Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 793 (2003) 167–179
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Labelling saccharides with phenylhydrazine for electrospray and matrix-assisted laser desorption–ionization mass spectrometry

Erika Lattova[a,b], Hélène Perreault[a,*]

[a] *Chemistry Department, University of Manitoba, 144 Dysart Road, Winnipeg, Manitoba, Canada R3T 2N2*
[b] *The Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia*

Abstract

A well-known reaction of carbonyl compounds with phenylhydrazine has been applied to saccharides, providing increased sensitivity for mass spectrometric (MS) and ultraviolet (UV) detection during high-performance liquid chromatographic (HPLC) separations. After a simple derivatization procedure for 1 h at 70 °C and purification of the reaction mixture from excess reagent by extraction, the sugar derivatives were characterized by direct injection or on-line HPLC/electrospray ionization (ESI) and by matrix-assisted laser desorption/ionization (MALDI) MS. Because no salts are used or produced upon reaction, this procedure is very simple and suitable for the tagging of saccharides. The reaction allows for on-target derivatization and products are very stable. The derivatization procedure has been applied to commercially-obtained small saccharides and standard *N*-linked oligosaccharides. Lastly, hen ovalbumin *N*-glycans were detached enzymatically and characterized by MALDI–MS as their phenylhydrazone derivatives.

Keywords: Saccharides; Phenylhydrazone

1. Introduction

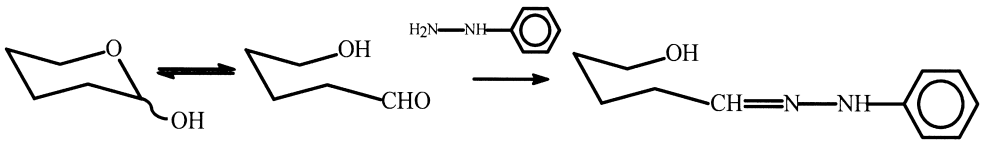
In recent years, a large number of new methods for saccharide analysis have been introduced and although the identification of oligosaccharides has reached a high degree of development, the challenge in introducing new techniques to help elucidate glycoconjugate structure is still very real [1].

For enhanced ultraviolet (UV) or mass spectrometric (MS) detection, carbohydrates are often derivatized at their reducing ends. Most of these so-called tagging methods convert saccharides into derivatives which can be detected at lower concentrations than their native analogs. The most common derivatization technique is reductive amination with aromatic amines [2–7]. A recent review reported on the preparation and comparative electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) detection sensitivity of a number of these derivatives and their subsequent collision-induced dissociation (CID) patterns [8]. Other research groups including ours have used derivatization techniques based on the reaction of reducing sugars with 1-phenyl-3-methyl-5-pyrazolone (PMP) under basic conditions to give disubstituted PMP derivatives [9–11].

Phenylhydrazones (PNH), formed during the reaction of carbonyl compounds with arylhydrazines (Scheme 1), feature among the oldest derivatives in carbohydrate chemistry and have been used for

*Corresponding author. Tel.: +1-204-474-7418; fax: +1-204-474-7608.
E-mail address: perreau@cc.umanitoba.ca (H. Perreault).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00374-X



Scheme 1. Labelling of a saccharide with phenylhydrazine, showing reaction with the acyclic form of a monosaccharide.

various purposes (e.g. Ref. [12]). Recently, 2,4-dinitrophenylhydrazine and dansylhydrazine have been used for labelling neutral monosaccharides and high-performance liquid chromatographic (HPLC) analysis [13–15]. In this work, we used phenylhydrazine because of the simplicity of the reaction and the fact that no salts are used or produced upon reaction. Phenylhydrazine also reacts with *N*-acetylated glycans without cleavage of the glycosidic bonds, acetyl groups or losses of sialic acid moieties.

In this paper, we report on the formation of PNH derivatives of small saccharides and of larger *N*-glycan standards for HPLC and MS purposes. Also, *N*-glycans detached enzymatically from hen ovalbumin were derivatized. Hen ovalbumin was chosen as a model glycoprotein for this study because of the wide availability of data in the literature pertaining to its structural characterization (e.g. Refs. [16–20]). Harvey et al. [21,22] recently carried out MALDI–MS analyses on glycans detached from ovalbumin by hydrazinolysis and enzymatic digestion, with no derivatization. They reported 37 glycans, found in glycoproteins separated by reversed-phase HPLC.

2. Experimental

2.1. Materials

Phenylhydrazine, small saccharides D-galactose, D-glucose, D-mannose, *N*-acetyl-D-galactosamine (GalNHAc), *N*-acetyl-D-glucosamine (GlcNHAc), *N*-acetyl-D-mannosamine (ManNHAc), β-lactose, 2′-fucosyl-lactose (2-FL), sialyllactose (a mixture of 3′- and 6′-*N*-acetyl-neuramin-lactose, SL), hen ovalbumin (Grade VII) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma (St Louis, MO, USA). *N*-Acetyllactosamine (LacNHAc) was prepared according to a procedure published in the literature [23] and the arabinosazone also according to a published procedure [24]. *N*-linked oligosaccharide standards NGA2, NGA2F, NA2F, AI, A2F (Table 1) were obtained from Oxford GlycoSciences (Oxford, UK). Peptide-*N*-glycosidaseF (PNGase F) deglycosylation kits were purchased from Glyko (Novato, CA, USA). Solvents (acetonitrile, methanol, ethanol) were HPLC-grade and purchased from Fisher (Fair Lawn, NJ, USA). HPLC-grade deionized, distilled water was obtained with a Milli-Q® plus TOC water purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of phenylhydrazones of small saccharides

The saccharides (3 μmol) were dissolved in distilled water (20 μl) and phenylhydrazine (1 μl) was added. The mixture was heated at 70–80 °C for 1 h. During incubation time, the samples were periodically mixed. After cooling to room temperature, unreacted phenylhydrazine was removed by extraction with ethylacetate (100 μl water and 2×50 μl EtOAC). The aqueous portion was diluted to desired concentrations using acetonitrile–water (1:1).

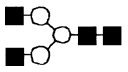
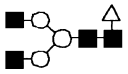
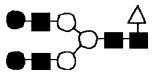
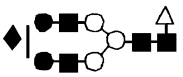
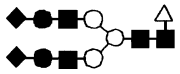
2.3. Derivatization of large oligosaccharides

To a water solution of *N*-glycans (1 μl, 50–76 pmol) was added a 10% methanolic solution of phenylhydrazine (0.3 μl) and distilled water (1 μl). The mixtures were then processed as described above.

