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Strategy for the investigation of O-linked oligosaccharides from mucins based on the separation into neutral, sialic acid- and sulfate-containing species

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A method for the separation of O-linked oligosaccharides into neutral, sialic acid-containing and sulfated species was applied to oligosaccharides released by alkaline borohydride from mucin glycopeptides from porcine small intestine. The released mixture of reduced oligosaccharides was applied to an anion exchange column, and the neutral oligosaccharides were collected as the unretarded fraction. A mixture of dimethyl sulfoxide and iodomethane was passed through the column to convert the sialic acid-containing oligosaccharides into methyl esters that were eluted and converted to methyl amides by methyl amine. Finally the sulfated oligosaccharide fraction was eluted with salt. The neutral and the derivatized sialic acid-containing oligosaccharides were analysed by gas chromatography-mass spectrometry after permethylation and the sulfated oligosaccharide fraction was analysed by high performance anion exchange chromatography.

Keywords: Gas chromatography-mass spectrometry, oligosaccharide, mucin, high performance anion exchange chromatography, sialic acid, sulfate

Abbreviations: GC, gas chromatography, GC/MS. gas chromatography-mass spectrometry; HPAEC-PAD, high performance anion exchange chromatography-pulsed amperometric detection; Hex, hexose; HexNAc, *N*-acetyl hexosamine; HexNAcol, *N*-acetyl hexosaminitol; Fuc, Fucose; NeuAc, *N*-acetyl neuraminic acid; NeuGc, *N*-glycolyl neuraminic acid

Introduction

Mucins are large glycoproteins which cover mucosal surfaces and usually have a carbohydrate content of more than 80% by mass [1, 2] . Their oligosaccharides are very heterogeneous in structure and size, and are O-linked to the peptide backbone primarily via *N*-acetyl-galactosamine to serine or threonine residues. Methods to investigate the structures of the oligosaccharides usually rely on their release by alkaline borohydride, followed by chromatographic techniques for separation into individual components. Further structural analysis can then be performed by use of mass spectrometry and proton-NMR spectroscopy [3–5]. Our group has successfully used the method of high-temperature gas chromatography-mass spectrometry (GC/MS) for analysing neutral and sialic acid-containing oligosaccharides from mucin glycopeptides after permethylation [6–10]. The permethylated *N*,*N*-dimethyl amide derivatives of the sialic acid-containing oligosaccharides were then created by ammonolysis and permethylation of the internal esters (lactones), which were formed from the sialic acid-containing oligosaccharides by peracetylation [9]. Formation of the uncharged lactones was used for the separation of sialic acid-containing and sulfated oligosaccharides by anion exchange chromatography; the sulfated compounds were retarded. The main drawback of this method is that the formation of the lactone is structurally dependent [11, 12] and that the acidic oligosaccharides are obtained as their unstable peracetylated species.

The method for modification of ganglioside sialic acid residues to methyl esters by dimethyl sulfoxide and iodomethane [13] and concomitant reaction with methyl amine [14] has now been adapted to create the *N*-methyl amide of the sialic acid-containing oligosaccharides. These were then converted to the permethylated *N*,*N*-dimethyl amide derivatives. The esterification was carried out on-column on an anion exchange column and the methyl ester derivatives were directly eluted. This method enabled a quick separation of oligosaccharides into neutral, sialic acid-containing and sulfated species by applying a mixture on the column, and esterifying the sialic acid-residues after elution of the neutral oligosaccharides.

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Materials and methods

Materials Sialyl-lactose, glucose-6-sulfate, glucosamine-6-sulfate, glucosamine-2-sulfate and glucosamine-3-sulfate were purchased from Sigma (St Loius, MO), iodomethane and methyl amine were from Fluka (Buchs, Switzerland), solid sodium hydroxide was from EKA (Bohus, Sweden), 50% sodium hydroxide solution from J. T. Baker Chemicals B. V. (Deventer, Netherlands), water used for permethylation was double glass distilled water kept over chloroform. For other purposes water was from a Millipore system. All other chemicals were from Merck (Darmstadt, FRG). DEAE-Sephadex A-25 was from Pharmacia (Uppsala, Sweden) and AG 50W-X8 was from Bio-Rad (Richmond, VA). Mucin glycopeptides of porcine small intestine were purified and oligosaccharides released as described before [7, 9].

Reduction and N*-acetylation of sulfated monosaccharides* Sulfated monosaccharides (100–700 μg) were reduced overnight by 200 μl of 1 M $NaBH_4$. The reaction was stopped by the addition of 1 drop of glacial acetic acid and samples were lyophilized. Methanol and a few drop of glacial acetic acid were added. The solvent was evaporated in a vacuum centrifuge (Heto, Alleød, Denmark). This procedure was repeated four times before applying the sample to a column of AG 50W-X8 resin (1 ml) and the sugars were eluted with water (5 ml). The samples were recovered by lyophilization and analysed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (conditions described below). The decrease in retention times compared to the unreduced sugars was taken as proof for the creation of the sulfated monosaccharide alditols. No unreduced sugars were observed in the chromatograms.

