc residue on each arm (m/z 2992.1). A new acidic biantennary glycan with fucose on the reducing GlcNAc and bearing NeuGc residue (m/z 2206; the structure can be seen also in Fig. 9a) was observed in the cells after exposure to Herceptin only. This undesirable non-human NeuGc has been discussed as being potentially immunogenic and unacceptable as a therapeutic agent (Jefferis, 2005). The evaluation of cell viability demonstrated that lipoplex targeted samples generally resulted in a more efficient delivery of the antibody into cancer cells and produced a higher growth-inhibitory effect than the monoclonal antibody administrated alone (Bartusik et al., 2010a,b). The results in a combination with MS evaluation of profiles supported the hypothesis that some N-glycans detected in cells exposed to antibody without Lipoplex may be associated with the development of cancer cells’ resistance against the antibody.

Another research group made an effort in including PHN-labeling for an automated MS analysis aimed for the routine clinical diagnostic work. Yamada et al. (2010) demonstrated a procedure for the O-glycans release using an automatic device. In this approach, an alkaline solution of free glycans is immediately cooled and neutralized by passing through a cartridge packed with cation-exchange resin. The system is connected to an auto spotter; the sample is mixed with matrix (DHB) and loaded on the MALDI target. To enhance MS sensitivity for released glycans, on-plate derivatization with PHN (Lattová et al., 2006, 2007) was applied and coupled with the device. This approach of analysis was successfully applied for the release and MS analysis of O-glycans obtained from MKN45 cells derived from human stomach adenocarcinoma and supported the abundant presence of trisialo-O-glycans (Yamada et al., 2010).

Over the last decade, the hydrazone-linkage principle has been extensively used in preparative procedures to isolate glycans from digested glycoproteins (Kaji et al., 2003; Zhang et al., 2003; Tian et al., 2007; Li et al., 2011; Taga et al., 2012; Yang & Zhang, 2012). This approach is based on an oxidation of sugars to generate aldehyde groups by means
FIGURE 10. Representative MALDI-QqTOF mass spectra of N-glycans obtained from breast carcinoma MCF-7 cells. N-glycans were released with PNGaseF and labeled with PHN: (a) glycans from original untreated cells; the inset shows an expansion of the spectrum of the fraction eluted prior to that shown here in regular size; (b) glycans obtained from cells after treatment with Herceptin for 6 hr; and (c) glycans obtained from cells after treatment with Herceptin for 72 hr. All peaks are \([M + Na]^+\). Reprinted with permission from Lattová et al. (2010). Copyright 2010, American Chemical Society.
glycosylated peptides are captured on the hydrazine resins through hydrazone bonds. After washing and removing non-binding peptides, the glycans are released from beads and can be then investigated. Although glycans here are not directly examined as hydrazone derivatives, still hydrazide bond plays a key role in their isolation aimed for another studies. This approach became popular as a preparative procedure and helped in identification of a large scale of new glycosylation sites in glycoproteins (Pan et al., 2006; Zhang et al., 2006; Sun et al., 2007; Klement et al., 2010; Tian et al., 2010).

V. CONCLUSION

Last two decades different types of hydrazine derivatives have been applied for MS analysis of carbohydrates. This simple type of reducing-end labeling based on the hydrazide linkage showed the usefulness not only in regard to enhanced sensitivity, but also in the assignment of new isomeric structures. Very recently, the successfulness has been presented on the analysis of glycans isolated from complex biological samples such as serum or cancer cells. Along the comparative studies, new glycan structures were identified. In the literature, more articles are becoming devoted to the total proteome analysis through glycopeptides instead of glycans. The rising interest for this type of analysis is not surprising, since direct glycopeptide investigation by tandem MS is fast and sensitive also for the site-specific characterization of glycosylation. It can provide informations on glycan compositions, glycan attachment site and peptide sequence. Besides that, the more lengthy procedure of glycan analysis involving separation and derivatization technique are avoided. However, the glycosylation is still not beyond full recognition and to understand this process, a combination of more approaches during investigation can provide more knowledgeable insights. Thus, using and improving labeling techniques for glycan analysis is still very important. Nevertheless, there is no doubt that results reported in this review constitute the positive contributions in the field of glycomics.

VI. ABBREVIATIONS

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AB</td>
<td>2-aminobenzamide</td>
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<tr>
<td>ATT</td>
<td>2-aza-2-thiothymine</td>
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<tr>
<td>CID</td>
<td>collision-induced dissociation</td>
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<tr>
<td>DHB</td>
<td>dihydroxybenzoic acid</td>
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<tr>
<td>ESI</td>
<td>electrospray ionization</td>
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<tr>
<td>GGT</td>
<td>gamma glutamyl transferase</td>
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<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption ionization</td>
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<td>MS/MS</td>
<td>tandem mass spectrometry</td>
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<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
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<tr>
<td>PNGaseF</td>
<td>peptide N-glycosidase F</td>
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<td>PHN</td>
<td>phenylhydrazine</td>
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<td>PMP</td>
<td>1-phenyl-3-methyl-5-pyrazolone</td>
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<tr>
<td>PTM</td>
<td>post-translational modification</td>
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<tr>
<td>QqTOF</td>
<td>MS quadrupole-quadrupole time-of-flight mass spectrometer</td>
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<tr>
<td>UVPD</td>
<td>ultraviolet photodissociation</td>
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