2.4. On-target reaction

A water solution of *N*-glycan (0.5 μl, 20–38 pmol), DHB (0.5 μl, 3 nmol) and 5% methanolic solution of phenylhydrazine (0.5 μl) were thoroughly mixed and the reaction mixture (0.5 μl) was spotted on a target and allowed to dry at room

Table 1
Structures of *N*-glycan standards used in this study

		M_w (Da)
1. NGA2		1316.5
2. NGA2F		1462.5
3. NA2F		1786.6
4. A1		1931.7
5. A2F		2368.8

No anomericity or optical isomericity was taken into account, neither were linkage characteristics; ○, Man; ●, Gal; △, Fuc; ■, GlcNAc; ♦, Neu5Ac.

temperature for 40 min. The matrix was shown not to react with phenylhydrazine.

2.5. Deglycosylation—PNGase F digestion [25,26]

Ovalbumin (100 μg) was dissolved in deionized water (36 μl) in a microcentrifuge tube and supplied Buffer 5× from the Glyko deglycosylation kit was added (9 μl). Then, ovalbumin was denatured by boiling in a hot water-bath (5–10 min). After cooling, the OLIGO profiling enzyme (4 μl) was added. Following incubation (37 °C, 18 h), the protein was precipitated by adding three volumes of cold ethanol and the mixture was kept in ice. The protein was centrifuged down to a pellet and the supernatant containing the oligosaccharides was pipetted out into another microcentrifuge tube. Ethanol was evaporated and the white residue was derivatized with phenylhydrazine.

2.6. HPLC

A System Gold HPLC chromatograph equipped with a System Gold 166 UV Detector and 32 Karat Software (Beckman-Coulter Canada, Missisauga, ON, Canada) was used for separation of derivatized small saccharides. A Zorbax 300-SB C_8 (4.6×150 mm) column was used (Chromatographic Specialties, Brockville, ON, Canada). Elution was carried out at a flow-rate 0.5 ml/min with solvents A (0.1 *M* acetic acid in 10:90 acetonitrile–water) and B (0.1 *M* acetic acid in 25:75 acetonitrile–water). The proportion of solvent B was linearly increased from 30 to 50% over 6 min, and kept at 50% until the end of the run. UV detection was carried out at 245 nm.

2.7. Electrospray mass spectrometry

The spectrometer used for ESI-MS experiments was a Quattro-LC (Micromass, UK) equipped with a Z-Spray™ ESI source and a triple quadrupole analyzer. Direct injections were carried out with a 20-μl Rheodyne loop and the carrying solvent was acetonitrile–water (1:1). The samples were sprayed using a 3.5 kV needle voltage, and the cone voltage was set from 20 to 60 V. The source block and desolvation temperatures were set at 110 and 130 °C, respectively. Mass spectra were recorded in the positive and negative ion modes with a scan rate of 300 U/s. CID tandem MS (MS–MS) experiments were conducted with argon at a gas pressure of 3×10^{-3} Torr and collision energy of 28 eV. For most experiments, 5-μl

aliquots were injected and diluted into the 20-μl loop. For on-line HPLC/ESI-MS, the total flow-rate was adjusted to 0.2 ml/min and a splitter was used, with the same gradient as described above. The instrument was used in the positive mode, with a cone voltage of 20 V. Desolvation and source block temperatures were set at 200 and 130 °C, respectively.

2.8. MALDI mass spectrometry

MALDI mass spectra were recorded on a Biflex-IV spectrometer (Bruker Daltonics, Billerica, MA, USA). The accelerating voltage was 20 kV. Profiling of the glycans was carried out in the positive and negative ion polarities, using reflectron and linear time-of-flight (TOF) modes. For sample preparation, the glycan solution (0.5 μl containing up to 10 pmol of glycan) was loaded onto a stainless steel target with the same volume of saturated matrix solution (2,5-DHB in acetonitrile–water 7:3) and allowed to dry. Spectra presented here result from the addition of data from several pulses with no further data processing. Peak widths at half height were generally ≤10 U.

3. Results and discussion

The conversion of mono- and oligosaccharides to the corresponding phenylhydrazones was quantitative. When the reaction mixture was heated at 70–80 °C for 1 h, no unreacted sugars were observed in the mass spectra. Also, full conversion was reached even when phenylhydrazine was added to the sugars in the presence of DHB matrix for on-target reaction at room temperature. There was no reaction between DHB and phenylhydrazine and thus the presence of matrix does not hamper the sugar labelling reaction on the MALDI target. Phenylhydrazones were prepared from small and large oligosaccharides. Sialyllactose was derivatized to show that sialic acid linkages remain intact during the reaction, even in large excess of reagent.

3.1. Electrospray mass spectrometry

All PNH derivatives prepared with small saccharides produced characteristic signals in ESI-MS (Table 2). When using a low cone voltage, mainly $[M+H]^+$, $[M+Na]^+$ and $[M-H]^-$ ions were produced in the positive and negative modes, with limited fragmentation. ESI-MS–MS spectra of $[M+H]^+$ ions featured mainly losses of monosaccharide residues from the nonreducing end (Y-ions [27]). For example, when $[M+H]^+$ ions of phenylhydrazine-lactose (m/z 433) were investigated by MS–MS, Y_1 fragment ions corresponding to loss of a galactosyl residue (m/z 253) plus loss of the label (Y_1/B_2, m/z 162) were observed.

The ESI-MS and MS–MS spectra of derivatized 2′-fucosyllactose are shown in Fig. 1. Loss of the fucosyl residue from the nonreducing end produced Y_2 ions at m/z 415 and Y_1 ions due to further loss of

Table 2
Saccharides used in ESI-MS studies, and ions observed for their PNH derivatives

Phenylhydrazone derivative of	Calculated molecular mass (Da)	Molecular ions (m/z)	
		$(M+H)^+$	$(M+Na)^+$
Arabinose	240.2	241.2	
Galactose	270.3	271.1	
Glucose	270.3	271.3	
Mannose	270.3	271.2	
Ga1NAc	311.3	312.3	
Ga1NAc	311.3	312.4	
ManNAc	311.3	312.3	
Lacoste	432.4	433.4	
LacNAc	473.5	474.4	
2′-Fucosyllactose	578.5	579.2	601.6
3′-Sialyllactose	723.7	724.7	746.7