One ml of 50 μg ml^{-1} of glucosaminitol-3-sulfate and 50 μg ml^{-1} glucosaminitol-6-sulfate were *N*-acetylated by adding 1 ml saturated sodium bicarbonate and 20 μl of acetic acid anhydride every 5 min for 25 min with stirring. Salts were removed by applying the reaction mixtures to a column of AG 50W-X8 resin (10 ml) and the sugars were eluted with 50 ml of water. The samples were concentrated by vacuum rotary evaporation at 40°C and lyophilized. The completeness of the reactions was confirmed by analysis with HPAEC-PAD (conditions described below).

Separation of neutral, sialic acid-containing, and sulfated O-linked reduced oligosaccharides released from mucin glycopeptide of porcine small intestine Reduced oligosaccharides released from mucin glycopeptides of porcine small intestine (7.5 mg) with alkaline borohydride treatment were separated on 4 ml DEAE-Sephadex A-25 in its acetate form, which had been pre-treated with 20 ml DMSO/MeI 10:1, 40 ml 1.0 M pyridinium acetate, pH 5.4, and 40 ml methanol. The neutral oligosaccharides were eluted with 40 ml dry methanol. The sialic acid-containing oligosaccharides were converted to their methyl esters by the addition of 0.7 ml of DMSO/MeI 5:1 which was allowed to sink into the anion exchange gel. The flow was turned off and the column was incubated for 5 min at room temperature. The procedure was repeated two times, The methyl esters were eluted with 40 ml dry methanol, and the sulfated oligosaccharides were then eluted with 40 ml 1.0 M pyridinium acetate, pH 5.4. Methanol was evaporated from the neutral oligosaccharide fraction by a stream of nitrogen gas at 45°C. Methanol and iodomethane were similarly evaporated from the sialic acid methyl ester-containing fraction and the remaining DMSO was removed by lyophilization.

Conversion of sialic acid methyl esters to methyl amides The oligosaccharide fraction containing sialic acid methyl esters was dissolved in 0.75 ml dry methanol, 300 μl of 25% methyl amine in methanol were added, the reaction mixture was stirred for 10 min at room temperature, and the reaction was stopped by placing the sample in a freezer at –20°C. Solvent was removed by vacuum centrifugation.

Permethylation Permethylation was carried out on the reduced neutral and *N*-methyl amide derivatives of sialic acid-containing oligosaccharides as described [15, 16] with minor modifications. Oligosaccharides were dried with a stream of nitrogen and further dried in a vacuum for 30 min; 0.5 ml DMSO, 0.1 ml iodomethane and approximately 25 mg of powdered NaOH were added and the reaction mixture was stirred with a magnetic bar for 10 min. The reaction was quenched by the addition 2 ml 0.1 M HCl followed by 1 ml chloroform. The water phase was removed after centrifugation, and the chloroform phase was washed five times with 2 ml of water before moving to new tubes. The fifth water phase was washed with 1 ml of chloroform. The chloroform phases were pooled and the solvent removed by a stream of nitrogen. The permethylated oligosaccharides were dissolved in ethyl acetate for analysis by GC and GC/MS.

GC and GC/MS of permethylated neutral and N, N*-dimethyl sialic acid-containing oligosaccharides* High temperature columns for GC and GC/MS were prepared as described [6–10]. Fused silica capillaries (10 m × 0.25 mm i.d. HT-polyimide coated, Chrompack, Middelburg, The Netherlands) were D4-deactivated and statically coated with 0.03 μm of PS 264 (Fluka, Buchs, Switzerland), and crosslinked at 145°C for 30 min with dicumyl peroxide as the initiating agent. The columns were extracted with 5 ml dichloromethane, before conditioning at 390°C for 4 h with hydrogen as the carrier gas. Capillary GC was performed on a Hewlett-Packard 5890A-II gas chromatograph with hydrogen as carrier gas (linear velocity of 123 cm s^{-1} at 80°C) including an oxygen trap (Oxypurge, Alltech) in the carrier gas line. The flame ionization detector was kept at 385°C. For GC/MS helium was used as carrier gas (linear velocity 65 cm s^{-1} at 80°C) and the Hewlett-Packard 5890A-II gas chromatograph was used in a constants flow mode. Samples were injected on to the column (1 μl, 1–100 ng per component) at 80°C (1 min). The temperature was programmed from 80°C to 200°C in increments of 50°C per min and then by 10°C per min to 390°C. The gas

chromatograph was coupled to a JEOL SX-102A mass spectrometer. The condition for the mass spectrometer were interface temperature, 385°C; ion source temperature, 370°C; electron energy, 70 eV; trap current, 300 μA; acceleration voltage, 10 kV; mass range scanned, *m/z* 100–1600; total cycle time, 1.4 s; resolution, 1000; pressure in the ion source region, 3×10^{-4} Pa.

