ELSEVIER

Carbohydrate Research 311 (1998) 51–59

CARBOHYDRATE RESEARCH

Determination of the sialylation level and of the ratio α-(2→3)/α-(2→6) sialyl linkages of *N*-glycans by methylation and GC/MS analysis

Didier Cointe, Yves Leroy, Frédéric Chirat *

Laboratoire de Chimie Biologique et Unité Mixte de Recherche du CNRS No 111, Université des Sciences et Technologies de Lille, F-59655 Villeneuve d'Ascq, France

Received 23 February 1998; accepted 15 July 1998

Abstract

A methodology for the determination of the sialylation pattern of *N*-glycans, extent of sialylation and the ratio between α-(2→3) and α-(2→6) sialyl linkages, is presented based on the labelling of the C-3 and C-6 hydroxyl groups of Gal residues obtained after permethylation, saponification, selective desialylation of sialylated oligosaccharides and methanolysis. Deuteromethylation and GC/MS analysis of Gal derivatives allow to determine the sialylation level of glycans. *O*-Ethyl ether labelling followed by GC analysis of the resulting Gal derivatives allows to obtain the ratio between α-(2→3) and α-(2→6) sialyl linkages. The method was applied to LNT ($LcOse_4$: β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p*), LSTa ($IV^3NeuAcLcOse_4$: α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p*), LSTc ($IV^6NeuAcn$ $LcOse_4$: α-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p*) and a bisialylated biantennary *N*-glycan in which sialic acid is bound to Gal residues via an α-(2→6) linkage. Using this method, it was found that 92.8% of *N*-glycans in bovine fetuin is sialylated and that the ratio of α-(2→6) versus α-(2→3) sialyl linkages was 31:19.

Keywords: Sialic acid; Methylation; Sialylation level; Sialyl linkage; Mass spectrometry

1. Introduction

Several methods [1–3] have been described for the quantitative and qualitative analysis of sialic acids. There are also different methods to discriminate between α-(2→3) and α-(2→6) linkages. For instance, monoclonal antibodies directed against the Neu5Ac-α-(2→6)-Gal epitope have been used for the study of glycosphingolipid sialylation [4]. Sialidases, in particular sialidase isolated from Newcastle Disease Virus (E.C. 3.2.1.18) which is specific of α-(2→3) sialyl linkage [5], can be used for this purpose. However, since there is no sialidase specific of the α-(2→6) sialyl linkage, the direct determination of the proportion of such linkage is not possible. Moreover, some peculiar sialic acid residues (e.g., *O*-acetylated) can be resistant to enzymatic cleavage making this

* Corresponding author. Fax: + 33-3-20-43-65-55; e-mail: frederic.chirat@univ-lille1.fr

0008-6215/98/$—see front matter
PII S0008-6215(98)00196-7

approach non quantitative. An HPLC method, based on the use of three successive HPLC columns [6] was proposed to structurally characterize the different oligosaccharide chains of a glycoprotein. The authors were able to clearly differentiate closely related Neu5Ac containing oligosaccharides involving α-(2→3) and α-(2→6) linkages. A discrimination between these two kinds of sialyl linkage can also be obtained by collisional-activation tandem mass spectrometry of methylated oligosaccharides [7]. All these methods require either purified glycans or to proceed with two separate analyses. In contrast, two other methods NMR spectroscopy and methylation analyses can be carried out on a mixture of oligosaccharides. The first one, which involves high field ^{1}H NMR spectroscopy, was successfully introduced by Vliegenthart et al. [8] to characterize glycoprotein structures. Among the structural-reporter groups which are clearly distinguishable for the determination of the structures, the H-3ax and the H-3eq atom resonances of Neu5Ac or Neu5Gc allow to define directly the linkage type and position in the carbohydrate chain. Such analyses generally require at least 10 nM of oligosaccharide or glycopeptide. However, it is possible to reduce this amount down to only a few micrograms of material through the use of microprobes. The second method, which involves methylation analysis, has been routinely used in many laboratories to study the carbohydrate glycosidic linkages [9]. When applied to classical sialylated *N*-glycans, in which terminal Gal residues are only substituted by sialic acid residues, it allows the assignation of both the sialylation level and the α-(2→3)/α-(2→6) sialyl linkage ratio of a glycoprotein. However, for some *N*-glycan chains, monosaccharides other than sialic acid (e.g., GlcNAc [10,11] or Gal [12]) can be linked to the terminal Gal residue. In this case, the method cannot be used for this purpose. In this paper, we propose a novel strategy to overcome this problem. The latter (Scheme 1) is based on deuteromethylation or ethylation of free Gal residue hydroxyl groups to determine unambiguously the sialylation level of a glycoprotein and the ratio of α-(2→3) to α-(2→6) sialic acid linkages.

A similar strategy was used to locate sulfate groups present in some oligosaccharide chains of recombinant human tissue plasminogen activator [13]. However, to our knowledge, it has never been applied to the study of sialic acid-containing oligosaccharides.

