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Profiling of *N*-linked oligosaccharides using phenylhydrazine derivatization and mass spectrometry

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Abstract

N-linked oligosaccharide standards obtained from commercial sources were derivatized with phenylhydrazine (PHN) and analyzed by on-line reversed-phase high performance liquid chromatography (HPLC)/electrospray ionization mass spectrometry (ESI-MS). This procedure was then applied to mixtures of *N*-glycans enzymatically released from hen ovalbumin. Under ESI-MS conditions, phenylhydrazones of asialylated oligosaccharide standards and ovalbumin glycans produced mainly $[M + 2H]^{2+}$ molecular ions at low cone voltage values, while minimal fragmentation was observed. Reversed-phase HPLC/ESI-MS total and selected ion chromatograms obtained for derivatized *N*-glycans from ovalbumin showed partial but useful separation. Overall glycan profiles obtained by ESI-MS were compared with results obtained by matrix-assisted laser desorption/ionization (MALDI)-MS. Qualitatively, profiles were similar from one technique to the other in terms of relative abundance of glycans versus composition. Post-source decay (PSD) analysis of the $[M+Na]^+$ ions of PHN–glycans showed dominant B, C and internal B/Y, C/Y cleavages. These patterns were helpful in relating fragmentation to proposed structures. Cross-ring cleavage fragment ions (A-type) were also observed in most cases. The PHN derivatization method is fast and simple. It produces abundant parent ions in both MALDI-MS and ESI-MS, while avoiding the presence of salt contaminants during the labeling procedure.

Keywords: Derivatization; LC; Mass spectrometry; Electrospray ionization; Matrix-assisted laser desorption ionization; Oligosaccharides; Phenylhydrazone; Glycans

1. Introduction

Altered protein glycosylation constitutes a characteristic modification within cancer cells, although only certain specific changes in glycans are frequently associated with tumors [1]. This is only one of many examples showing the importance of oligosaccharide analysis as an emerging field to which many research groups have already contributed (e.g. [2]). In recent years, the technologies amenable to the analysis of small amounts of glycoprotein oligosaccharides have improved considerably in the areas of sample preparation, derivatization, chromatographic and electrophoretic separations, and mass spectrometry (MS) [3]. Mass spectrometric methods are now among the most sensitive for the characterization these compounds, although one of their main drawbacks is the inability to differentiate isomers [4].

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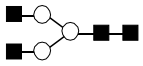
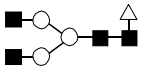
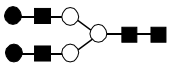
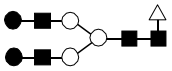
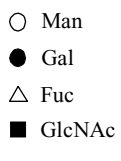
Consequently, the combination of MS with high performance liquid chromatography (HPLC) using an electrospray (ESI) interface has become invaluable for obtaining total glycan profiles from glycoproteins. In order to facilitate detection of the saccharides, it is common practice to label these compounds at their reducing termini with chromophores to enhance ultraviolet (UV) absorption, fluorescence or mass spectrometric detection (e.g. [5–13]). Recent reports have been shown that reducing end tagging can influence fragmentation patterns of oligosaccharides and can greatly simplify structural analysis of unknowns (e.g. [14–19]).

In a recent article we have shown that phenylhydrazine (PHN) labeling is a very simple derivatization procedure, which can be used successfully for MS detection and characterization of oligosaccharides [19]. Mass spectra of PHN–lactose and PHN analogs of lactose (*N*-acetyllactosamine, sialylactose, fucosyllactose) and PHN–*N*-glycan standards (NA2, NA2F, NGA2F, see Table 1) showed enhanced sensitivity under electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization (MALDI)-MS conditions. Also, fragment ions obtained by post-source decay (PSD) experiments provided useful structural information [19].

The present paper describes the development and application of on-line HPLC/electrospray ionization mass spectrometry and matrix-assisted laser desorption/ionization methods involving our PHN derivatization technique. Firstly, *N*-glycan standards from a commercial source are investigated, and secondly, the characterization of oligosaccharides enzymatically released from ovalbumin is attempted and compared to results from previous reports. We chose hen ovalbumin as a model because it is one of the best characterized glycoproteins, having been the object of extensive studies over the past two decades (e.g. [20–25]). Recently, Harvey et al. [26] have shown that commercial samples of hen ovalbumin may contain other glycoproteins with different glycosylation profiles, and that several of the glycans previously attributed to ovalbumin, in fact, may originate from these other glycoproteins. These authors observed 37 glycans (including all isomers and glycoprotein contaminants) using a combination of hydrazinolytic cleavage and MALDI-MS. Characterization of the 1-phenyl-3-methyl-5-pyrazolone (PMP)-derivatized ovalbumin glycans by on-line HPLC/ESI-MS had previously confirmed some glycan compositions found in Ref. [27]. Reversed-phase HPLC conditions for PMP derivatives were more useful for desalting glycan pools than for properly separating sample components. Moreover, PMP derivatives of large *N*-glycans were relatively labile during HPLC experiments, with fragmentation occurring either on-column or in-source. PMP derivatives could be separated more efficiently by normal-phase HPLC, with reduced sensitivity due to eluent conditions [27]. Results shown in the present paper demonstrate that PHN derivatives are stable under reversed-phase HPLC conditions and ionize in a predictable manner, depending on acetic acid concentration in the eluent and declustering (cone) voltage used. As for MALDI-MS, PHN-labeled glycans are very simple to prepare directly on-target, and ionize very consistently as $[M + Na]^+$ ions. Post-source decay experiments on the parent ions of PHN–glycans from hen ovalbumin also produce very consistent and predictable fragmentation patterns. All results combined emphasize the simplicity and usefulness of PHN derivatization for the analysis of complex *N*-glycan pools.

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

		MW (Da)
1. NGA2		1316.5
2. NGA2F		1462.5
3. NA2		1640.6
4. NA2F		1786.6



2. Experimental

2.1. Materials

Phenylhydrazine, hen ovalbumin (Grades V and VII) and 2,5-dihydroxybenzoic acid (DHB) matrix

were purchased from Sigma (St. Louis, MO). *N*-linked oligosaccharide standards—NGA2, NGA2F, NA2, NA2F (see Table 1)—were obtained from Oxford GlycoSciences (Oxford, UK). Peptide-*N*-glycosidaseF (PNGaseF) deglycosylation kits were purchased from Glyko (Novato, CA, USA). Solvents (acetonitrile, methanol, ethanol) were HPLC-grade and purchased from Fisher Scientific (Fair Lawn, NJ). HPLC-grade deionized, distilled water was obtained from a Milli-Q® plus TOC water purification system (Millipore, Bedford, MA).

2.2. Derivatization of oligosaccharides [19]

A mixture of oligosaccharide standards NGA2, NGA2F, NA2, NA2F (approximately 200 pmol of each) and a 10% methanolic solution of phenylhydrazine (5 μl) were combined and heated at 70–80 °C for 1 h. Incubation time involved some frequent mixing. Glycans released from 200 μg of ovalbumin (a 40 μl solution) were derivatized according to this procedure. In both cases, no extraction of unreacted phenylhydrazine was performed. For on-target derivatization, a solution of phenylhydrazine (0.5 μl), prepared by dissolving of 1 μl of phenylhydrazine in 10 μl of 8:2 water-methanol, was spotted directly onto the mixture of oligosaccharides and matrix on the target. The mixture was allowed to dry at room temperature for approximately 45 min.

2.3. Deglycosylation by PNGaseF digestion

Incubation of hen ovalbumin was carried out at 37 °C for 20 h using PNGaseF [28,29]. Hen ovalbumin (200 μg) was dissolved in deionized water (36 μl) in a microcentrifuge tube, and Buffer 5× supplied with the Glyko™ deglycosylation kit was added (9 μl). Boiling in a hot water bath for 5–10 min then denatured the glycoprotein. After cooling, the OLIGO profiling enzyme (4 μl) was added. Following incubation, adding three volumes of cold ethanol and keeping the mixture in ice precipitated the protein. The *N*-deglycosylated 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 as described above.