Preparation of sulfated oligosaccharides The fraction containing sulfated oligosaccharides was desalted by passing over 40 ml of AG 50W-X8 and eluted with 200 ml of water. The sample was concentrated with vacuum rotary evaporation at 40°C and passed through a Sep-Pak C_{18} cartridge pretreated with 5 ml 1:1 chloroform: methanol, 5 ml methanol and 10 ml water. The sulfated oligosaccharides were eluted with 5 ml water, recovered by lyophilization, dissolved in water and analysed by HPAEC-PAD (conditions described below).

High performance anion exchange chromatography of sulfated saccharides HPAEC-PAD analysis was carried out using an LKB 2150 HPLC pump (Pharmacia) equipped with an LKB 2152 HPLC controller and low pressure mixture for running gradients. The column was a CarboPac PA1 (4 × 250 mm) (Dionex Corp., Sunnyvale, CA with a guard column CarboPac PA1 (4 × 50 mm). Detection was carried out on a pulsed amperometric detector (PAD-II; Dionex). The signal was recorded and integrated by a Hewlett-Packard 3396-II integrator. Sulfated monosaccharide alditols, used for optimizing conditions described in this paper, were run isocratically with 0.15 M NaOH/0.35 M NaAc, with a flow rate of 1.0 ml min^{-1}. Sulfated oligosaccharides released from mucin glycopeptides of porcine small intestine were run isocratically for 3 min with 25 mM NaOH followed by a linear gradient of NaAc from 0 to 0.20 M over 22 min. Finally, the NaAc concentration was increased to 0.90 M over 5 min. The final solvent proportion was maintained for 10 min. The NaOH concentration was kept constant during the run and the flow rate was 1.0 ml min^{-1}. All solvents were degassed before use and continuously bubbled with helium gas. Standard potentials on the pulsed amperometric detector were E_1 = 0.05 V (300 m), E_2 = 0.60 V (120 ms) and E_3 = –0.80 V (60 ms). The potentials were measured against an Ag/AgC reference electrode.

Results

The approach for analysing O-linked oligosaccharides alditols The method devised for analysing O-linked oligosaccharide alditols released from mucins relies on the separation of the oligosaccharide mixture into three fractions containing the neutral, sialic acid-containing and sulfated oligosaccharides. Each fraction is then pre-treated for further analysis (Fig. 1). Test substances have been used to optimize the method.

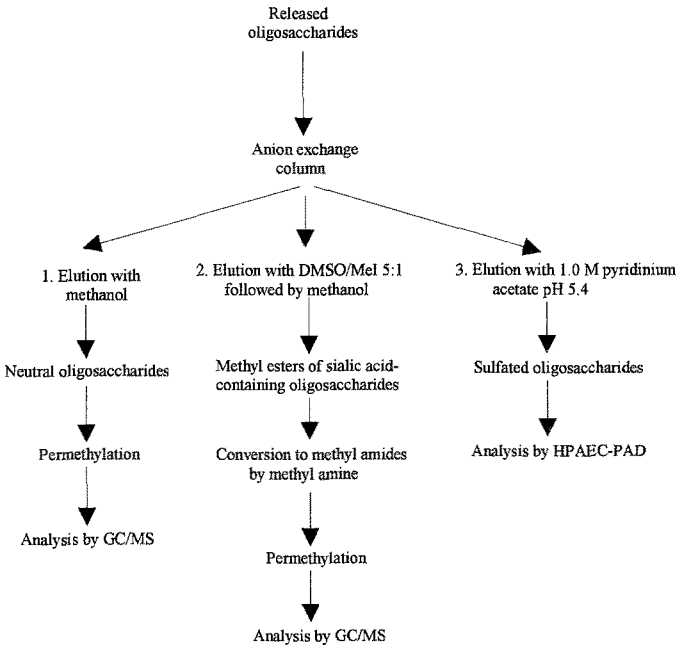

Figure 1. The approach for analysing O-linked oligosaccharide mixtures released from mucins by alkaline borohydride. The neutral oligosaccharides were eluted with methanol. The sialic acid containing oligosaccharides were converted to their methyl esters by addition of DMSO/MeI to the column and eluted with methanol. Finally, the sulfated oligosaccharides were eluted with pyridinium acetate. GC/MS was used to analyse the permethylated neutral oligosaccharides and the *N,N*-dimethyl amide derivative of the sialylated oligosaccharides. Sulfated oligosaccharides were analysed by HPAEC-PAD.

Conversion of sialic acid residues to methyl esters The method for converting sialic acid residues into methyl esters by reaction on a DEAE-Sephadex column, was applied to a mixture of 3′- and 6′-sialyl-lactose. The yield for the mixture was obtained by comparing the gas chromatograms after permethylation with nonesterified permethylated sialyl-lactose (Table 1). The total yield obtained for the mixture was approximately 90%. As the 3′- and 6′-sialyl-lactose peaks were not completely resolved a separate calculation of the yields was not possible. When the DEAE-Sephadex column was eluted with pyridinium acetate and the fraction permethylated, no intact sialyl-lactose was detected (result not shown).