The study of *N*-glycan chain sialylation of fetuin was chosen as a first application of this strategy.

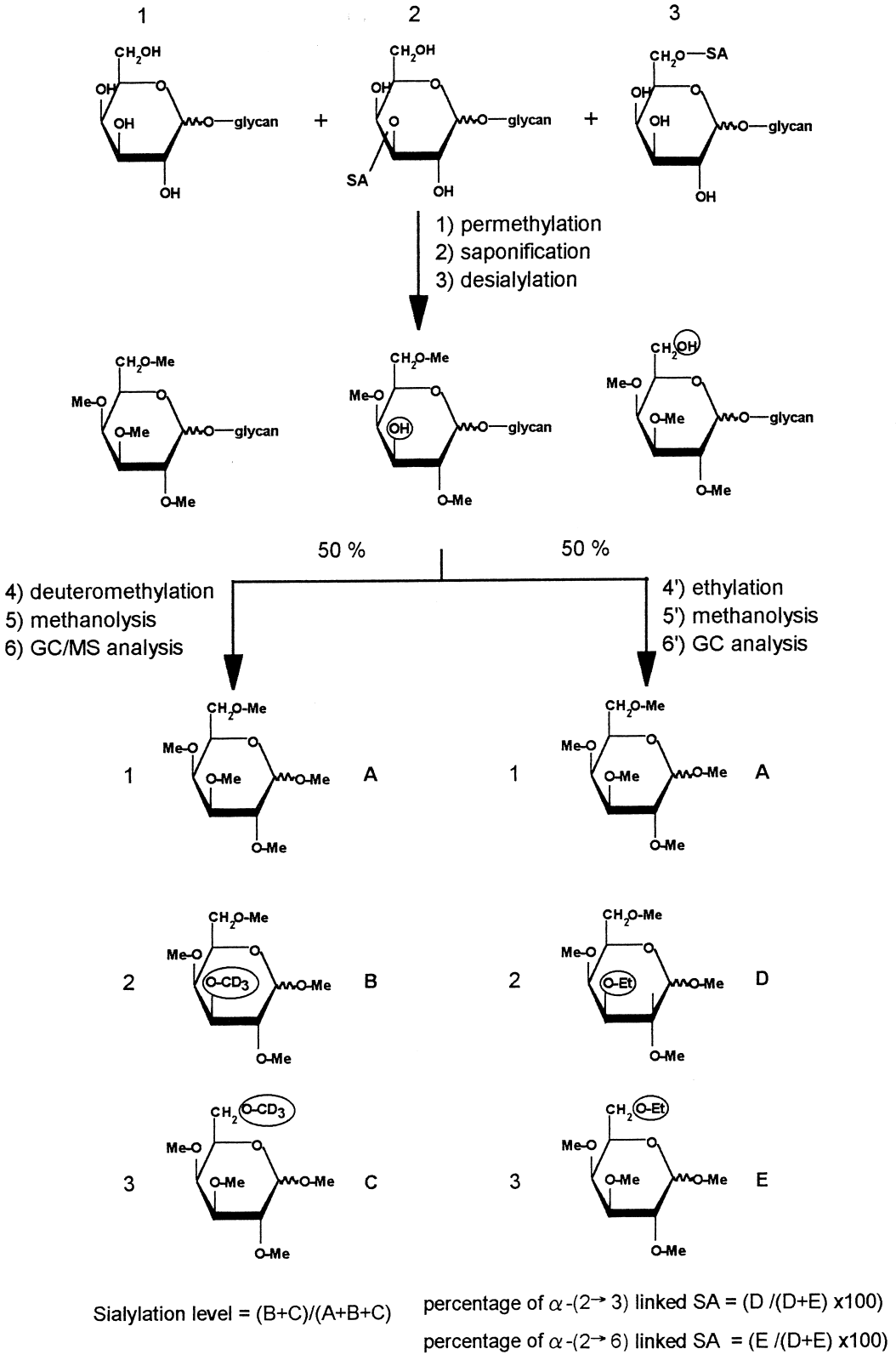
2. Results and discussion

Evaluation of the strategy with reference oligosaccharides.—In order to establish the optimal conditions for each step of the strategy presented in Scheme 1, the protocols were developed using four reference compounds: LNT, (β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p*); LSTa, (α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p*); LSTc, (α-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p*), and a bisialylated biantennary *N*-glycan in which sialic acid is bound to Gal residues through an α-(2→6) linkage.

Methylation, saponification and selective desialylation.—LSTa and LSTc were permethylated and subsequently checked for complete methylation by MALDI-TOF. Application of a mild acid treatment (methanol–0.05 M HCl at 80 °C for 1 h) in order to desialylate permethylated oligosaccharides lead, as observed by MALDI-TOF analysis, to several peaks corresponding to oligosaccharide fragments. Decreasing the temperature from 80 to 60 °C induced no change.