2.4. On-line HPLC/ESI-MS

HPLC analyses were performed on a Hewlett-Packard 1100 quarternary delivery system (Agilent Technologies, Mississauga, ON). The analytical column was a Vydac 218-TP54 Protein & Peptide C18 (Separation Group, Hesperia, CA, USA) for reversed-phase separations. The chromatograph was equipped with a Rheodyne injector (20 μl loop). The samples were separated at flow rates varying from 0.3 to 1.0 ml/min. The gradient used for *N*-glycan standards (200 pmol) was programmed as follows: 5% acetonitrile in water maintained for 5 min, up to 15% acetonitrile over 10 min, and to 40% acetonitrile over 40 min. Acetic acid concentration was kept constant at 0.1 M throughout the separation. For ovalbumin glycans, two series of separations were performed. In the first series, Eluent A was 0.05 M acetic acid in water, and Eluent B was 0.05 M acetic acid in acetonitrile. In the second series, A was water and B was 0.1 M acetic acid in 80% acetonitrile in water. In both cases, the proportion of B was increased from 0 to 10% over 30 min and then to 15% over the next 30 min. The column effluent was split and introduced directly in the Z-Spray™ ESI source of a Quattro-LC triple quadrupole mass spectrometer (Micromass, UK). The ESI needle voltage was 3.6 kV, and the instrument was operated in the positive mode with a declustering (cone) voltage of 20 V. The source block and desolvation temperatures were set at 110 and 250 °C, respectively. The scan rate was 300 u/s. For each HPLC/MS experiment, a 5 μl aliquot was injected, representing oligosaccharides from ca. 200 μg of hen ovalbumin. Injections of *N*-linked oligosaccharide derivatized standards contained ca. 200 pmol of each standard.

2.5. Off-line reversed-phase HPLC with UV detection

Because of the low concentrations of some *N*-glycans the pool of PHN–glycans was purified by HPLC prior to post-source decay experiments. For this purpose, a System Gold HPLC chromatograph equipped with a System Gold 166 UV Detector and with 32-Karat software (Beckman-Coulter, City, ST) was used. Elution was performed at a flow rate 1 ml/min with solvents A (5:95 water-acetonitrile)

and B (water). The proportion of A was linearly increased from 5 to 50% over 15 min, and then kept at this concentration until the end of the run. UV detection was performed at 245 nm. Fraction were collected manually, and the solvent was evaporated.

2.6. MALDI–TOF mass spectrometry

MALDI spectra were recorded on a Biflex-IV spectrometer (Bruker Daltonics, Billerica, MA). The accelerating voltage was 20 kV. Profiling of the molecular ions of derivatized glycans was achieved in the positive mode, using linear and reflective TOF. Glycan solutions (0.5 μl containing approximately 10–20 pmol of glycan) were loaded onto a stainless steel target with the same volume of saturated matrix solution (2,5-dihydroxy benzoic acid in acetonitrile:water 7:3) and allowed to dry in ambient air. PSD spectra were recorded in 10–14 segments, each corresponding to 50–100 laser shots.

3. Results and discussion

3.1. Formation of phenylhydrazones

The reaction conditions used for carbohydrate labeling in this work led to formation of phenylhydrazones, without osazone by-products. Osazones are usually prepared by reacting sugars with large excess amounts of phenylhydrazine (or its hydrochloride) in aqueous acetic acid (or sodium acetate) upon heating (e.g. [30]). No osazones were detected in the mass spectra of derivatives, e.g. spectra presented in this article and in our first publication on this subject [19].

3.2. Reversed-phase HPLC/ESI-MS of PHN N-glycan standards

In a recent article we discussed PHN derivatization of *N*-linked glycan standards (NGA2, NGA2F, NA2F, see Table 1) and of hen ovalbumin glycans, showing the usefulness of this method for MALDI-MS

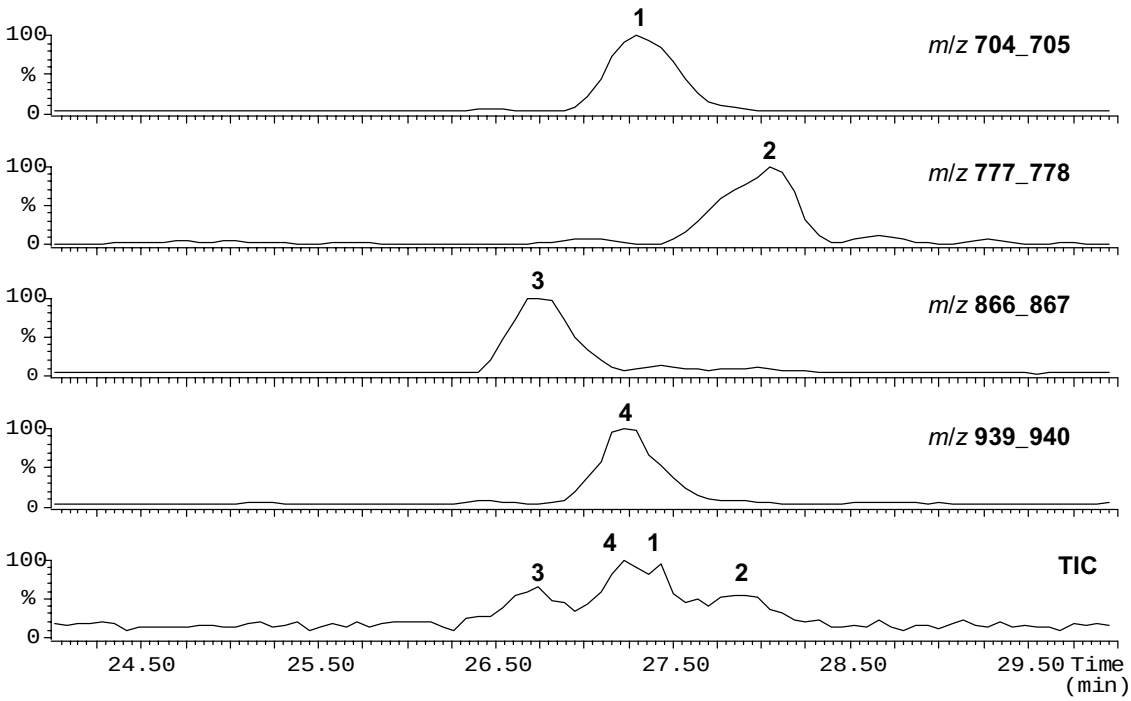


Fig. 1. Reversed-phase HPLC/MS selected and total ion chromatograms obtained for standard *N*-glycans labeled with phenylhydrazine: NGA2 (1), NGA2F (2), NA2 (3), NA2F (4). Selected ions are $[M + 2H]^{2+}$ species. See Table 1 for structures.

experiments [19]. However ESI-MS and on-line HPLC/MS, as well as MALDI–PSD of these PHN–*N*-linked glycans were not explored, and thus results from these experiments will be presented here. The mixture of standards investigated used for method development this paper is the same as mentioned above, with the addition of PHN–afucosyl digalactosyl glycan (NA2).

Reversed-phase HPLC/MS conditions allowed separation of PHN-NA2 and PHN-NGA2F, as shown in Fig. 1. Glycans NGA2 and NA2F eluted together, although the apex of the NA2F peak occurred slightly earlier than that of the NGA2 peak. Interestingly, the glycans did not elute in particular molecular weight order, but non-fucosylated forms eluted prior to their fucosylated analogs, and galactosylated analogs eluted prior to their non-galactosylated analogs (global elution order NA2, NA2F, NGA2, NGA2F). We had also observed the limited ability of reversed-phase HPLC to separate of *N*-linked glycans derivatized with PMP, and had found normal phase conditions to yield more efficiency [27]. PHN derivatives are relatively polar compared with PMP and thus reversed-phase elution overlapping was expected. Traces in Fig. 1 are reconstructed ion chromatograms for the $[M + 2H]^{2+}$ species. Pyridylaminated *N*-linked oligosaccharides and PMP–oligosaccharides (e.g. [18,31]) were also reported to produce abundant $[M + 2H]^{2+}$ molecular ions, although $[M + Na + H]^{2+}$ and $[M + 2Na]^{2+}$ are frequently observed with sialylated sugars and other types of derivatives (e.g. [9,18,31]). With the asialylated standards, PHN derivatives gave rise mostly to $[M + 2H]^{2+}$ ions as shown in the spectra of Fig. 2. While the preparation of aminopyridyl, PMP, aminobenzoyl and 2-aminoacridine derivatives of oligosaccharides require desalting prior to MS, no salts are used or produced upon reaction with phenylhydrazine, thus minimizing the formation of salt adducts in the ion source. HPLC here was used not for desalting, but rather to separate unreacted phenylhydrazine from the derivatized glycans. Using a low cone voltage of 20 V, fragmentation of these derivatives was minimized and good molecular weight profiling conditions were in place. Spectra of agalactosyl and galactosylated standards (Fig. 2) featured low abundance Y fragment ions (nomenclature suggested