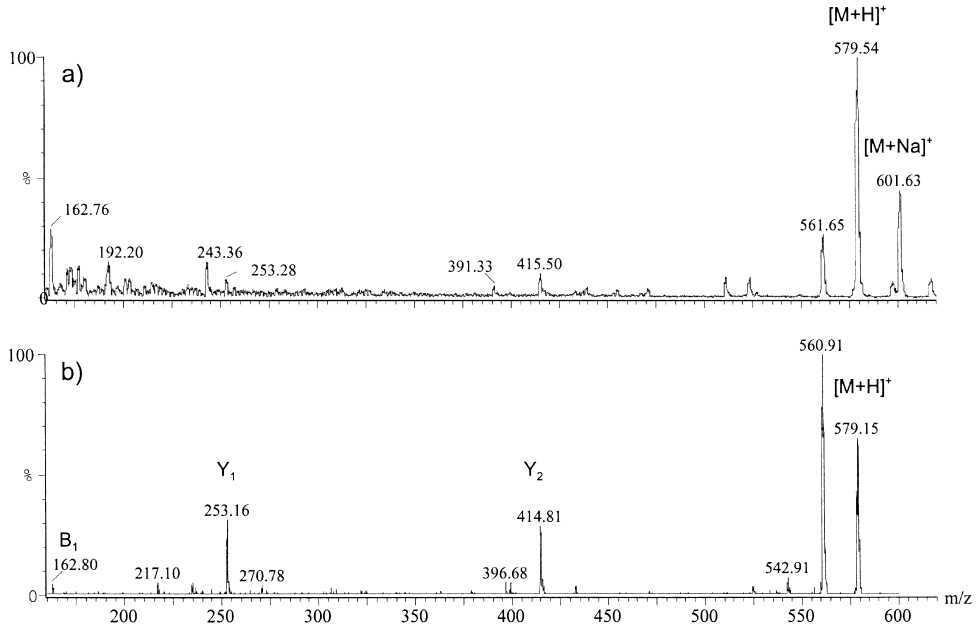


Fig. 1. ESI spectra of PNH-fucosyllactose: (a) positive ESI-MS, (b) ESI-MS–MS spectrum of the $[M+H]^+$ ions.

a galactosyl residue at m/z 253. Fig. 2 displays: (a) positive and (b) negative ESI-MS data obtained for PNH-sialyllactose. In (c), the MS–MS spectrum of $[M+H]^+$ ions, fragment ions at m/z 415 (Y_2) correspond to the cleavage of sialic acid and fragment ions at m/z 253 (Y_1) to the subsequent loss of

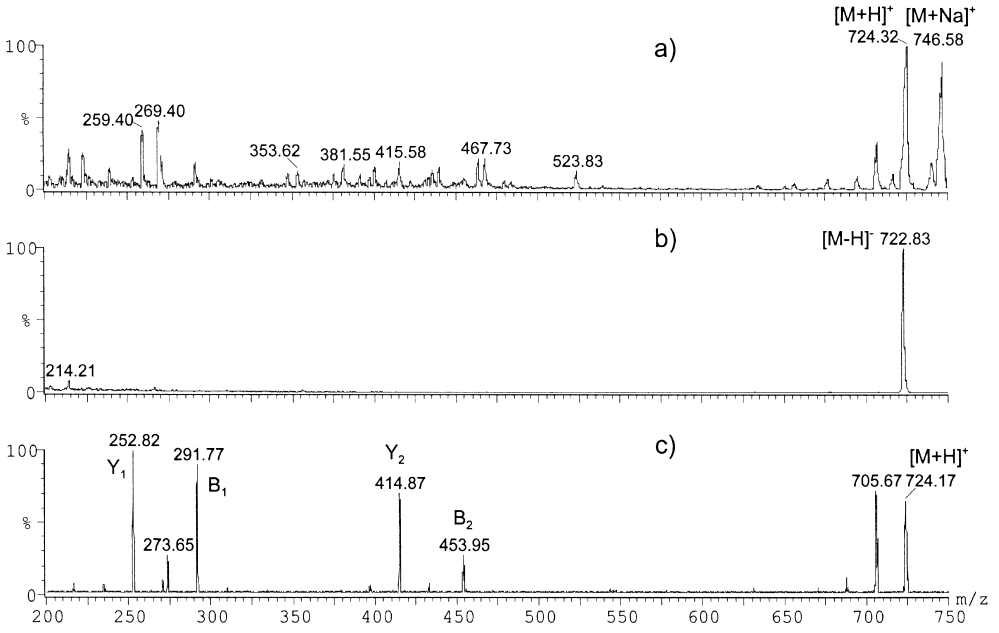


Fig. 2. ESI spectra of PNH-sialyllactose: (a) positive mode, (b) negative mode, (c) ESI-MS–MS spectrum of the $[M+H]^+$ ions.

a galactosyl residue. For these experiments, the resolution of the triple quadrupole analyser was of the order of 800 and the mass accuracy was ±0.3 U. In this view, peaks in Fig. 1 labelled at m/z 415.50 (a) and 414.81(b) both represent Y_2 ions, suggesting that low extent in-source fragmentation occurred (a), even if the cone voltage was adjusted at only 20 V, which is minimal in the case of our instrument.

In order to assess sensitivity enhancement provided by phenylhydrazine derivatives in ESI-MS, an equimolar mixture of PNH-lactose and lactose was tested (Fig. 3a). In the positive mode, a significant difference was observed in relative ion abundances. Thus m/z 433 ions (derivatized) were about 15-fold more abundant than m/z 343 ions (native). In the negative mode, ion abundances were comparable (not shown). Although the negative-ion spectrum of Fig. 2b is fairly exempt of noise and ions due to prompt fragmentation, the overall abundance of $[M-H]^-$ ions is lower than that of $[M+H]^+$ and $[M+Na]^+$ also seen in Fig. 2a, in spite of observed prompt fragmentation observed there. As was expected due to the presence of an *N*-acetyl group, derivatized LacNHAc had a lower detection limit in positive ESI-MS than its neutral analog PNH-lactose. PNH-LacNHAc (at m/z 474) yielded a twofold signal enhancement over non-derivatized LacNAc (at m/z 384). While for lactose the detection limits were 300 pmol for the native form and 20 pmol for the PNH form, for LacNHAc the detection limits were 100 pmol (native) and 10 pmol (PNH). Here, the detection limit was defined as an amount of material necessary to produce $[M+H]^+$ ions with a 4:1 S/N ratio. These values are rather conservative, although they approach those reported for PMP derivatives of neutral sugars [31] and for amino-benzoic ethyl acid ester (ABDEAE) derivatives [32].

3.2. Reversed-phase HPLC of PNH-saccharides

A mixture of arabinose, galactose, glucose, GalNAc, GlcNAc and lactose (3 μmol of each) was reacted with phenylhydrazine as described in the Experimental section. Aliquots corresponding to 20 nmol of each derivative were used for chromatographic analyses on an analytical scale C_8 column.