Conversion of sialic acid methyl esters to methyl amides The reaction for converting sialic acid methyl ester residues into *N*-methyl amides using anhydrous methyl amine in methanol, was tested with the methyl esters of 3′- and 6′-sialyl-lactose, obtained by the on-column derivatization described above. The samples were permethylated and analysed by GC. The shift in retention times of the permethylated methyl ester and *N,N*-dimethyl amide enabled the estimation of the reaction yield by comparing the unreacted methyl esters with nonesterified permethylated sialyl-lactose (Table 1). The yield for

Table 1. Yields of sialyl-lactose on DEAE-Sephadex columns in fractions eluted with DMSO/MeI 5:1 and MeOH.

Compound	*Amount applied* (μg)	*Yields* (%)
Sialyl-lactose, methyl ester derivative, permethylated[a]	20	90
Sialyl-lactose, methyl amide derivative, permethylated[a,b]	20	86

[a]The yields were estimated from the mean of four experiments with maltotriose as internal standard (10 μg) added to the fractions after elution from the columns. The samples were permethylated and analysed by GC by injecting 1 μl (100 ng) on-column. Maltotriose and sialyl-lactose chromatograph as doublets, and the ratio of sialyl-lactose/maltotriose was calculated by summation of the area under each doublet. The ratios were compared with those from permethylated mixtures of the compounds without derivatization on the column.

[b]The methyl amide derivative was prepared as described in the text. The permethylated methyl amide was eluted approximately 1 min after the methyl ester derivative, and the proportion of unreacted methyl ester calculated as described in a.

[c]This figure includes both the on-column methyl derivatization and the conversion to the methyl amide derivative.

the mixture of about 86% includes both the on-column esterification and the conversion to the *N*-methyl amide derivative. Compared to the 90% yield for the esterification only, the yield for the N-methyl amide derivatization should be about 96%. The yield did not increase when the reaction time with methyl amine was extended for up to 1 h.

Elution of sulfated monosaccharide alditols Sulfated monosaccharide alditols were used to test the possible degradation of sulfated oligosaccharide alditols during the on-column methyl esterification. A mixture of the test substances was applied to a DEAE-Sephadex column and subjected to the esterification procedure described for the sialic acid-containing oligosaccharides. The fraction eluted with pyridinium acetate was analysed with HPAEC-PAD (Table 2) which showed a recovery of at least 89% of the sulfated monosaccharides alditols. When applying the method to unreduced and de-*N*-acetylated sulfated monosaccharides the yields were lower (results not shown).

Separation of oligosaccharides released from mucin glycopeptides into three fractions The oligosaccharides were released from glycopeptides derived from mucins of porcine small intestine using the alkaline borohydride treatment. The obtained oligosaccharides were desalted and applied to a DEAE-ion exchange column. The neutral oligosaccharides were directly eluted and the oligosaccharides bound to the column were treated with iodomethane in dimethyl sulfoxide to convert carboxylic groups into their methyl esters. This converted the sialic acid species into neutral ones, no longer retarded on the column. The oligosaccharides that were sulfated, with or without sialic acid, were eluted with salt. The three fractions obtained were analysed.

Table 2. Yields of different sulfated monosaccharides of DEAE-Sephadex columns in fractions eluted with 1.0 M pyridinium acetate pH 5.4.

Compound[s]	*Amount applied* (μg)	*Yields* (%)
Glucosaminitol-2-sulfate	0.5	89%
N-acetyl glucosaminitol-6-sulfate	0.5	103%
N-acetyl glucosaminitol-3-sulfate	0.5	98%
Glucitol-6-sulfate	10	95%

[a]The yields were estimated by adding 0.5 μg glucosaminitol-3-sulfate to the sample after elution with pyridinium acetate as internal standard, and by injecting a quarter of each sample on to HPAEC-PAD as described in the text. Yields were calculated by comparing the peak heights in the chromatograms of the same compounds treated and not treated by the methyl esterification procedure. Figures are from the mean of three experiment.

Analysis of the neutral reduced oligosaccharides fraction by GC/MS The neutral oligosaccharide fraction was permethylated and analysed by GC/MS. The oligosaccharide structures deduced from their mass spectra are indicated in the total ion chromatogram (Fig. 2). The spectra of permethylated neutral oligosaccharides are dominated by sequence oxonium ions, and α-cleavages between C-4 and C-5 of the permethylated *N*-acetyl galactosaminitol residues. The 21 different structures found here were described previously from this mucin glycopeptide sample [7]. The permethylated *N*-acetyl-galactosaminitol was eluted early and was not recorded.

Analysis of the reduced oligosaccharide fraction eluted with MeI-DMSO The oligosaccharides that became neutral upon methyl esterification were treated with methyl amine and permethylated. The obtained permethylated *N,N*-dimethyl amide derivatives of the sialylated oligosaccharides were analysed by GC/MS. The structures interpreted from the mass spectra of individual peaks are indicated in the total ion chromatogram (Fig. 3) and were identical to 28 sialylated oligosaccharides previously identified from the same source [9]. A mass spectrum (Fig. 4) indicated by * in Fig. 3 from scan 794–798 revealed a pentasaccharide and exemplifies the common pattern of fragmentation [9]. The dimethyl amides are characterized by intense fragment ions $[M\text{-}72]^+$, due to α-cleavage of the amide side group of the sialic acid [9, 12]. Other features in the mass spectra were oxonium fragment ions and fragment ions from α-cleavages of the permethylated *N*-acetyl galactosaminitol residues.