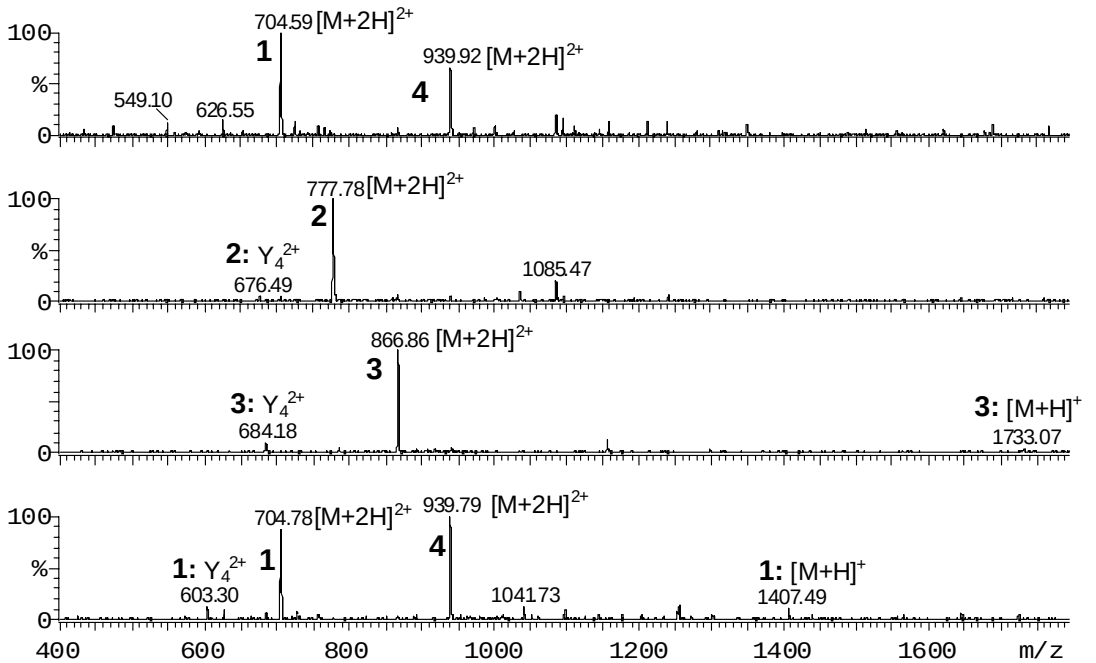


Fig. 2. ESI mass spectra of phenylhydrazones of *N*-glycan standards, obtained by reversed-phase HPLC/MS from Fig. 1. NGA2 (1), NGA2F (2), NA2 (3), NA2F (4). See Table 1 for structures.

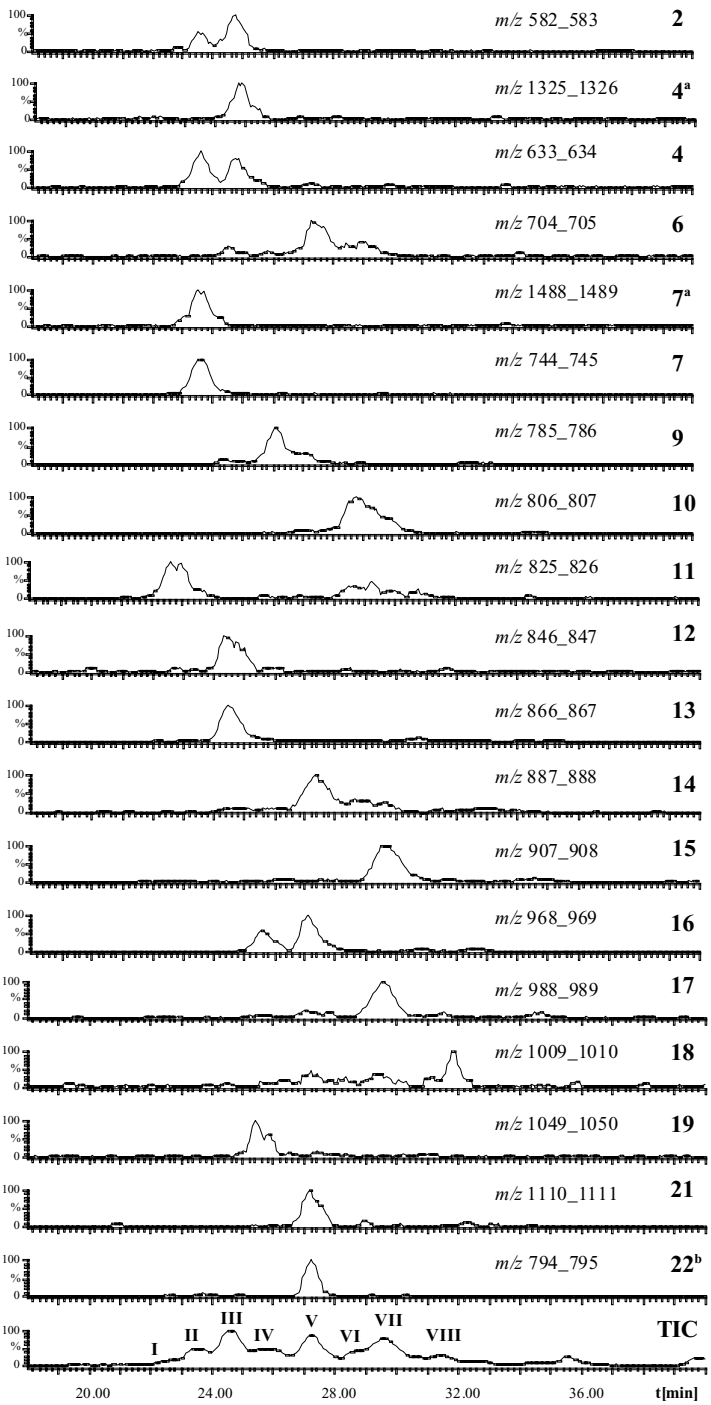


Fig. 3. Reversed-phase HPLC/MS selected and total ion chromatograms obtained for *N*-glycans detached from Sigma Grade VII hen ovalbumin and labeled with phenylhydrazine. See Table 2 for proposed assignments. In most cases $[M + 2H]^{2+}$ ions were monitored, and in some cases [a] $[M + H]^{+}$ and [b] $[M + 3H]^{3+}$ were measured. Constant 0.1 M acetic acid concentration throughout the separation.

by Domon and Costello [32]), among other fragments. Limited amounts of these compounds prevented us to study their behavior under collision-induced dissociation (CID) tandem MS (MS/MS) conditions. Emphasizing that reducing-end labeling with PMP, 2-AB, and 2-AMAC among other derivatives produces fragments mostly containing the labeled end of the molecule, it would be interesting to compare ESI–CID–MS/MS fragmentation patterns in a systematic fashion among derivatives, including phenylhydrazine. Our previous spectra of PHN-labeled smaller saccharides [19] and studies on other derivatized oligosaccharides (e.g. [14,17,18]) certainly call for the importance of establishing fragmentation trends among different oligosaccharides and different derivatives.

3.3. Reversed-phase HPLC/ESI-MS of PHN–glycans from ovalbumin

The *N*-glycans enzymatically released from hen ovalbumin by PNGase were also derivatized with phenylhydrazine and analyzed as their corresponding phenylhydrazones. Using reversed-phase conditions, it was again not possible to separate species individually, however different levels of separation were achieved upon slight modifications of the mobile phase and flow rate. Fig. 3 shows the total and some reconstructed chromatograms for $[M + H]^{2+}$ ions (Grade VII ovalbumin), obtained at a flow rate of 0.5 ml/min with constant acetic acid concentration (0.05 M, see Section 2). As unreacted phenylhydrazine eluted earlier than the glycans, it did not interfere with MS detection of the latter. In order to characterize global oligosaccharide pool compositions, data were averaged between the retention times of 21 and 33 min, producing the spectra shown in Fig. 4. HPLC/MS allowed the mass identification of individual saccharide compositions in the pool (see Table 2), and these compounds corresponded to native analogs reported earlier for Grades V and VI ovalbumin [26]. Spectra of individual PHN–oligosaccharides were not possible to obtain, as limited separation was achieved.