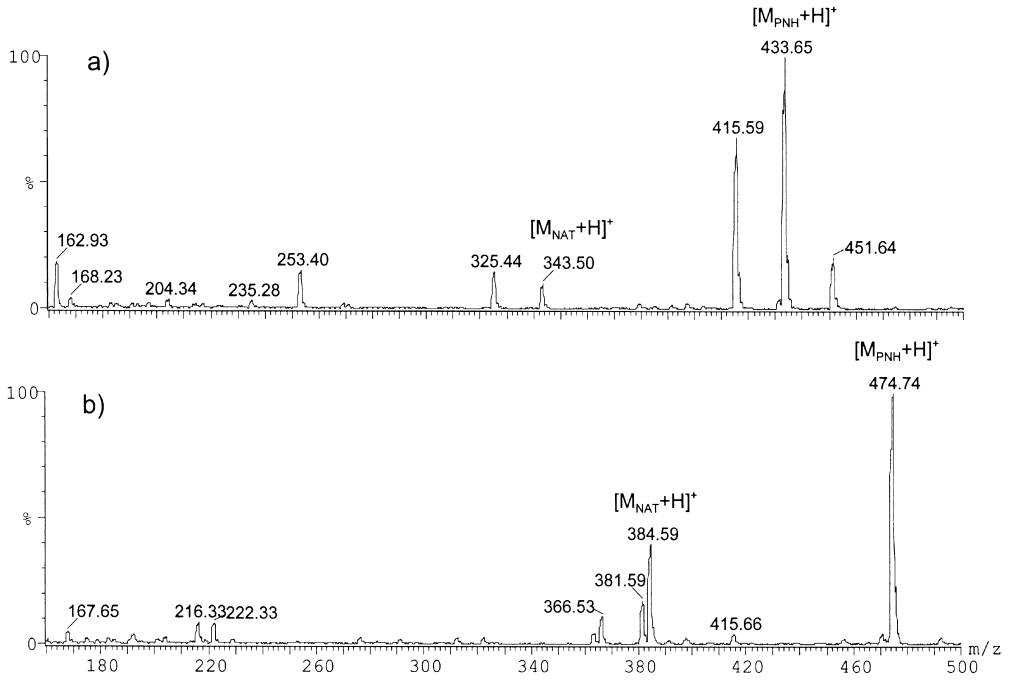


Fig. 3. Positive ESI-MS spectra of an equimolar mixture, 10^{-4} *M* of each compound. (a) Lactose and its PNH-derivative, (b) *N*-acetyllactosamine and its PNH-derivative.

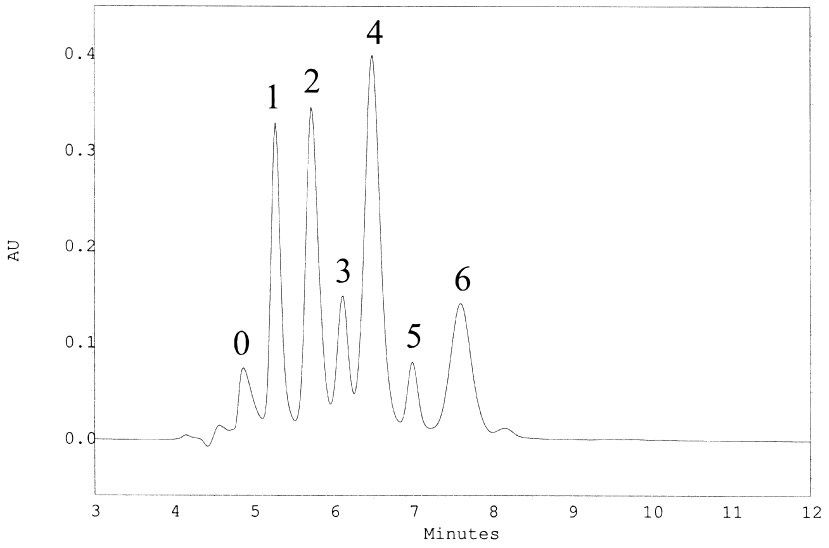


Fig. 4. HPLC–UV chromatogram of the mixture of PNH derivatives of lactose (**1**), glucose (**2**), galactose (**3**), *N*-acetylglucosamine (**4**), arabinose (**5**) and *N*-acetylgalactosamine (**6**). Detection: 245 nm.

Initially, the best separation conditions were achieved (not shown) when solutions of acetonitrile–water in 0.1 *M* ammonium acetate were used, as previously described for PMP-derivatives of small sugars [11]. Because on-line HPLC/ESI-MS is not acetate-tolerant, the ammonium salt was replaced by

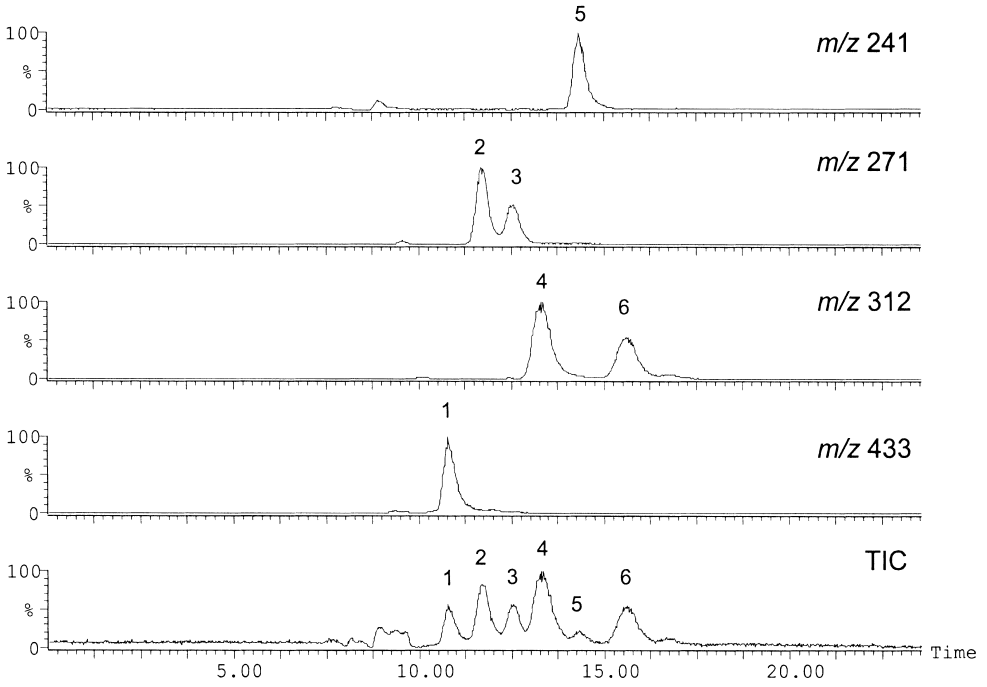


Fig. 5. HPLC/ESI-MS reconstructed and total ion chromatograms obtained for a mixture of PNH derivatives of lactose (**1**), glucose (**2**), galactose (**3**), *N*-acetylglucosamine (**4**), arabinose (**5**) and *N*-acetylgalactosamine (**6**).