In order to quantitatively and selectively remove sialic acid residues, permethylated LSTa and LSTc oligosaccharides were saponified by alkali treatment with 0.1 M KOH in 4:1 ethanol–water for 4 h at 60 °C. The MALDI-TOF mass spectrum obtained with LSTa (Fig. 1a) showed two major ions at *m*/*z* 1327.9 and *m*/*z* 928.7 corresponding respectively to the $[M-H+2K]^+$ ion of the free acid and to the $[M'+K]^+$ ion of the desialylated product (M′). This last compound is probably generated by the acidic matrix (2,5-dihydroxybenzoic acid) used for the MALDI-TOF analysis as suggested by Powell and Harvey [14]. This result confirms the stabilisation of the sialyl bond when the carboxyl group of sialic acid is esterified and justifies the modification introduced at the end of the methylation procedure (i.e., cooling of the reaction mixture, centrifugation to discard insoluble compounds (e.g., excess of NaOH, NaI) and acidification by a 5% acetic acid solution) in order to avoid saponification of the carboxylmethyl group of sialic acids.

Treatment of saponified sialylated oligosaccharides with formic acid leads to their quantitative and



Scheme 1. Strategy proposed for the determination of the rate of sialylation and the ratio α-(2→3)/α-(2→6) sialyl linkages. Samples (containing sialylated or unsialylated) were permethylated, saponified and selectively desialylated (steps 1, 2 and 3 respectively). Free hydroxyl groups of Gal residues were then labelled; 50% of the reaction mixture was deuteromethylated (step 4) whereas the remaining 50% was ethylated (step 4′). Partially deuteromethylated Gal residues, obtained by methanolysis (step 5), were analyzed by GC/MS (step 6) to determine the sialylation level of the glycoprotein. Partially ethylated galactose residues, obtained by methanolysis (step 5′), were analyzed by GC (step 6′) to determine the ratio of α-(2→3) to α-(2→6) sialyl linkages.