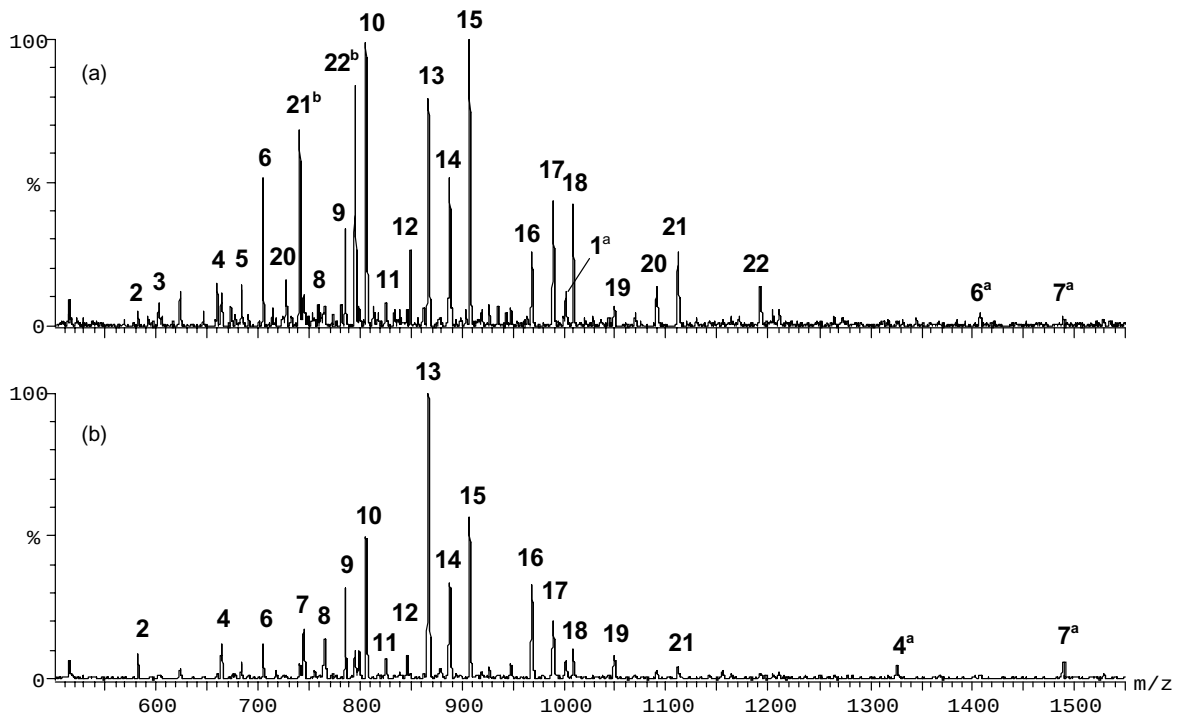


Fig. 4. Integrated ESI mass spectra representing phenylhydrazine-labeled *N*-glycan pools from hen ovalbumin: (a) Grade V and (b) Grade VII. See Table 2 for proposed assignments.

Table 2
Peak assignments for HPLC/MS and MALDI-MS of PHN–glycans released from hen ovalbumin

Number	*m/z* values of PHN−glycans measured by HPLC/ESI-MS[a]	*m/z* values of $[M + Na]^+$ ions of PHN−glycans MALDI-MS[b]	Calculated *m/z* values for $[M + Na]^+$ ions of reported native compositions [26][c,d]
1	1001.6[e]	1023.5	933.3 (H_3N_2)*
2	582.6	–	1095.4 (H_4N_2)*
3	602.9	1226.7	1136.4 (H_3N_3)*
4	663.7, 1325.9[e]	1347.7	1257.4 (H_5N_2)
5	683.9	1388.6	1298.5 (H_4N_3)*
6	704.5, 1407.9[e]	1429.7	1339.5 (H_3N_4)
7	744.6, 1488.0[e]	1509.8	1419.5 (H_6N_2)
8	764.1	1550.8	1460.5 (H_5N_3)
9	785.3, 1569.9[e]	1591.7	1501.5 (H_4N_4)
10	806.3	1632.8	1542.6 (H_3N_5)
11	825.3	1671.7	1581.5 (H_7N_2)
12	846.1	1712.6	1622.6 (H_6N_3)
13	866.7	1753.8	1663.6 (H_5N_4)
14	887.1	1794.8	1704.6 (H_4N_5)
15	907.8	1835.9	1745.6 (H_3N_6)
16	968.3	1956.9	1866.7 (H_5N_5)
17	988.7	1998.0	1907.7 (H_4N_6)
18	1009.3	2038.9	1948.7 (H_3N_7)*
19	1049.2	2118.7	2028.7 (H_6N_5)*
20	1090.3, 728.2[f]	–	2110.8 (H_4N_7)*
21	1110.7, 741.2[f]	2241.9	2151.8 (H_3N_8)*
22	1192.0, 794.9[f]	2404.2	2313.9 (H_4N_8)*

[a] Observed *m/z* values in HPLC/ESI-MS spectra of PHN–glycans from hen ovalbumin, Grades V and VII.
[b] Observed *m/z* values in HPLC/ESI-MS spectra of PHN–glycans from hen ovalbumin, Grade VII.
[c] H: hexose (mannose, galactose); N: *N*-acetylglucosamine.
[d] PHN labeling adds 90.1 Da to the mass of native oligosaccharides.
[e] $[M + H]^+$ ions.
[f] $[M + 3H]^{3+}$ ions.
* Peaks identified as possibly originating from glycoprotein impurities.

Fig. 5 shows ESI-MS spectra corresponding to different elution times, obtained by on-line HPLC/ESI-MS. Electrospray produced mainly $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ parent ions, with some $[M + H]^+$ ions.

Attempts were made to improve separation by varying the concentration of acetic acid in time (0–0.015 M) as part of the gradient (see Section 2). Very good separation of individual oligosaccharides was achieved at 1 ml/min. PHN–oligosaccharides were eluted over a 17–36 min time range. ESI-MS sensitivity was decreased and the chromatograms and spectra displayed more noise than in the former conditions. With a decreased flow rate to 0.5 ml/min again sensitivity improved, but only partial separation was achieved (Fig. 6). It seems that the varying concentration of acetic acid influenced ionization and a large number of PHN–oligosaccharides were detected as $[M + H]^+$ species (Fig. 7). The order of elution was different relative to that observed with constant acid concentration.

3.4. MALDI-MS of PHN–glycans from ovalbumin

The oligosaccharide content of hen ovalbumin observed by HPLC/ESI-MS spectrum is in good agreement with the MALDI data shown in Fig. 8. Table 2 compiles and compares results from both types of experiments. The spectrum in Fig. 8 was obtained after conducting *N*-deglycosylation directly on the target, followed by PHN derivatization, also on-target. Ethanol precipitation was not used to precipitate the deglycosylated protein, which was not detected due to