0.1 *M* acetic acid. Solvents A and B were made of 0.1 *M* acetic acid in 10:90 and 25:75 acetonitrile–water, respectively. These eluents allowed separation of the mixture in its individual components, as shown in Fig. 4. This UV trace, obtained at 245 nm, shows that most peaks are baseline resolved. Residual unreacted phenylhydrazine was eluted first (Peak 0) at ca. 4.7 min. Peaks corresponding to the derivatives were assigned by individual injections and HPLC/ESI-MS.

Although equimolar quantities of the materials were used, PNH derivatives of arabinose, galactose and GalNAc produced smaller peaks than their gluco epimers. According to an NMR study of several sugar phenylhydrazones, their ratios of cyclic to acyclic forms depend on the solvent used and on sugar configuration [33]. Hence, the difference in UV response of individual saccharides could be explained by different concentrations of acyclic forms in solution. Moreover, discrepancies in the extent of electron delocalization around the phenylhydrazone groups, depending on saccharide structure, could cause differences in the absorption patterns. Lactose and glucose have three secondary hydroxyl groups at Carbons 2, 3 and 4 in the gauche arrangement in their zigzag conformations [34]. The same applies to *N*-acetylglucosamine, where a hydroxyl is replaced by an *N*-acetyl function. These groups could take part in phenylhydrazone delocalization through hydrogen bridges. The lower UV absorptivity observed for galactose, *N*-acetylgalactosamine and arabinose could result from only two such groups in *cis* and with therefore less bridging taking place. A more accurate explanation for this phenomenon would require a more detailed study.

On-line HPLC/ESI-MS was carried out on the same mixture. PNH-derivatives gave rise to $[M+H]^+$ ions, which produced the reconstructed and total ion chromatograms (RIC) shown in Fig. 5. As two pairs of isomers were present (glucose/galactose, GlcNHAc/GalNHAc)), only four traces were necessary to monitor the elution of all components. These isomers produced the ESI-MS in-source fragmentation patterns (Fig. 6b,c) and could not be distinguished by MS only, whereas retention time was a good identification tool for these compounds. Small-

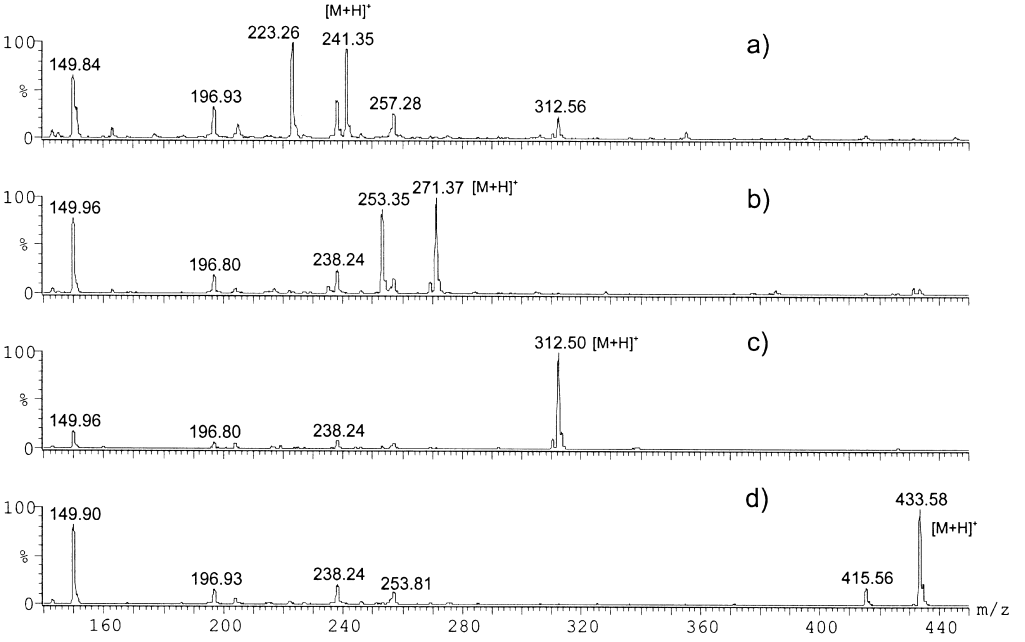


Fig. 6. Positive ESI-MS spectra obtained from the HPLC/ESI-MS experiment of Fig. 5. (a) Arabinose (**5**), (b) galactose (**3**) and glucose (**2**), (c) *N*-acetyl-galactosamine (**6**) and -glucosamine (**4**), (d) lactose (**1**).

er saccharides were prone to losing a water molecule in the source, even with a cone voltage of 20 V. This loss was not as pronounced with GlcNAc, GalNAc and lactose.

These experiments are preliminary to separations in micro-LC mode, which will require less material than analytical conditions and improve the overall sensitivity. Micro-LC/UV and micro-LC–MS results will be presented in a future article.

3.3. MALDI mass spectrometry

Under MALDI–MS conditions, PNH derivatives of *N*-linked oligosaccharide standards consistently