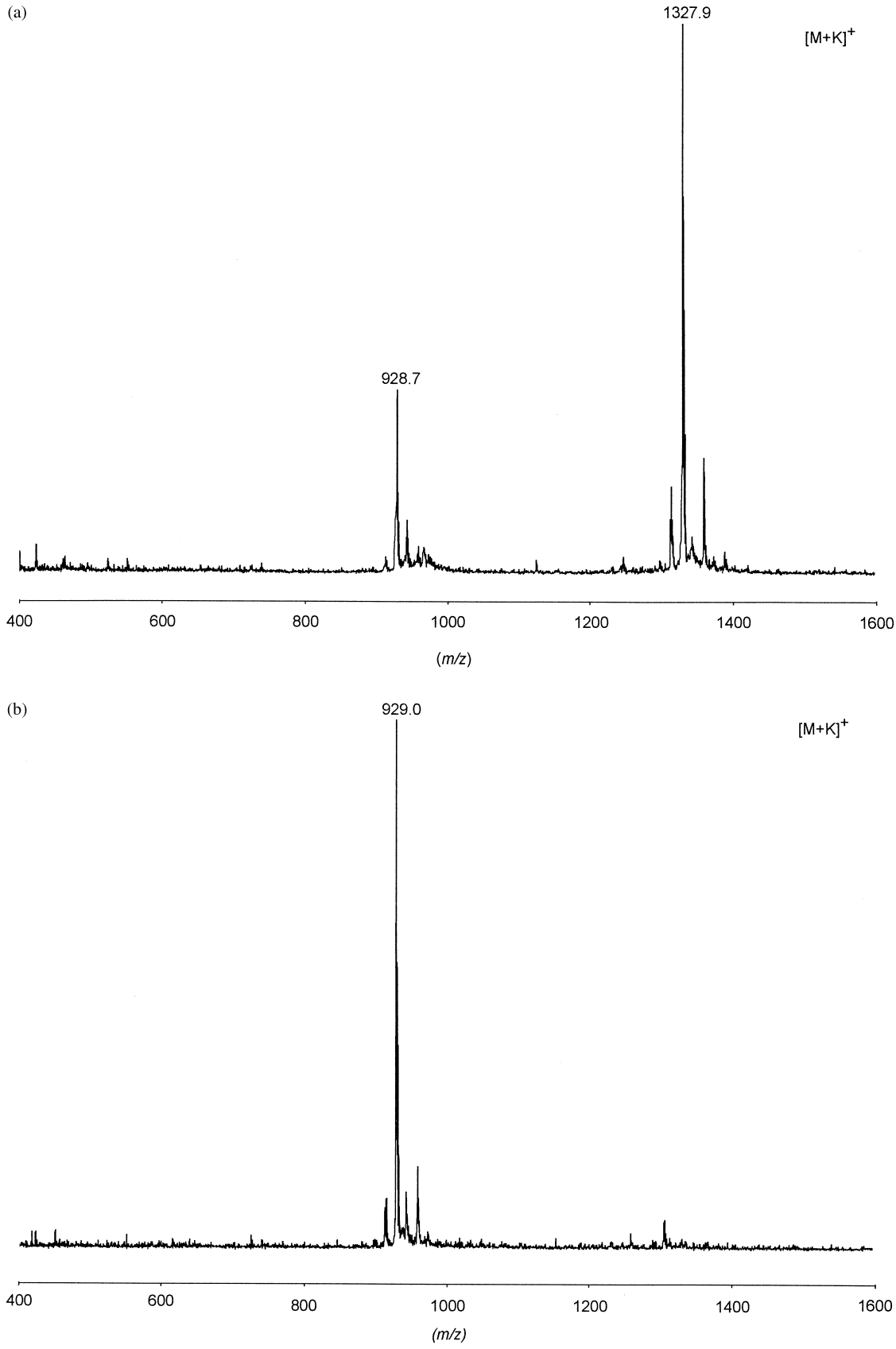


Fig. 1. MALDI-TOF mass spectra of (a) permethylated LSTa after alkali treatment and (b) after acidic desialylation.

selective desialylation as illustrated in Fig. 1b. The mass spectrum displays only one peak at m/z 929 which corresponds to the calculated molecular mass of desialylated methylated LST (a or c). The free hydroxyl groups of the terminal galactose residue, generated during the desialylation step, were alkylated. For this purpose, one half of the sample was trideuteromethylated whereas the remaining part was ethylated. Samples were then treated with methanol–0.5 M HCl for 24 h at 80 °C, and the released methylglycosides were analyzed either by gas chromatography (GC) or by GC coupled to a mass spectrometer (GC/MS).

Analysis of partially methylated and ethylated methyl galactopyranoside residues by gas chromatography.—Several GC-columns were tested in order to separate the following methyl galactopyranosides: methyl tetra-*O*-methyl-D-galactopyranoside (Me4-Gal), methyl 3-*O*-ethyl-2,4,6-tri-*O*-methyl-D-galactopyranoside (3-Et-Gal) and methyl 6-*O*-ethyl-2,3,4-tri-*O*-methyl-D-galactopyranoside (6-Et-Gal). By using either a non-polar column (DB1 column), a weakly polar column (OV17 column) or a polar column (DB Wax column), coelution not only between some methyl galactopyranosides but also between the latter and other methyl glycopyranosides was observed. Complete separation of these three methyl galactopyranoside derivatives was achieved by coupling a DB Wax column to a DB1 column (Fig. 2).

Ethylated derivatives were identified on the basis of their molecular mass by GC/CIMS whereas the position of the ethyl group in the molecule was determined by GC/EIMS. The presence of an ethyl group at the C-6 hydroxyl group of a Gal residue leads to an H fragment [15] at m/z 88, whereas on the C-3 hydroxyl group of a Gal residue, the H fragment is observed at m/z 102. The m/z 14 difference is due to the additional CH_2 group in this fragment.

Quantitative evaluation of ethylation labelling.—To check the quantitative nature of the proposed strategy with respect to the α-(2→3) versus α-(2→6) sialyl linkage ratio, we prepared and analyzed three different mixtures of LSTa and LSTc containing 2/1, 1/1 and 1/2 α-(2→3) to α-(2→6) sialyl linkage ratios. Results are presented in Fig. 3. From the area measured for each ethylated compound (α and β anomers), the ratio of 3-Et-Gal to 6-Et-Gal was inferred. Results listed in Table 1 are in good agreement with the theoretical values although the level of 6-Et-Gal is slightly larger than the theoretical value. This difference is probably due to a higher quantity of LSTc in the 3 mixtures since the ratio of methyl 3-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucopyranoside (specific of LSTa) to methyl 4-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucopyranoside (specific of LSTc), calculated for each mixture, leads to similar values to those presented in Table 1.

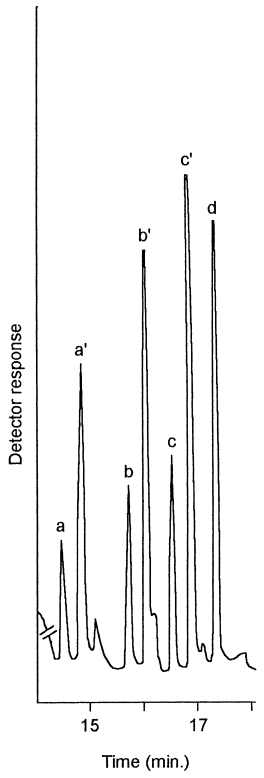


Fig. 2. Chromatogram corresponding to the separation (anomeric forms) of Me4-Gal (peaks a and a′), 3-Et-Gal (peaks b and b′), 6-Et-Gal (peaks c and c′) and methyl 2-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannopryanoside (peak d) residues co-injected on a DB Wax column coupled to a DB1 column. These monosaccharide derivatives were obtained as follows: Me4-Gal, methyl 2-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannopryanoside and 6-Et-Gal were generated from bisialylated biantennary *N*-glycan whereas 3-Et-Gal was generated from LST a.