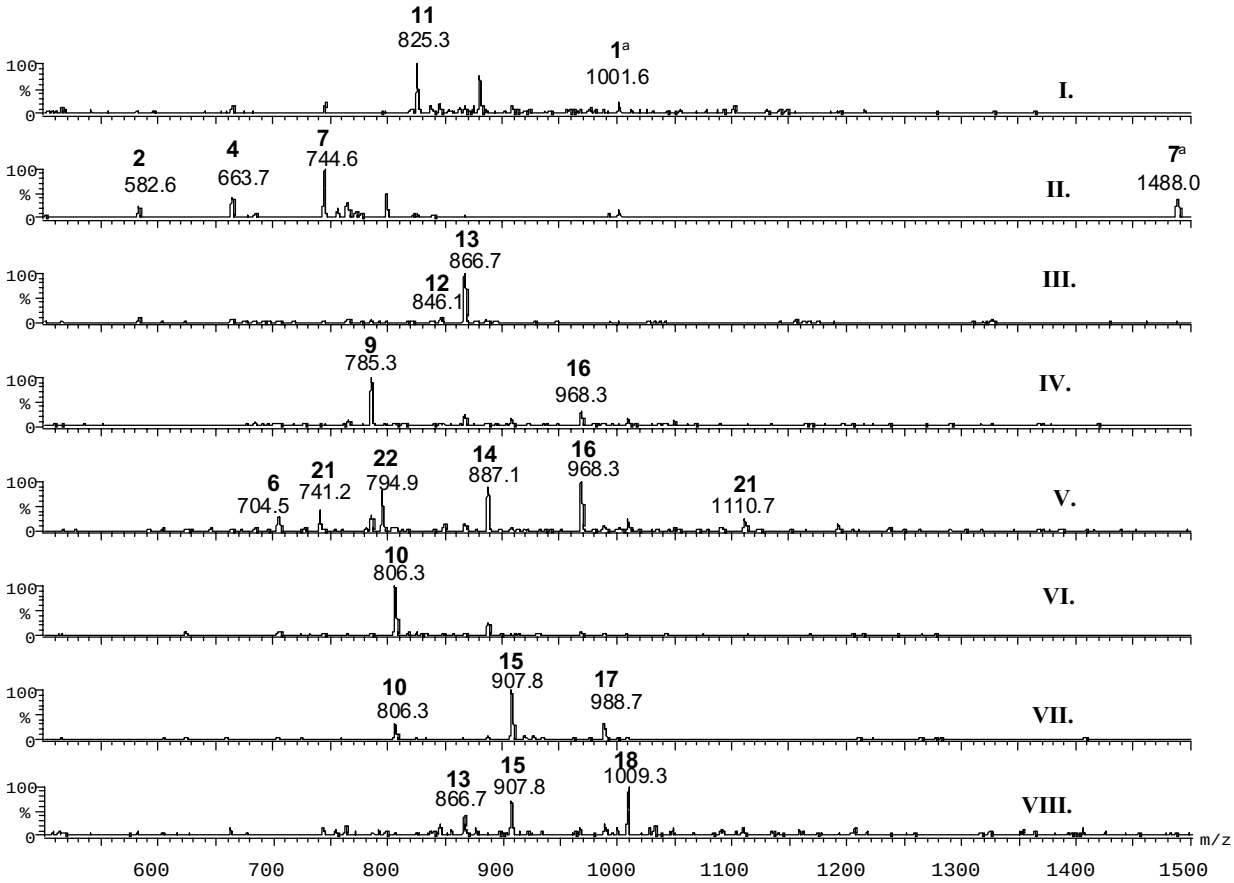


Fig. 5. ESI mass spectra corresponding to specific peaks in Fig. 3. Roman numbers correspond to total ion current peaks. Numbers in mass spectra refer to assignments in Table 2.

the restricted *m*/*z* range used. The presence of protein did not seem to affect ionization of PHN derivatives as mainly $[M + Na]^+$ ions. Comparing the MALDI-MS glycan profiles of Grades V and VII ovalbumin, there are visibly some glycans in lower abundance or missing in the Grade VII pool, especially those with masses over 2000 Da. It was earlier reported that most complex glycans in Grade V ovalbumin might originate from other glycoproteins present as contaminants, particularly chicken riboflavin binding protein [26]. Therefore, the lower abundances or absence of some oligosaccharides could be explained by lower amounts of glycoprotein contaminants in Grade VII ovalbumin. In Table 2, such species are labeled with an asterisk.

3.5. MALDI–PSD of PHN–glycans from ovalbumin

The PHN derivatives of enzymatically-released glycans were examined under MALDI post-source decay conditions. One high-mannose and several complex glycans were investigated using this method. In our previous paper [19], the PSD spectra of PHN–asialylated *N*-glycan standards showed predominant B fragments and internal B/Y fragments. The MALDI spectra discussed here are similar. Generally, B, C and internal B/Y and C/Y were the most abundant fragment ions for complex glycans. The internal fragments B/Y formed by loss of the chitobiose core and the 3-antenna, designated as D-ions in the studies of native *N*-linked oligosaccharides [33],

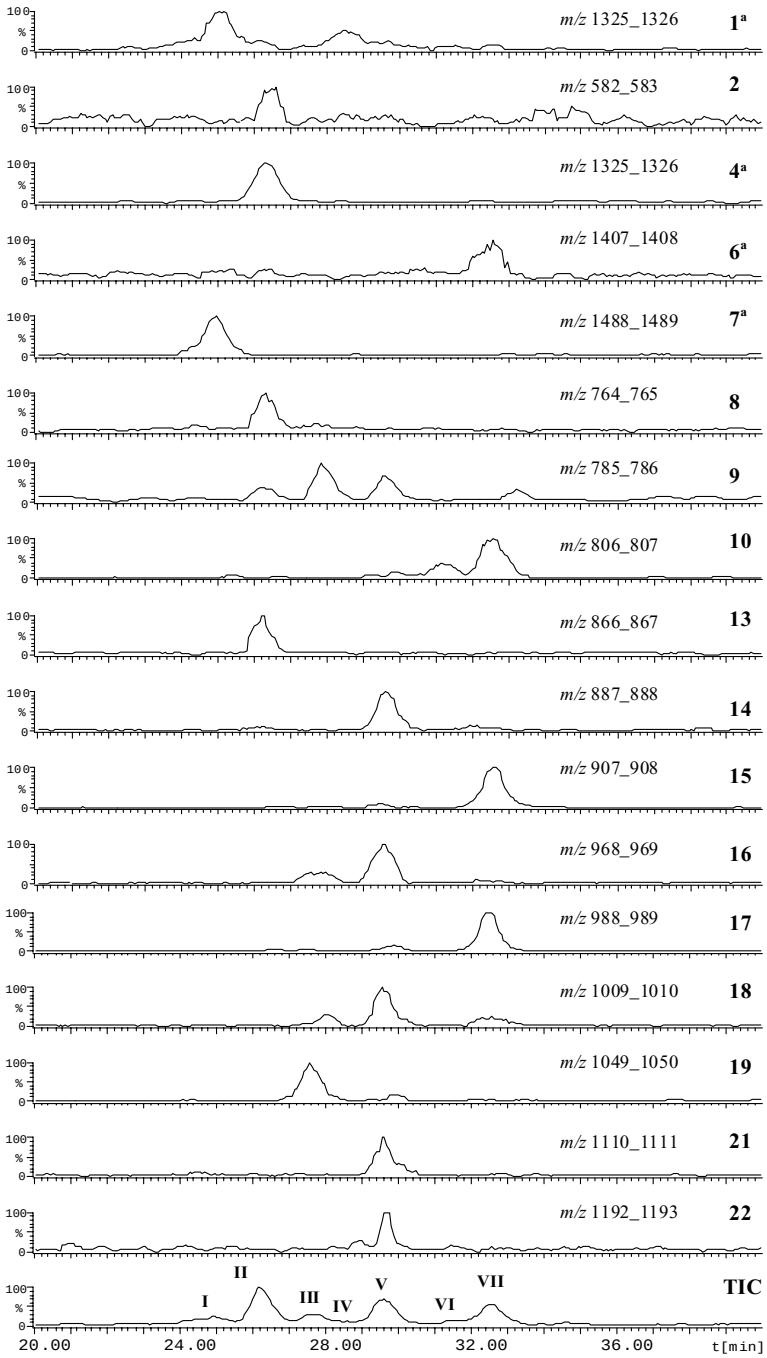


Fig. 6. Reversed-phase HPLC/MS selected and total ion chromatograms obtained for *N*-glycans detached from Sigma Grade VII hen ovalbumin and labeled with phenylhydrazine. See Table 2 for proposed assignments. In most cases $[M + 2H]^{2+}$ ions were monitored, and in some cases [a] $[M + H]^{+}$ ions were measured. Variable acetic acid concentration during separation, $\leq$0.15 M.