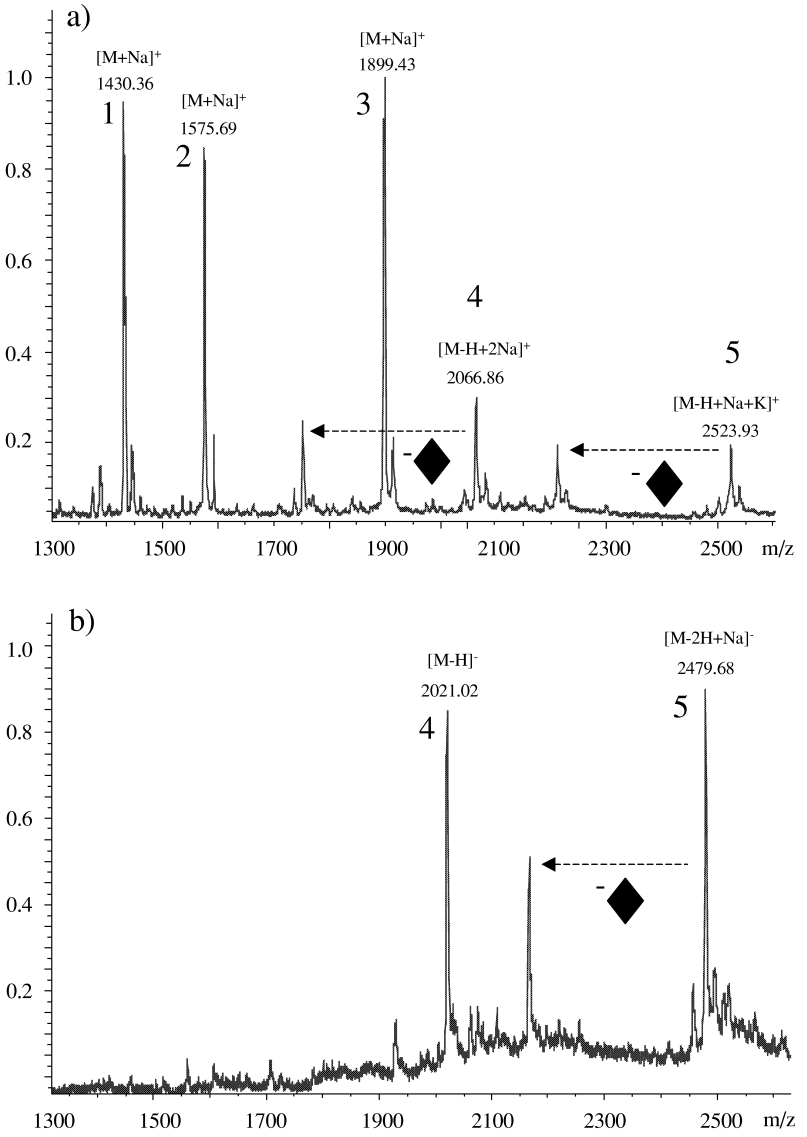


Fig. 7. MALDI–MS spectrum of an equimolar mixture of derivatized asialyl and sialylated *N*-linked oligosaccharides, 5 pmol of each, (a) positive mode, (b) negative mode. Symbols as in Table 1.

produced $[M+Na]^+$ and $[M+K]^+$ ions, characteristic of the technique. Positive mode ionization efficiency did not seem to be affected by addition of either fucose and galactose residues onto NGA2 (see Peaks **1**, **2** and **3** in Fig. 7a, corresponding to Compounds **1**, **2** and **3** in Table 1). Addition of sialic acid residues cut the efficiency down, which discriminated against sialylated oligosaccharides in the positive mode (see higher m/z low-abundance ions **4** and **5**, Fig. 7a). Asialyl *N*-glycans **1** to **3** were observed only in the positive mode (Fig. 7a). In the negative mode, sialylated compounds **4** and **5** clearly dominated (Fig. 7b) due to their acidic nature. These results suggest the difficulty of using a desorption method such a MALDI in quantitative analysis; however, it is practical and versatile for qualitative analysis. Other investigators have exploited this type of observation [28] in the case of immunoglobulin glycans.

In order to try and minimize sialic acid (SA) losses during the MALDI process, arabinosazone was used as a matrix [24] and spectra (not shown) were compared to those obtained using DHB. There were still ions that corresponded to detachment of SA, for both the derivatized and native acidic *N*-glycans, in proportions similar to those of Fig. 7. Most probably, the lost of SA occurs promptly in the source [30], because the ion abundances resulting from this cleavage were even greater for native acidic oligosaccharides than for their PNH derivatives.

A post-source decay (PSD) spectrum of $[M+Na]^+$ and $[M+K]^+$ ions of PNH asialylated *N*-glycan standards showed dominant B-fragments. Fig. 8a presents a PSD spectrum of agalactosyl asialyl *N*-glycan (NGA2 in Table 1) with a molecular mass (M_r) after derivatization of 1406.6 Da. PSD of the precursor ions, $[M+Na]^+$ and $[M+K]^+$, produced B_4(Na) ions at m/z 1118, C_4(Na) ions at m/z 1134, C_3(Na) ions at m/z 933, and Y_3(Na) ions at m/z 550. Very similar types of fragmentations were observed in both other PSD spectra of asialylated fucosylated (NGA2F in Table 1, Fig. 8b) and digalactosyl fucosyl *N*-glycans (NA2F in Table 1, Fig. 8c).

In order to compare ionization efficiencies for PNH derivatives of *N*-glycans in MALDI, PNH-NA2F was mixed with its non-derivatized analog in

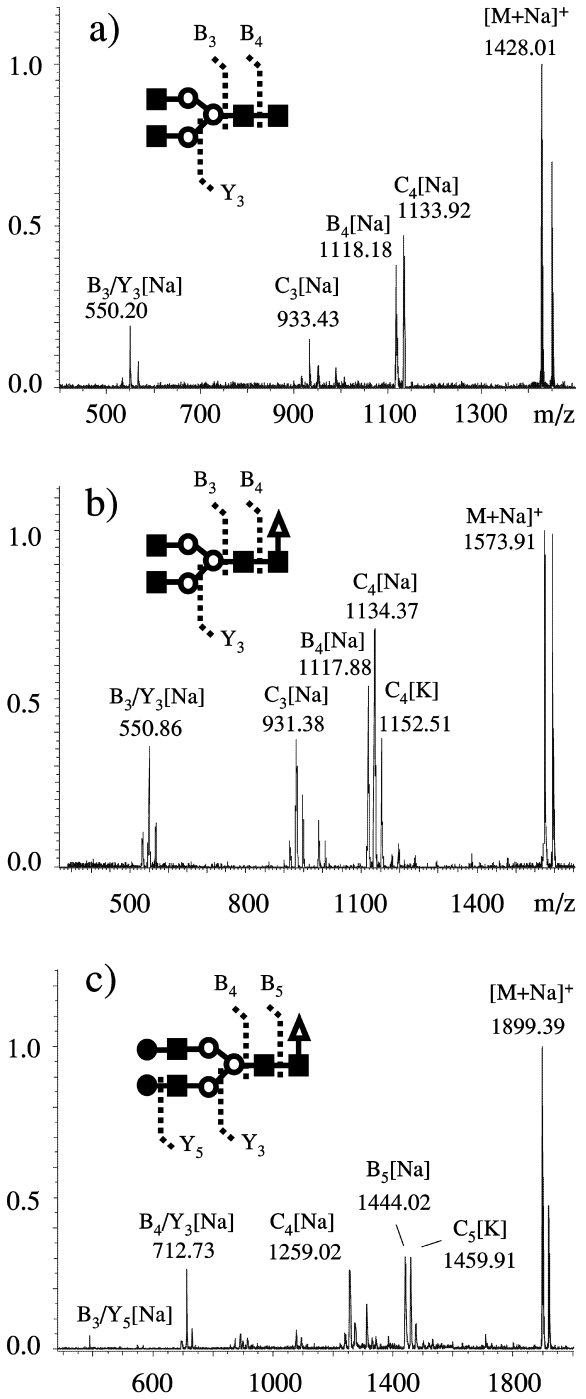


Fig. 8. MALDI-PSD spectra of (a) PNH-biantennary asialyl agalactosyl *N*-glycan (NGA2), (b) PNH-biantennary fucosyl asialyl agalactosyl *N*-glycan (NGA2F), (c) PNH-biantennary fucosyl asialyl digalactosyl *N*-glycan (NA2F).

equimolar proportions. With 10 pmol of each compound, the ion abundances were comparable. With the amount decreased to 5 pmol of each saccharide, ion abundances favoured the PNH-glycan (Fig. 9a). A more significant difference can be seen in Fig. 9b. This spectrum was acquired from an equimolar mixture of PNH-NGA2F and its derivatized analog, 1 pmol of each. All MALDI results discussed so far were obtained after labelling was carried out in solution and not on-target.