From these data, it can be concluded that the method allows an accurate quantification of sialic acid residues bound to galactose through an α-(2→3) and/or an α-(2→6) linkage.

Determination of the degree of glycan sialylation.—To determine the sialylation level of *N*-glycan-containing glycoproteins, the strategy described above was followed except for the labelling of the free hydroxyl group. Trideuteromethylation was used in place of ethylation. Following methanolysis, partially methylated and trideuteromethylated

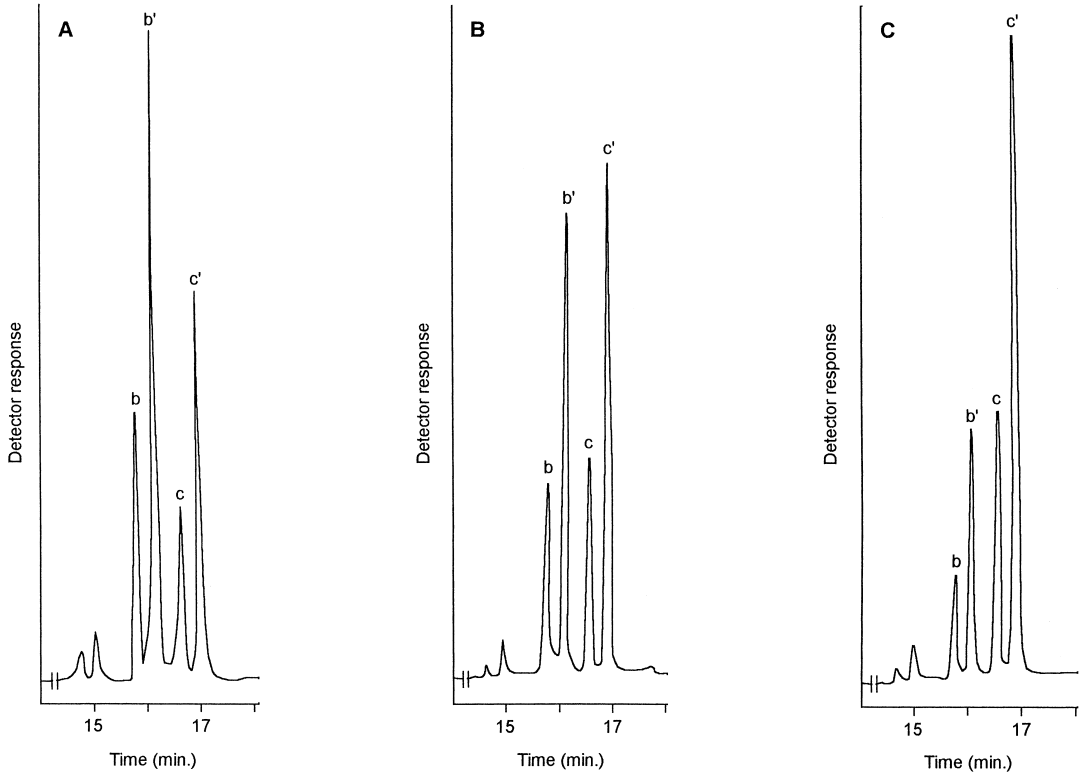


Fig. 3. Gas-phase chromatograms of partially methylated and ethylated (3-Et-Gal or 6-Et-Gal) methyl galactopryanoside generated from synthetic mixtures of LSTa and LSTc in 2/1 ratio (A), 1/1 (B) and 1/2 (C). Identification of peaks is indicated in Fig. 2.

methyl galactopyranoside and Me4-Gal were analyzed by ammonia CIMS. Ammonia was chosen rather than methane or isobutane because ammonia CI spectra display predominantly $[M+NH_4]^+$ and very few fragment ions [16]. This feature is particularly important to precisely quantify the partially trideuteromethylated galactose residue (sialylated in the native glycan) and the permethylated galactose (not sialylated in the native glycan). To check our strategy, a 2:1 LNT-LSTc mixture was tested. Peak areas of $[M+NH_4]^+$ ions at m/z 271 and 268, named respectively A (139,415) and B (262,146) and peak areas of $[M+H]^+$ ions at m/z 254 and 251, named respectively A′ (213,379) and B′ (384,987) were recorded by ammonia GC/CIMS. From these data, sialylation levels of 34.7% (A/(A + B)) and 35.7% (A′/(A′ + B′)) were determined. These values are in good agreement with the theoretical value of 33.3%. As previously, the 2:1 LNT-LSTc weight ratio was confirmed by quantifying the methyl 3-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucopyranoside (generated from LNT) and the methyl 4-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucopyranoside (generated from LSTc).