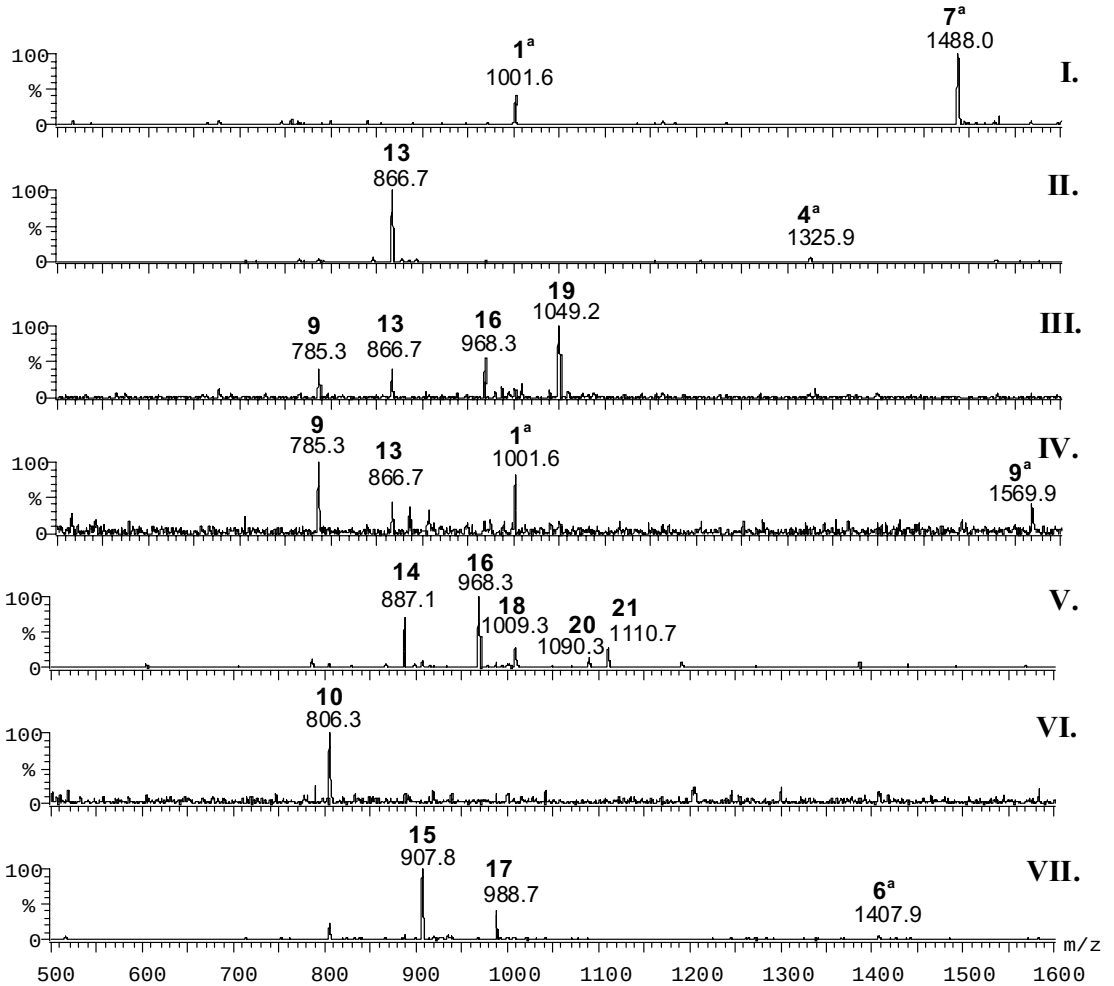


Fig. 7. ESI mass spectra corresponding to specific peaks in Fig. 6. Roman numbers correspond to total ion current peaks. Numbers in mass spectra refer to assignments in Table 2.

were observed in PSD spectra of PHN derivatives too. These ions were used to determine the 6-antenna structure and thus assess the presence of bisecting GlcNAc units [33]. Native *N*-linked oligosaccharides tend to produce more abundant A-type fragments [33], which were observed here but only in some cases. In general, PHN–glycans produced much simpler PSD fragmentation patterns than their corresponding native analogs. For parent ions corresponding to more than one isomeric possibility, spectral interpretation was compared with structures suggested in previous MS and ^{1}H NMR studies of ovalbumin glycans [34,35].

Fig. 9a presents a PSD spectrum and a proposed fragmentation scheme [35] for the complex glycan of composition $(GlcNAc)_4(Man)_3PHN$ (MW 1406.6 Da). For both proposed structures $[M + Na]^+$ precursor ions at *m/z* 1429 produced B_4 fragment ions at *m/z* 1119, C_4 ions at *m/z* 1136, and B_3 ions at *m/z* 918. A further loss of GlcNAc caused abundant B_3/Y_4 ions at *m/z* 714. Fragment ions at *m/z* 552 correspond to additional loss of a Man residue (B_3/Y_3). For Structure 1, this would indicate the presence of a residual $(GlcNAc)_1(Man)_2$ branch made up of the bisecting GlcNAc moiety and a Man residue linked in the 6-position of the chitobiose core Man unit. A

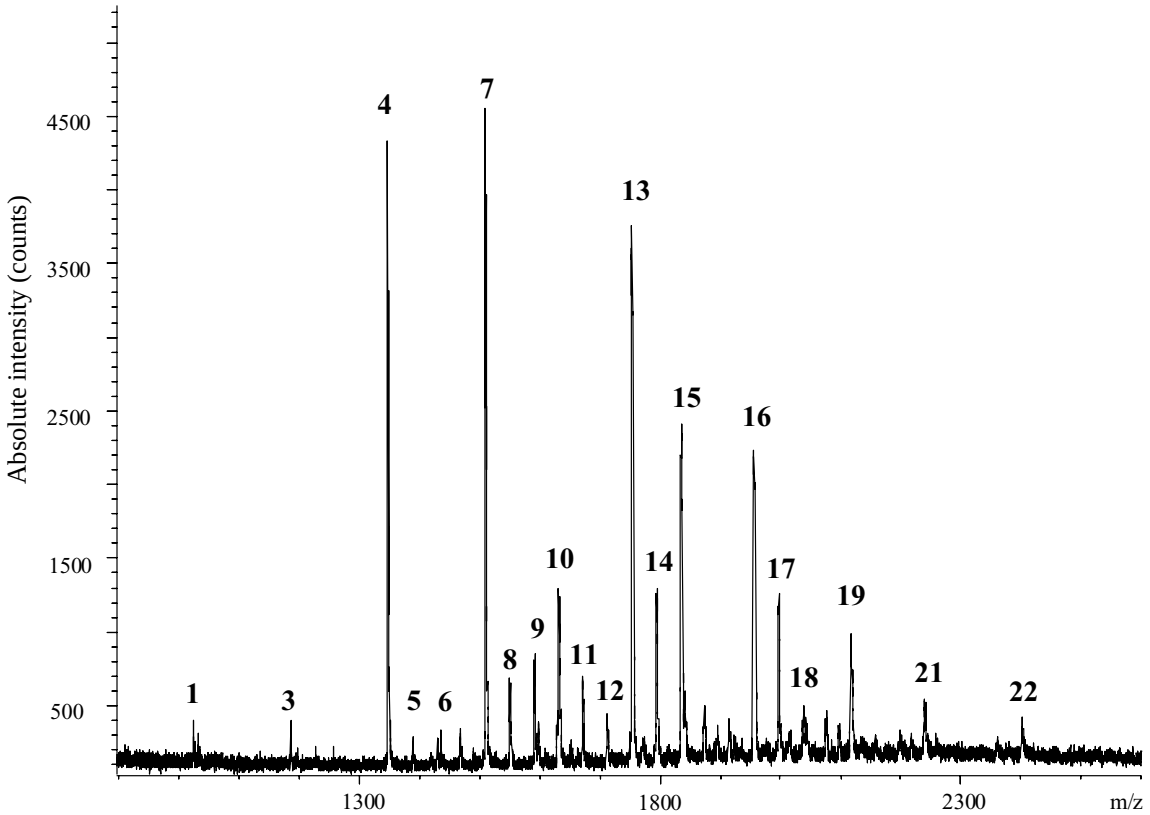


Fig. 8. MALDI spectrum obtained for the PHN–oligosaccharide pool from Sigma Grade VII hen ovalbumin. Numbers refer to assignments in Table 2.

preferential loss of Man from position 3 can be supported by the observation of low abundance ions at *m/z* 534, produced by loss of water from $B_3/Y_{3\alpha}$ ions [36]. Lastly, very low abundance $B_3/(Y_3)_2$ ions were observed at *m/z* 389 to support the presence of Structure 1. However, there are no Z_3 ions (−221 U) to support a structure with a bisecting GlcNAc [36]. The spectrum of Fig. 9a most probably is a superposition of fragmentation patterns associated with Structures 1 and 2.