On-target derivatized samples did not benefit extraction of excess phenylhydrazine and no large crystals were observed in the camera image, only dark spots. The ion signals were however as intense as when crystals were observed. Overall, on-target labelling providing MALDI spectra of equal quality and showing the same features as when derivatization was carried out in solution.

3.4. Profiling of derivatized N-glycans from ovalbumin

Glycans released from hen ovalbumin by PNGase were examined by MALDI–MS with DHB as the matrix. The resulting profile from Grade VII hen

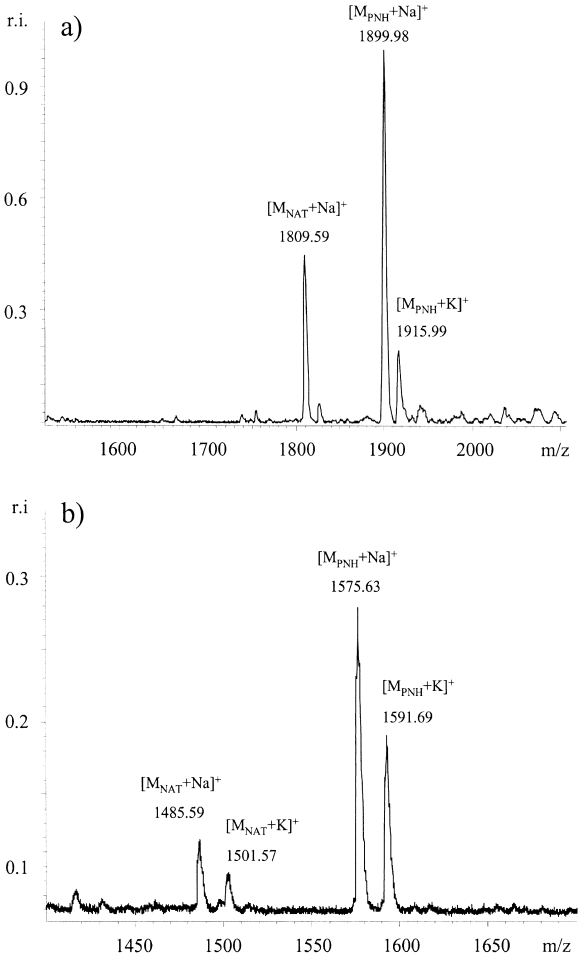


Fig. 9. MALDI–MS spectra of an equimolar mixture of (a) *N*-glycan (NAF2) and its PNH-derivative, 5 pmol each, (b) PNH-NAG2 and its native analog, 1 pmol of each.

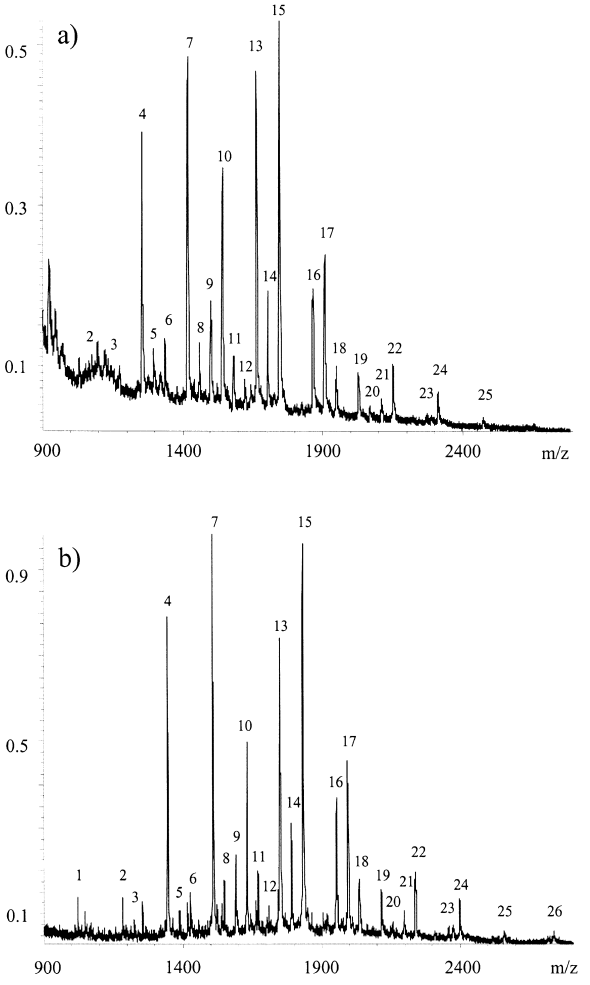


Fig. 10. Positive MALDI–MS spectra of *N*-linked glycans released with PNGase-F from hen ovalbumin: (a) native form, (b) labelled with phenylhydrazine. See Table 3 for peak assignments.

ovalbumin is shown in Fig. 10a. All ions are sodium cationized. The peaks (Table 3) correspond to compositions which are in good agreement with data already published by Harvey and colleagues [21,29], for the Sigma Grade V and VI ovalbumin. Using oligosaccharides from Grade VII ovalbumin, PNH-labelling was applied. After derivatization of the mixture, a similar MALDI spectrum of *N*-glycans was obtained with m/z values increased by 90.1 u for all glycans (Fig. 10b). Individual peaks were not identified as possibly originating from different isomers and only suggested compositions were given. The aim of this experiment was not to determine sensitivity improvement, but rather to establish the possibility to obtain a coherent pattern of *N*-glycans when labelled with PNH. Thus, the *y*-axis numbers in Fig. 10 are not meaningful in terms of quantitation. Each spectrum was obtained on a different day, using a different number of pulses and different laser powers. For a rigorous quantitative comparison by MALDI, samples of native and derivatized glycans would have to be prepared identically and run side by side and as a mixture under the same MALDI conditions. This will be the purpose of future work.