Table 1
Quantification of 3-Et-Gal and 6-Et-Gal derivatives generated from synthetic mixture of LSTa and LSTc in respective proportion 2/1, 1/1 and 1/2

Ratio LSTa/LSTc	Derivatives	Areas[a]	%	Theoretical %
2:1	3-Et-Gal	385.780	60.4	66.6
	6-Et-Gal	253.311	39.6	33.3
1:1	3-Et-Gal	413.051	48	50
	6-Et-Gal	446.680	52	50
1:2	3-Et-Gal	145.803	28.5	33.3
	6-Et-Gal	366.649	71.5	66.6

[a] Areas of 3-Et-Gal and 6-Et-Gal were obtained by summing the areas corresponding to their α and β anomers.

The case of sulfated and fucosylated oligosaccharides.—Sialic acid residues, and also fucose residues and sulfate groups, are known to be labile in acidic conditions. To check that our strategy can be applied to sialylated sulfated and/or fucosylated oligosaccharides, the following oligosaccharide, β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-6-*O*-SO_3^--(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p* were analyzed in the same conditions as sialylated oligosaccharides. As displayed by the

MALDI-TOF mass spectra (data not shown) of the permethylated oligosaccharide, before and after permethylation, saponification and acidic treatment with 2% formic acid in 4:1 acetone–water, no difference was observed. These results lead us to conclude that our strategy used to determine the sialylation extent and the ratio of α-(2→3)/α-(2→6) sialyl linkages, can also be applied to sulfated and/or fucosylated *N*-glycans. It must be noted that in the case of sulfated oligosaccharides, a particular attention must be paid to the end of the methylation procedure as described in [17]. The possible dephosphorylation of phosphorylated oligosaccharides was not checked in these experiments.

Application to the study of sialylation from bovine fetuin N-*glycans*.—After releasing of fetuin *N*-glycan chains by hydrazinolysis (see experimental section), the absence of sialylated *O*-glycans was demonstrated by GC analysis of monosaccharides (as their trimethylsilylated derivatives) which did not reveal the presence of GalNAc residues, present in all fetuin *O*-glycans [18]. In the case of polysialylated oligosaccharides containing more than 2 sialic acids per molecule, the solubility in dimethyl sulfoxide was very low resulting in a poor methylation yield. To overcome this problem, counter-ions (Na^+ or K^+) of sialic acids had to be replaced by triethylammonium ions as described in the experimental section.

Determination of the level of fetuin glycan chain sialylation.—Partially methylated and trideuteromethylated methyl galactopyranoside and permethylated galactose generated from the *N*-glycan chains of fetuin were analyzed by GC/CIMS as described for the 2:1 LNT-LSTc mixture. From the respective peak are, as (A = 212,192; B = 16,354; A′ = 180,015; B′ = 14851), the A/(A + B) ratio gave 92.8% of sialylation (based on $[M + NH_4]^+$ ions) and the A′/(A′ + B′) ratio gave 92.4% of sialylation (based on $[M + H]^+$ ions). These sialylation levels are in good agreement with the sialylation level of *N*-glycan chains of fetuin (91.6%) as calculated from published data [19].

Determination of the α-*(2→3) versus* α-*(2→6) sialic acid linkages of fetuin* N-*glycans*.—Following permethylation, saponification and selective desialylation of glycans, the free hydroxyl groups of Gal residues were ethylated before methanolysis. As shown in Fig. 4, the GC analysis of partially methylated and ethylated methyl galactopyranoside indicate that 62% of sialic acids are bound to

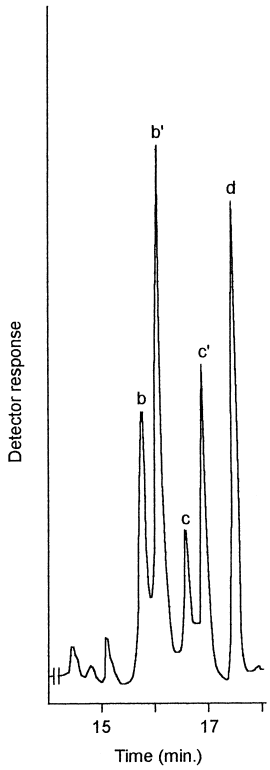


Fig. 4. Gas-phase chromatogram of partially methylated and ethylated monosaccharides generated from bovine fetuin *N*-glycans. Identification of peaks is indicated in Fig. 2.

galactose via an α-(2→6) linkage whereas 38% are bound via an α-(2→3) linkage. This proportion differs from earlier results obtained through NMR spectroscopy analysis which gave 50% for each linkage [19]. To check the possibility that another compound could coelute with one of the two peaks of 6-Et-Gal, leading to an increase of the corresponding peak area, quantification of 3-Et-Gal and 6-Et-Gal was carried out by GC/CIMS. According to the peak areas of ions $[M + NH_4]^+$ at *m*/*z* 282 and $[M + H]^+$ at *m*/*z* 265, the proportion of sialic acids linked to a Gal residue through an α-(2→6) linkage was 64 and 61% respectively (data not shown). These two results confirmed those obtained by GC analysis.