Analysis of the sulfated oligosaccharide fraction The third fraction eluted with salt analysed with high performance anion exchange liquid chromatography coupled to a pulsed ampero-

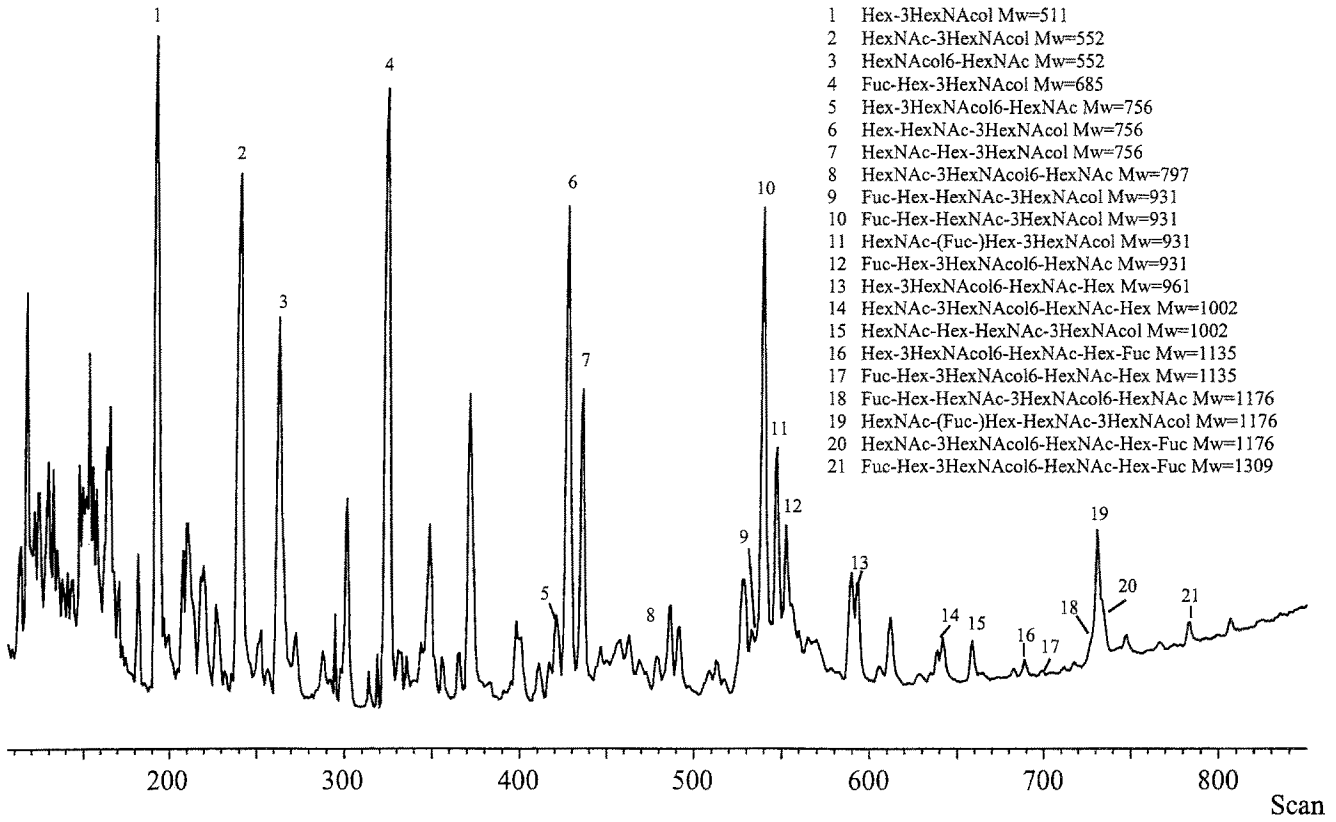

Figure 2. Total ion chromatogram with interpreted structures from GC/MS of permethylated neutral oligosaccharide alditols released from mucin glycopeptides obtained from porcine small intestine. See Materials and methods for GC/MS conditions.

metric detector, a proven technique for analysing acidic O-linked oligosaccharides [17]. A modified gradient was devised for the efficient separation of the sulfated oligosaccharides alditols, with or without sialic acids, obtained from intestinal mucins. The chromatogram of the sulfated fraction from oligosaccharides released from mucin glycopeptides of procine small intestine is shown in Fig. 5.

Discussion

The analysis of oligosaccharides by an informative mapping approach has been devised. This is based on a simple method for the direct separation of released oligosaccharides on-column into neutral, sialic acid-containing, and sulfated to allow for different analytical techniques for each oligosaccharide type. These fractions can then be analysed with chromatography-mass spectrometry techniques to obtain as much information as possible from each fraction. This approach will give a quick and sensitive mapping of the oligosaccharides found in different types of glycoconjugates, in our case mucins. The technique has already allowed us to find glycosylation alterations of the intestine upon parasite infection.