Fig. 9b shows the PSD spectrum of complex glycan(s) of formula $(GlcNAc)_5(Man)_3PHN$ (MW 1609.7, $[M + Na]^+$ ions at *m/z* 1633) and fragmentation patterns for two representative isomers, Structures 1 and 2 [35]. The loss of two GlcNAc residues from the labeled side (chitobiose core) produced B_3 ions at *m/z* 1120 and C_3 ions at *m/z* 1136. The cleavage of another GlcNAc residue yielded B_3/Y_4 ions, possibly from Structures 1 and 2, at *m/z* 917. Fragment ions at *m/z* 755 were assigned as $B_3/Y_{3\alpha}$ for Structure 2. Ions at *m/z* 552 possibly correspond to $B_3/Y_{3\alpha}$ fragments from Structure 1. Accordingly, the loss of the 3-positioned Man residue (Structure 1) would produce $B_3/(Y_3)_2$ ions at *m/z* 390. The prominent ion peak at *m/z* 534 involves a Z-fragmentation scheme and supports the presence of a bisecting GlcNAc residue in position 4 of the core mannose. This spectrum was compared to CID results obtained with an underivatized glycan standard of same composition and corresponding to Structure 2 [36]. The CID spectrum also contained a similar Z-ion peak, which was attributed to the loss of bisecting GlcNAc as 221 mass units, i.e. an intact GlcNAc molecule [36]. The latter *m/z* 534 ions lost the core Man residue (128 U) to produce abundant C_2 ions at *m/z* 406, which were also observed in the CID spectrum of native isomer 2

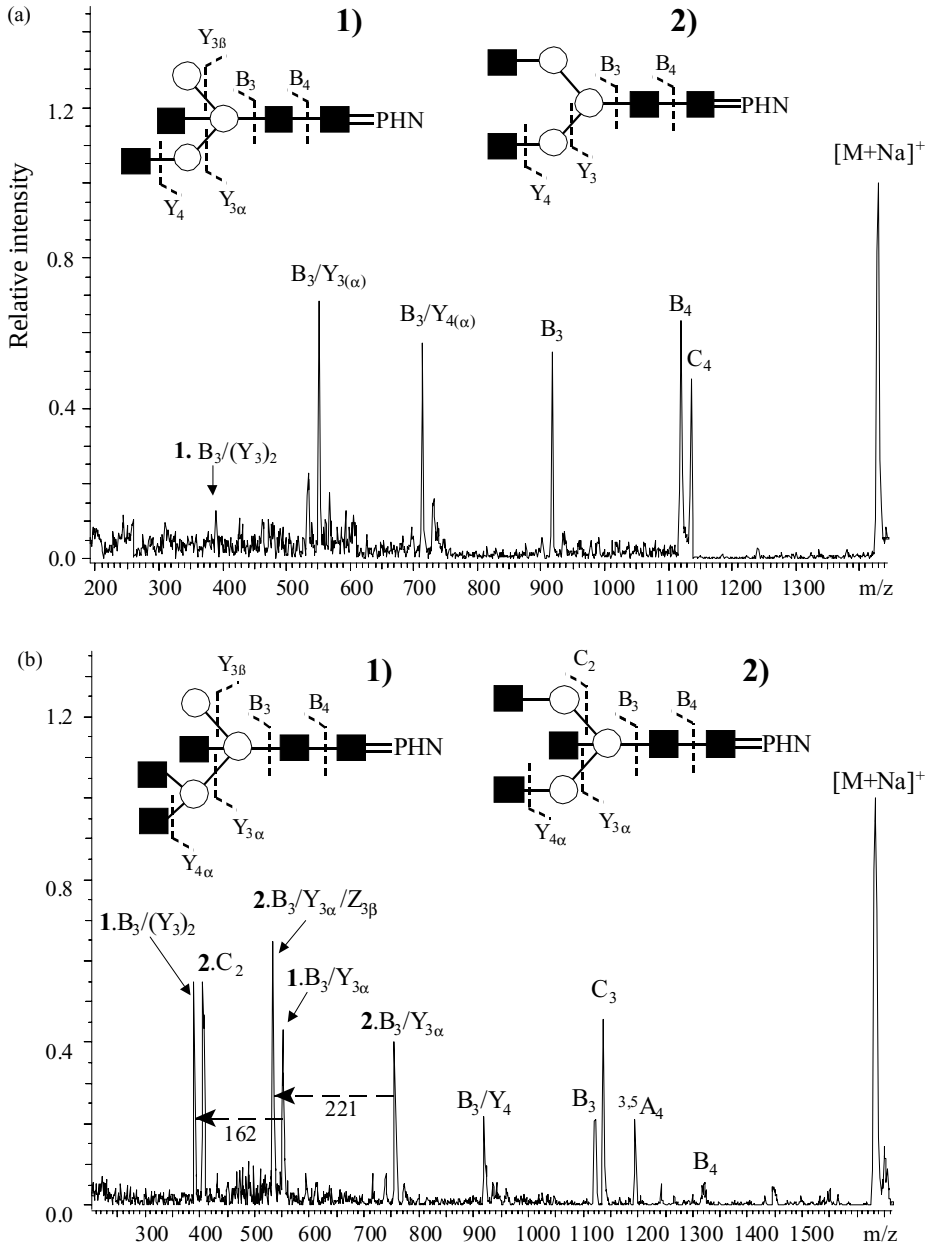


Fig. 9. MALDI–PSD spectra of the $[M + Na]^+$ ions of (a) $(GlcNAc)_4(Man)_3PHN$ and (b) $(GlcNAc)_5(Man)_3PHN$. All fragment ions include a sodium cation. See structural symbols in Table 1. Relative intensities are normalized to 1 (largest peak).

[36]. The important differences in fragmentation patterns observed in Fig. 9a and b possibly occur from the presence of a structure with no bisecting GlcNAc (Fig. 9a, Structure 2) while such unit is obviously present in Fig. 9b.

Glycans of composition $(GlcNAc)_6(Man)_3PHN$ (MW 1812.7, $[M + Na]^+$ ions at m/z 1836) were investigated by PSD next (see Fig. 10a). The fragmentation pattern consistent with a structure previously suggested by ^1H NMR data [35]. Here, B_3 and C_3

ions appeared at *m*/*z* 1323 and 1339, respectively. A B_3/Y_4 double cleavage produced the ions at *m*/*z* 1120. The next significant ion peak was observed at *m*/*z* 755 and may be attributed to $B_3/Y_{3\alpha}$ internal fragments. Low abundance *m*/*z* 405 ions have been assigned as $C_{2\beta}$ fragments. The low peak at *m*/*z* 534 could correspond to the loss of a bisecting GlcNAc residue (221 U) from $B_3/Y_{3\alpha}$ ions.

Fig. 10b shows a PSD spectrum obtained for glycan(s) of composition $(GlcNAc)_8(Man)_3PHN$ (MW