The slight divergence between both results could be explained by the fact that different methods were used. Using the Green et al. methodology [19], the proportion of α-(2→3) and α-(2→6) sialyl linkages was deduced from HPLC peak areas of different fractions of *N*-glycans which are not always well resolved followed by NMR spectroscopy analysis of these peaks. In our case, whole *N*-glycans were chemically treated simultaneously avoiding loss of glycan chains.

3. Conclusion

The strategy presented allows the accurate determination of the sialylation level of N-glycan chains by GC/CIMS analysis of partially methylated and trideuteromethylated methyl galactopyranoside and permethylated galactose. It allows furthermore quantification of sialic acids linked to galactose via an α-(2→3) or α-(2→6) linkage by GC analysis of 3-Et-Gal and 6-Et-Gal residues. It relies on the selective labelling of free hydroxyl group of galactose residue obtained after removal of the sialic acids. The saponification is the critical step of our procedure. Indeed, selective desialylation of permethylated glycans is only possible when the carboxyl group of sialic acid remains free.

This methodology presents three advantages: (i) all glycan chains are treated at the same time, allowing a more precise quantification; (ii) it constitutes a useful tool for the study of sialylation pattern of glycans since it can be applied to glycans containing *N*-acetyllactosaminidic units (bound to the C-3 hydroxyl group of terminal Gal residues), fucose residues and sulfate groups; (iii) this strategy could be extended to the study of sialylation of monosaccharides other than galactose (e.g., GalNAc or GlcNAc residues) (for review [20]).

4. Experimental

Materials.—Oligosaccharides, LNT, LSTa and LSTc, were purchased from Oxford Glycosystems (England).

Release of sialylated N-*glycans of fetuin*.—Lyophilised fetuin (2 mg) from fetal calf serum was subjected to hydrazinolysis on a glycoprep 1000 apparatus (Oxford Glycosystems, England) using the *N*-glycan program. The oligosaccharides were freeze-dried and applied to a Dowex 50WX12 column equilibrated by triethylammonium formate, 1 M, pH 5.0 in order to allow a complete solubilisation of polysialylated oligosaccharides in Me_2SO during the methylation step. Sialylated oligosaccharides were subsequently eluted by water and lyophilised.

Methylation.—Sialylated oligosaccharides were methylated according to the method of Ciucanu and Kereck [21]. The sialylated oligosaccharides (50 μg) were dissolved in Me_2SO (200 μL) containing NaOH (2 mg) and methyl iodide (100 μL) was added. After sonication for 1 h at room temperature, the reaction mixture was cooled to 0 °C and $CHCl_3$ (1 mL) was added. The tube was centrifuged at 2000 rpm for 3 min and insoluble compounds (excess of NaOH, NaI) were discarded. The supernatant was first washed with 5% acetic acid and then washed by cooled water (x 9). The $CHCl_3$ phase was evaporated under N_2 stream and freeze-dried. Two successive methylation cycles were performed for each sample.

Selective desialylation.—Permethylated sialylated oligosaccharides were dissolved in EtOH (40 μL) and saponified by a treatment with 0.1 M KOH (200 μL) in 4:1 EtOH–water at 60 °C for 4 h. The reaction mixture was neutralised by adding 2% formic acid (50 μL) in 4:1 acetone-water and immediately dried under a N_2 stream. Finally, the same formic acid solution (400 μL, 0.5 M final concentration) were added and the selective desialylation was carried out overnight at 80 °C. The mixture was divided in two equal portions which were dried under a N_2 stream before lyophilisation.

Deuteromethylation and ethylation procedures.—The first sample fraction was submitted to deuteromethylation and the second one to ethylation by using MeI-*d* and EtI respectively. In both cases, the methylation procedure described above was used, except that washing was carried out at room temperature. Two successive alkylation steps (deuteromethylation or ethylation) were carried out for each samples.

Gas chromatography analysis.—Partially methylated and trideuteromethylated methyl galactopyranosides and partially methylated and ethylated methyl galactopyranosides were obtained by methanolysis with MeOH–0.5 M HCl at 80 °C for 24 h. At the end of the methanolysis step, the mixture was concentrated under a N_2 stream until dried. Methylglycosides were acetylated by acetic anhydride (40 μL) in the presence of pyridine (10 μL) at 80 °C for 1 h in the dark. After evaporation under N_2 stream, partially acetylated and trideuteromethylated methyl glycosides were dissolved in $CHCl_3$ (40 μL) and analyzed by GC/MS by using a DI 700 chromatograph (Delsi France) fitted with a Ross injector and coupled to a Riber Mag 10–10 M (France) mass spectrometer. The column was a DB1 (SGE, 25 m×0.32 mm). The temperature program used was from 110 to 180 °C at the rate of 3 °C/min and from 180 to 240 °C at a rate of 5 °C/min. Helium pressure was 0.5 bar. The EI mass

spectrum was obtained by using an electron energy of 70 eV , an ionizing current of 0.2 mA and a source temperature of 150 °C. Masses were scanned from 41 to 400 amu during 1/2 s. For CI/MS analysis, ammonia was introduced into the source at a pressure of 0.1 mm Hg. The source temperature was 100 °C and the masses were scanned from 200 to 400 amu.