The formation of the methyl ester of sialic acid residues by dimethyl sulfoxide and iodomethane is structurally independent and allows the removal of the sialic acid containing oligosaccharides from the sulfated ones. The earlier method for creating the permethylated *N,N*-dimethyl amide derivative of sialic acid containing oligosaccharides was based on the ability of sialic acid residues to form lactones upon peracetylation, followed by ammonolysis before permethylation [9]. The method presented here created the same sialic acid derivative by a simpler procedure. This reaction is done directly during column chromatography of a total oligosaccharide fraction and decreases the number of separation steps to a minimum. The methyl esters are usually relatively unstable and the release of the carboxyl group-containing oligosaccharides with the reactants circumvents this problem.

The formation of the methyl amide derivatives of the sialic acid residues is essential for the permethylation, due to poor yields when attempting to permethylate the methyl esters of oligosaccharides containing a reduced GalNAc. However, the methyl esters of sialic acid-containing oligosaccharides having non-reduced Glc at the reducing end are stable upon permethylation [9]. The use of the permethylated *N,N*-dimethyl amide derivatives of sialic acid-containing oligosaccharides has another advantage as they give an intense fragment ion $[M-72]^+$ upon electron impact ionization, making them easier to interpret as the molecular masses are readily calculated [9,12].

The neutral oligosaccharides can be analysed by the high-temperature GC and GC/MS technique for up to about

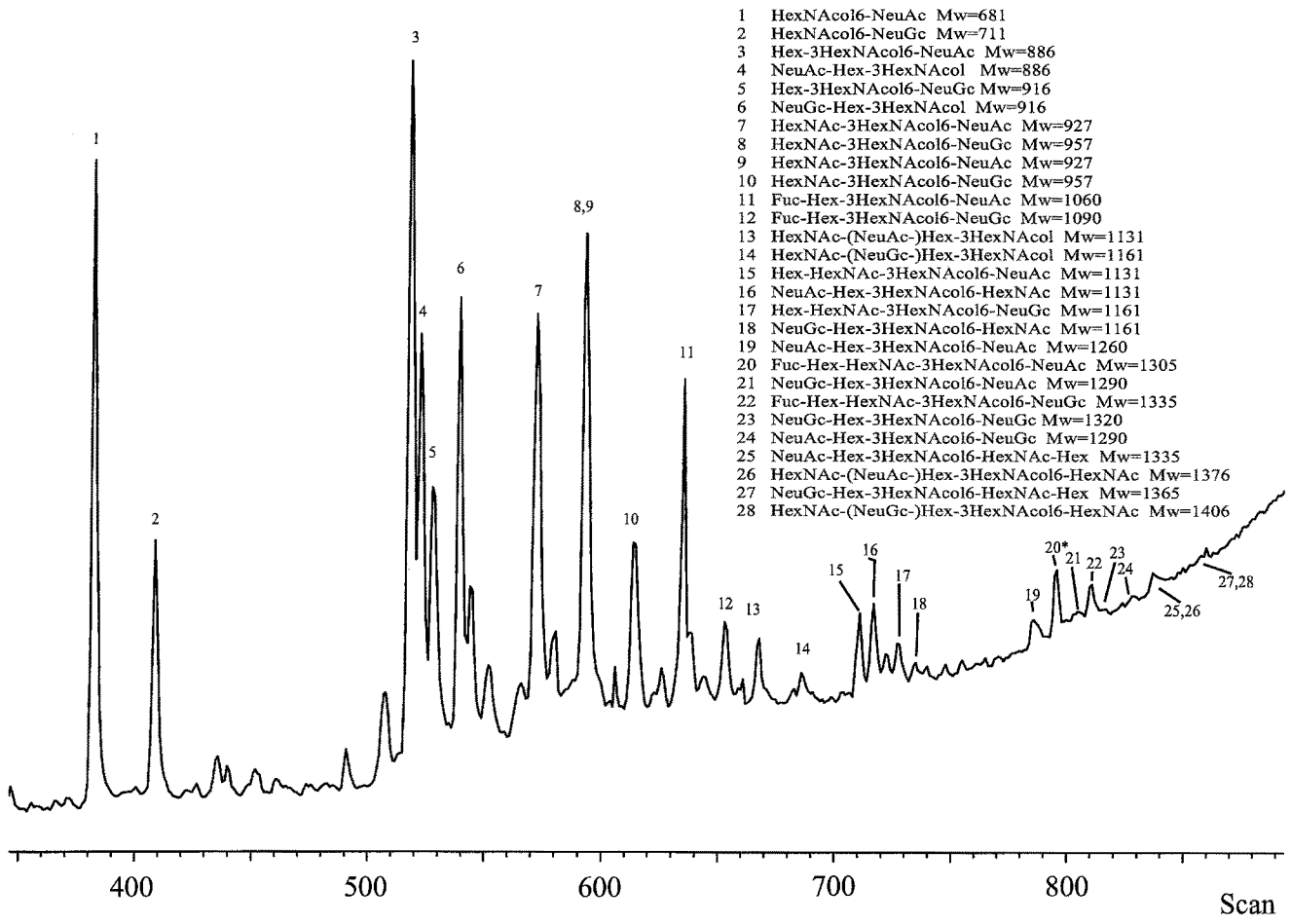

Figure 3. Total ion chromatogram and interpreted structures of permethylated sialic acid containing oligosaccharides as their dimethyl amides obtained from mucin glycopeptides in porcine small intestine. See Materials and methods for GC/MS conditions.