4. Conclusions

In this study, we have shown that phenylhydrazine-tagging can be used successfully for UV and MS detection of saccharides. ESI- and MALDI–MS sensitivities increase upon PNH-labelling, and no salts are used or produced during reaction. It was possible to separate the PNH derivatives of small sugars by reversed-phase HPLC with UV detection, and to apply the same conditions to on-line HPLC/ESI-MS experiments. The rapid and

Table 3
Peak assignments for MALDI–MS measurements of native and PNH-glycans detached from hen ovalbumin

No.	Observed m/z, PNH -glycans, $[M+Na]^+$	Observed m/z, native glycans, $[M+Na]^+$	Calc. M_r and composition (H = hexose, N = GlcNAc)[a]
1	1023.55	933.29	933.3 (H_3N_2)
2	1185.03	1094.98	1095.4 ($H_4N_{2)}$
3	1226.45	1136.36	1136.4 (H_3N_3)
4	1347.49	1257.33	1257.4 (H_5N_2)
5	1388.23	1298.77	1298.5 (H_4N_3)
6	1429.17	1339.42	1339.5 (H_3N_4)
7	1508.99	1419.63	1419.5 (H_6N_2)
8	1549.49	1460.38	1460.5 (H_5N_3)
9	1590.89	1501.26	1501.5 (H_4N_4)
10	1631.97	1542.21	1542.6 (H_3N_5)
11	1670.69	1581.18	1581.5 (H_7N_2)
12	1712.05	1622.26	1622.6 (H_6N_3)
13	1752.83	1663.04	1663.6 (H_5N_4)
14	1793.06	1704.16	1704.6 (H_4N_5)
15	1834.69	1745.18	1745.6 (H_3N_6)
16	1955.61	1866.01	1866.7 (H_5N_5)
17	1995.68	1907.07	1907.7 (H_4N_6)
18	2037.33	1948.12	1948.7 (H_3N_7)
19	2117.58	2027.96	2028.7 (H_6N_5)
20	2158.83	2069.24	2069.7 (H_5N_6)
21	2199.79	2110.35	2110.8 (H_4N_7)
22	2240.96	2150.88	2151.8 (H_3N_8)
23	2361.79	2272.03	2272.8 (H_5N_7)
24	2402.96	2312.41	2313.9 (H_4N_8)
25	2564.04	2474.89	2475.9 (H_5N_8)
26	2729.14	–	2638.0 (H_6N_8)

[a] From Ref. [21].

easy sample preparation makes this method suitable especially for MALDI analyses of oligosaccharides obtained from biological material.

Acknowledgements

The authors would like to thank the National Science and Engineering Science Council (NSERC), the Canadian Research Chair Program and the Canadian Foundation for Innovation for funding this research. Dr I.C.P. Smith from the Institute of Biodiagnostics of the National Research Council of Canada (NRC) is thanked for financial support. The authors are grateful to Dr J.C. Jamieson for providing some of the *N*-linked oligosaccharide standards.

References

[1] A. Varki, R. Cunemings, J. Esko, H. Freeze, G. Hart, J. Marth (Eds.), Essentials of Glycobiology, Book News Inc, Portland, OR, 1999, 583 pp.
[2] L. Poulter, A.L. Burlingame, Methods Enzymol. 193 (1990) 661.
[3] S. Hase, T. Ibuki, T. Ikenaka, J. Biochem. 95 (1984) 197.
[4] M. Okamoto, K.I. Takahashi, T. Doi, Rapid Commun. Mass Spectrom. 9 (1995) 641.
[5] J.C. Bigge, T.P. Patel, J.A. Bruce, P.N. Goulding, S.M. Charles, R.B. Parekh, Anal. Biochem. 230 (1995) 229.
[6] T.J.P. Naven, D.J. Harvey, Rapid Commun. Mass Spectrom. 10 (1996) 829.
[7] S. Hase, J. Chromatogr. A 720 (1996) 173.
[8] D.J. Harvey, J. Am. Soc. Mass Spectrom. 11 (2000) 900.
[9] S. Honda, E. Akao, S. Suzuki, M. Okuda, K. Kakehi, J. Nakamura, Anal. Biochem. 180 (1989) 351.
[10] D.J. Strydom, J. Chromatogr. A 678 (1994) 17.
[11] X. Shen, H. Perreault, J. Chromatogr. A 811 (1998) 47.
[12] W. Pigman, D. Horton (Eds.), The Carbohydrates, Vol. IB, Academic Press, New York, 1980, p. 929.
[13] N.K. Karamanos, T. Tsegenidis, C.A. Antonopoulos, J. Chromatogr. 405 (1987) 221.
[14] S. Honda, K. Kakehi, Carbohydr. Res. 47 (1976) 213.
[15] W.F. Alpenfels, Anal. Biochem. 114 (1981) 153.
[16] R.P. Rago, D. Ramirez-Soto, R.D. Poretz, Carbohydr. Res. 236 (1992) 1.
[17] K.L. Duffin, J.K. Welply, E. Huang, J.D. Henion, Anal. Chem. 64 (1992) 1440.
[18] T.J.P. Naven, D.J. Harvey, Rapid Commun. Mass Spectrom. 10 (1996) 1361.
[19] C. Sottani, M. Fiorentino, C. Minoia, Rapid Commun. Mass Spectrom. 11 (1997) 907.
[20] J.A. Saba, X. Shen, J.C. Jamieson, H. Perreault, J. Mass Spectrom. 36 (2001) 563.
[21] D.J. Harvey, D.R. Wing, B. Kuster, I.B.H. Wilson, J. Am. Soc. Mass Spectrom. 11 (2000) 564.
[22] D.J. Harvey, J. Am. Soc. Mass Spectrom. 11 (2000) 572.
[23] E. Lattova, L. Petrus, Carbohydr. Res. 235 (1992) 289.
[24] P. Chen, A.G. Baker, M. Novotny, Anal. Biochem. 244 (1997) 144.
[25] T. Muramutsu, in: N. Takahashi, T. Muramutsu (Eds.), CRC Handbook of Endoglycosidases and Glycoamidases, CRC Press, Boca Raton, FL, 1992.
[26] P. Jackson, Methods Enzymol. 227 (1995) 377.
[27] B. Domon, C.E. Costello, Glycoconj. J. 5 (1988) 397.
[28] T.S. Raju, J.B. Briggs, S.M. Borge, A.J.S. Jones, Glycobiology 10 (2000) 477.
[29] D.J. Harvey, Methods Mol. Biol. 61 (1996) 243.
[30] D.J. Harvey, Proteomics 1 (2001) 311.
[31] X. Shen, H. Perreault, J. Mass Spectrom. 34 (1999) 502.
[32] K.I. Yoshino, T. Takao, H. Muruta, Y. Shimonishi, Anal. Chem. 67 (1996) 4028.
[33] Y. Takeda, Carbohydr. Res. 77 (1979) 9.
[34] J.J. Angyal, Adv. Carbohydr. Chem. Biochem. 47 (1989) 1.