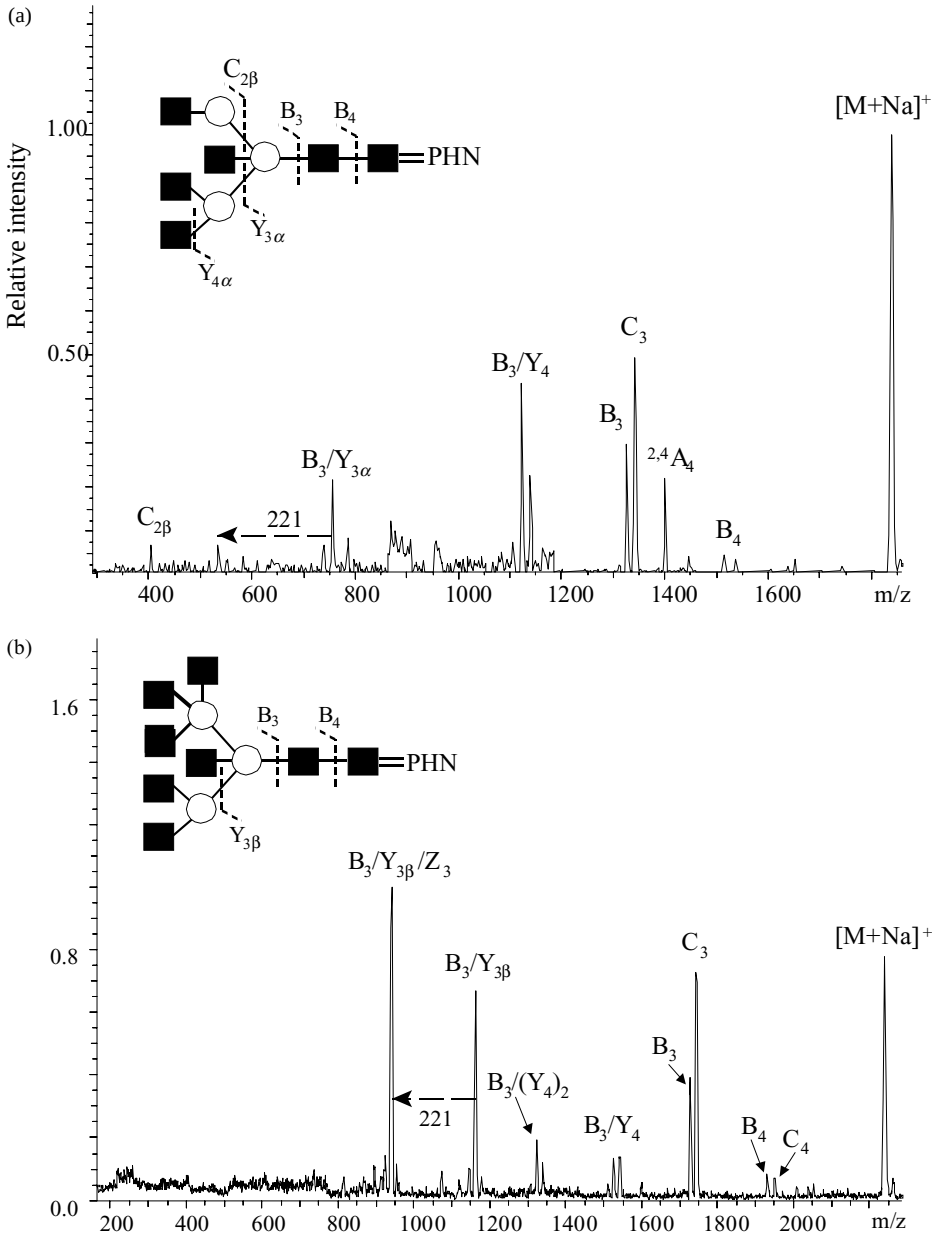


Fig. 10. MALDI–PSD spectra of the $[M + Na]^+$ ions of (a) $(GlcNAc)_6(Man)_3PHN$ and (b) $(GlcNAc)_8(Man)_3PHN$. All fragment ions include a sodium cation. See structural symbols in Table 1. Relative intensities are normalized to 1 (largest peak).

2218.9 Da, $[M + Na]^+$ ions at *m*/*z* 2242). As in almost all previous spectra, abundant B_3 and C_3 ions were present, in this case at *m*/*z* 1728 and 1745, respectively, were present. B_4 and C_4 ions were also observed. According to the structure shown on the spectrum, a loss of the 3-positioned $(GlcNAc)_2Man$ branch could produce $B_3/Y_{3\beta}$ ions at *m*/*z* 1163. Lower abundance ions at *m*/*z* 1527 (B_3/Y_4) and 1324 ($B_3/(Y_4)_2$) provided good evidence for the presence of terminal non-bisecting GlcNAc residues. The bisecting residue, when lost from $B_3/Y_{3\beta}$ ions (−221 U) gave rise to an ion peak at *m*/*z* 941. This spectrum was acquired with precursor of relatively low and unstable abundance (see Fig. 8, peak no. 21) when laser-irradiated during the MALDI process. This helps to explain variations in fragmentation patterns between Fig. 10a and b.

Lastly Fig. 11 presents a PSD spectrum obtained for galactosylated glycans of composition $(GlcNAc)_6(Man)_3(Gal)PHN$ (MW 1974.8, $[M+Na]^+$ ions at *m*/*z* 1998). Structures 1, 2 and 3 are possible and suggest that the position of the terminal Gal residue may vary. The observed fragmentation patterns suggest the occurrence of all three structures. Precursor $[M + Na]^+$ ions produced relatively abundant cross-ring A_5 fragments (at *m*/*z* 1527, 1544 and 1558). B_4 and C_4 ions at *m*/*z* values 1485 and 1502 were prominent, and a peak at *m*/*z* 1122 indicated the loss of a GlcNAc-hexosyl residue, which would satisfy all proposed structures ($B_4/Y_{3\,or\,4}$ ions). Low abundance ions at *m*/*z* 1325 seem to indicate a loss

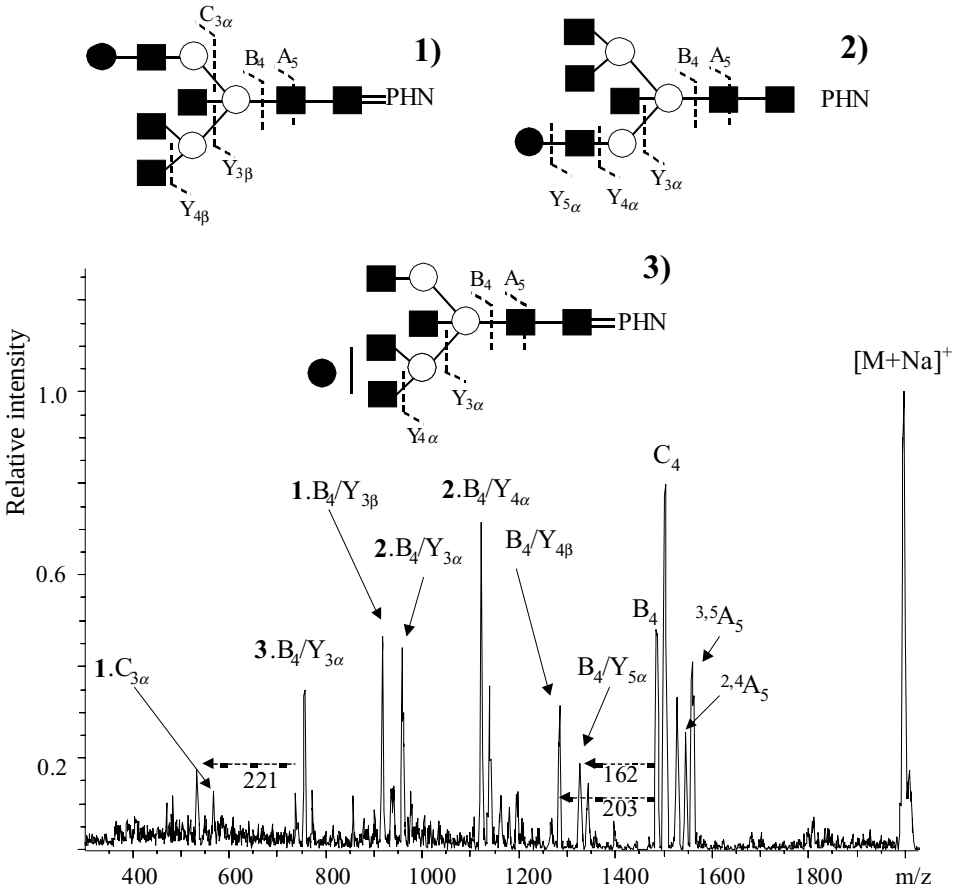


Fig. 11. MALDI–PSD spectrum of the $[M + Na]^+$ ions of $(GlcNAc)_6(Man)_3(Gal)PHN$. All fragment ions include a sodium cation. See structural symbols in Table 1. Relative intensities are normalized to 1 (largest peak).

of Gal along with a B_4 cleavage, also plausible for all three structures (B_4/Y_5). Accordingly, ions at *m*/*z* 1282 correspond to the cleavage of a GlcNAc residue from B_4 ions (B_4/Y_4). The peak at *m*/*z* 959 possible indicates the further loss of a $(hexosyl)_2(GlcNAc)$ branch from B_4 ions, which would be favored by Structure 2 ($B_4/Y_{3\alpha}$ cleavage). The ions at *m*/*z* 918 could result from $B_4/Y_{3\beta}$ fragmentations in Structure 1, which also produce $C_{3\alpha}$ ions at *m*/*z* 566. Ions at *m*/*z* 755 account for Structure 3 and could be characterized as $B_4/Y_{3\alpha}$ ions. Finally, ions at *m*/*z* 533 suggest the loss of a bisecting GlcNAc residue from the latter $B_4/Y_{3\alpha}$ fragments.

4. Conclusions

Phenylhydrazones of *N*-glycans were characterized using MALDI-MS and on-line HPLC/ESI-MS. Reversed-phase conditions allowed partial but useful separation of these derivatized oligosaccharides. The total profile of ovalbumin *N*-glycans found by electrospray was in good agreement with results obtained by MALDI-MS. Under post-source decay conditions, these derivatives produced relatively simple fragmentation patterns, allowing to speculate on predominant isomeric glycan structures present in hen ovalbumin glycan pools. All PSD spectra acquired using $[M + Na]^+$ ions of PHN-complex glycans as precursors showed B, C and internal B/Y, C/Y fragment ions. Although these ions were obtained in a predictable manner, they did not provide a means of singling out isomeric structures with certainty. In order to make this possible, comparisons with PSD spectra of standard glycans with diverse known compositions would be required. PHN derivatization is fast, simple and does not involve salts in the labeling procedure. For MALDI analyses, on-target derivatization avoids sample loss and gives rise to intense signals. PHN derivatives are also very easily amenable to ESI-MS analysis, either by flow-injection or HPLC coupling, as demonstrated in this article.

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