decasaccharides [7, 8]. The total ion chromatogram from the GC/MS reproduces the gas chromatogram very well without discrimination of higher molecular mass oligosaccharides or decreased resolution. The sialic acid-containing fraction can be analysed by the same technique after modification and permethylation. For some types of mucins the oligosaccharides are too large and well beyond the range of high-temperature GC, as is the case for bile mucins where the mean length of the oligosaccharides is in the range of 40–50 sugar residues [18].

The sulfated oligosaccharide fraction was further analysed by HPAEC-PAD. The high resolution with high performance anion exchange chromatography and the sensitive detection with a pulsed amperometric detector makes this ideal for analysing these components. Other sulfated oligosaccharide fractions prepared by the presented method have been analysed and show different patterns. Further analyses of the sulfated oligosaccharides from porcine small intestine glycopeptides have been done using FAB-MS and MS/MS. A series of sulfated structures with the sulfate group at the 6-position of GlcNAc residues were assigned (unpublished results). No unreacted sialylated structures lacking sulfate were detected in the mass spectra.

The approach of separating oligosaccharide fractions into three groups should also be of importance for other types of oligosaccharides, for example, free oligosaccharides present in milk or N-linked glycans released by enzymes or by hydrazinolysis. The N-linked oligosaccharides on glycoprotein hormones can occur as sialylated and sulfated species [19]. The separation of oligosaccharides into the three groups should also be useful in very sensitive cell biology experiments in which the components can be radioactively labelled, either by metabolic incorporation of radioactivity or by reducing the saccharides with $NaBT_4$.

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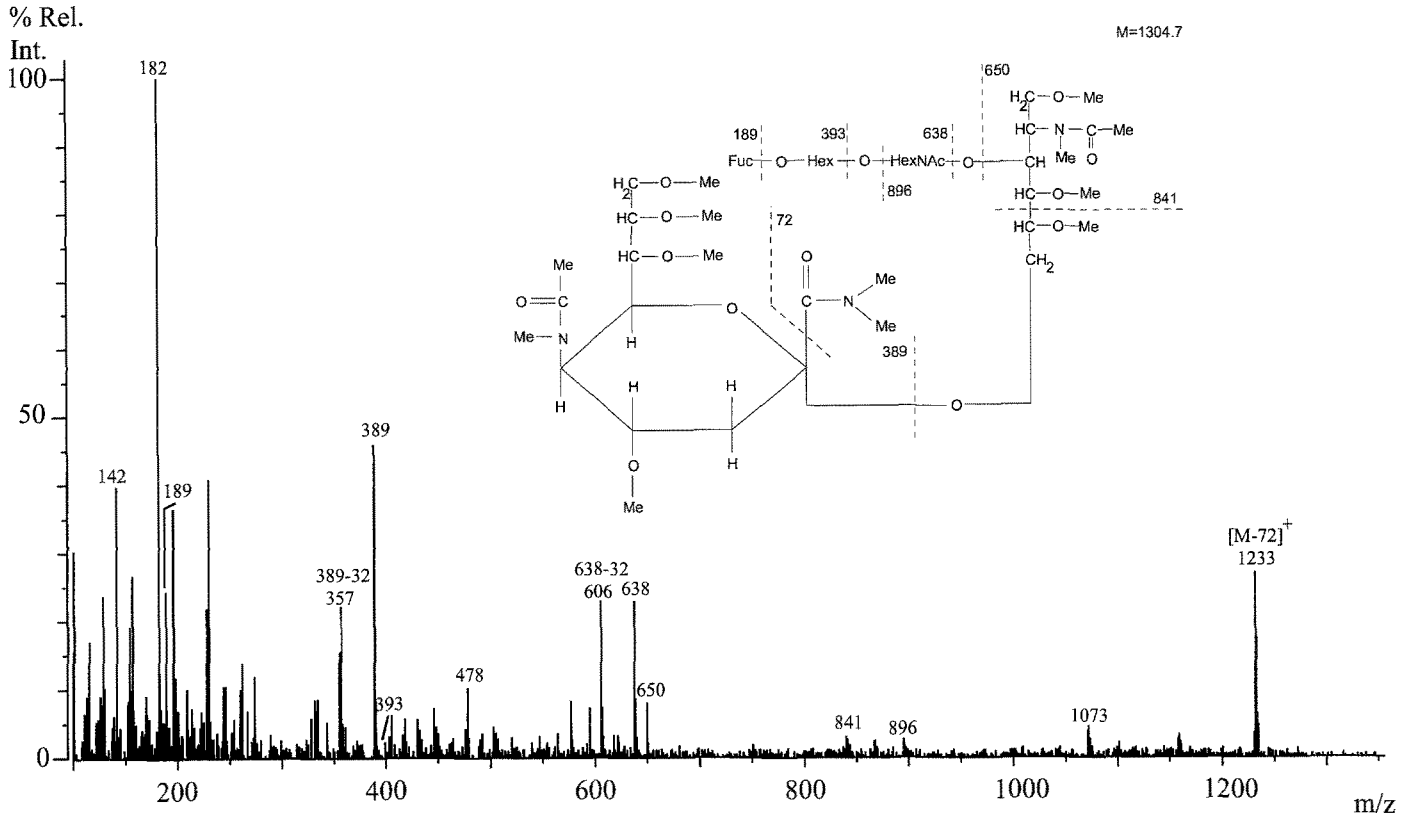