The partially acetylated, methylated and ethylated methylglycopyranosides were dissolved in $CHCl_3$ (1 mL). The $CHCl_3$ phase was washed with water (x 3) and concentrated under a N_2 stream. GC analysis was subsequently performed by using a GC-14A chromatograph (Shimadzu, Japan). A DB Wax column (30 m×0.32 mm, JW Scientific INC) was coupled to a DB1 column (25 m×0.32 mm, SGE) through a glass union (MOGF/005, SGE). The temperature program used was 140 °C→180 °C (2 °C/min); 180 °C (10 min); 180 °C→220 °C (4 °C/min); 220 °C (10 min); 220→240 °C (6 °C/min). For these three different conditions, the separation was monitored by FID.

MALDI-TOF analysis.—The molecular mass of the permethylated oligosaccharides was measured by MALDI-TOF on a vision 2000 instrument (Finnigan Mat, Bremen, Germany) equipped with a 337 nm UV laser. The mass spectra were acquired in reflectron mode under a 6 kV accelerating voltage and positive detection. 2,5-dihydroxybenzoic acid (10 mg/mL) was used as matrix.

Acknowledgements

This work was supported by the Centre National de la Recherche Scientifique (UMR No 111, Relations Structure-Fonction des constituants membranaires, Prof. André VERBERT) and by the Laboratoire Français du Fractionnement et des Biotechnologies, Lille (France). The authors thank Dr. G. Strecker and Dr. J.C. Michalski for the generous gift of the fucosylated and sulfated oligosaccharide and of the bisialylated biantennary *N*-glycan, respectively. The authors thank also Prof. A. Verbert, Dr. A. Harduin-Lepers, Dr. P. Talaga and Mr D. Stokes for their assistance in preparing this paper.

References

[1] G. Reuter and R. Schauer, *Methods Enzymol.*, 230 (1994) 168–200.
[2] S. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura, and Y. Ohkura, *Anal. Biochem.*, 164 (1987) 138–145.
[3] K.R. Anumula, *Anal. Biochem.*, 230 (1995) 24–30
[4] M. Iwamori, M. Noguchi, T. Yamamoto, M. Yago, and Y. Nagai, *FEBS lett.*, 233 (1988) 134–138.
[5] J.C. Paulson, J. Weinstein, L. Dorland, H. van Halbeek, and J.F.G. Vliegenthart, *J. Biol. Chem.*, 257 (1982) 12734–12738.
[6] N. Takahashi, K.B. Lee, H. Nakagawa, Y. Kawamura, Y.T. Li, and Y.C. Lee, *Anal. Chem.*, 230 (1995) 333–342.
[7] J. Lemoine, G. Strecker, Y. Leroy, and B. Fournet, *Carbohyd. Res.*, 221 (1993) 209–217.
[8] J.F.G. Vliegenthart, H. van Halbeek, and L. Dorland, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 209–374.
[9] B. Lindberg, *Methods Enzymol.*, 28 (1972) 178–195.
[10] C.H. Hokke, A.A. Bergwerff, G.W.K. Van Dedem, J.P. Kamerling, and J.F.G. Vliegenthart, *Eur. J. Biochem.*, 228 (1995) 981–1008.
[11] H. Yoshima, A. Matsumoto, T. Mizuochi, T. Kawasaki, and A. Kobata, *J. Biol. Chem.*, 256 (1981) 8476–8484.
[12] B. Coddeville, G. Strecker, J.M. Wieruszeski, J.F.G. Vliegenthart, H. van Halbeek, J. Peter-Katalinié, H. Egge, and G. Spik, *Carbohydr. Res.*, 236 (1992) 145–164.
[13] G. Pfeiffer, M. Schmidt, K.-H. Strube, and R. Geyer, *Eur. J. Biochem.*, 186 (1986) 273–286.
[14] A.K. Powell and D.J. Harvey, *Rapid Commun. Mass Spectrom.*, 8 (1996) 1027–1032.
[15] B. Fournet, G. Strecker, Y. Leroy, and J. Montreuil, *Anal. Biochem.*, 116 (1981) 489–502.
[16] R. Geyer and H. Geyer, *Methods enzymol.*, 230 (1994) 86–108.
[17] J.M. Lo-Guidice, J.M. Wieruszeski, J. Lemoine, A. Verbert, P. Roussel, and G. Lamblin, *J. Biol. Chem.*, 269 (1994) 18794–18813.
[18] A.S.B. Edge and R.G. Spiro, *J. Biol. Chem.*, 262 (1987) 16135–16141.
[19] E.D. Green, G. Adelt, J.U. Baenziger, S. Wilson, and H. van Halbeek, *J. Biol. Chem.*, 263 (1988) 18253–18268.
[20] A. Harduin-Lepers, M.A. Recchi, and P. Delannoy, *Glycobiology*, 5 (1995) 741–758.
[21] I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.