Figure 4. Mass spectrum and interpretation formula of the component in scan 794–98 (*- marked) from the GC/MS analysis showed in Fig. 3

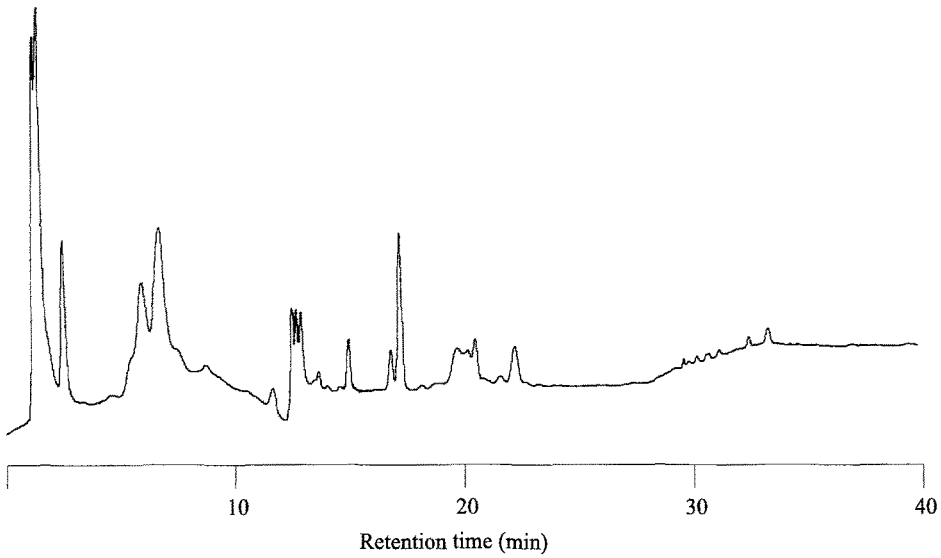

Figure 5. HPAEC-PAD from sulfated oligosaccharides alditols released from porcine small intestine. See Materials and methods for chromatographic conditions.

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References

1. Gum JR (1992) *Am J Respir Cell Mol Biol* **7**:557–64.
2. Strous GJ, Dekker J (1992) *Crit Rev Biochem Mol Biol* **27**:57–92.
3. Sangadala S, Bhat UR, Mendicino J (1992) *Mol Cell Biochem* **118**:75–90.

4. Klein A, Carnoy C, Lamblin G, Roussel P, vanKuik JA, Vliegenthart, JFG (1993) *Eur J Biochem* **211**:491–500.
5. Capon C, Laboisse CL, Wieruszeski JM, Maoret JJ, Augeron C, Fournet B, (1992) *J Biochem* **267**:19248–57.
6. Karlsson H, Carlstedt I, Hansson GC (1987) *FEBS Lett* **226**:23–27.
7. Karlsson H, Carlstedt I, Hansson GC (1989) *Anal Biochem* **182**:438–46.
8. Hansson GC, Karlsson H (1990) *Methods Enzymol* **193**:733–38.
9. Hansson GC, Bouhours JF, Karlsson H, Carlstedt I (1991) *Carbohdr Res* **221**:179–89.
10. Hansson GC, Karlsson H (1993) In *Methods Molecular Biology*, Vol. 14 (Hounsell EF, ed.) pp. 47–54. Totowa:Humana Press.
11. Levery SB, Salyan MEK, Roberts CE, Bouchon B, Hakomori S (1990) *Biomed Environ Mass Spectrom* **19**:303–10.
12. Levery SB, Roberts CE, Salyan MEK, Bouchon B, Hakomori S (1990) *Biomed Environ Mass Spectrom* **19**:311–18.
13. Handa S, Nakamura K (1984) *J Biochem* **95**:1323–29.
14. Nakamura K, Handa S (1986) *J Biochem* **99**:219–26.
15. Ciucanu I, Kerek F (1984) *Carbohydr Res* **131**:209–17.
16. Larson G, Karlsson H, Hansson GC, Pimlott W (1987) *Carbohydr Res* **161**:281–90.
17. Hayase T, Sheykhanazari M, Bhavanandan VP, Savage AV, Chuan Y (1993) *Anal Biochem* **211**:72–80.
18. Baeckström D, Karlsson N, Hansson GC (1994) *J Biol Chem* **269**:14430–37.
19. Green ED, Baenziger JU (1988) *J Biol Chem* **263**:25–35.