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Enhanced Detection of Sialylated and Sulfated Glycans with Negative Ion Mode Nanoliquid Chromatography/Mass Spectrometry at High pH

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Negative ion mode nanoliquid chromatography/mass spectrometry (nano-LC/MS) on porous graphitic carbon columns at pH 11 was studied and compared to capillary LC/MS at pH 8 for the analysis of neutral and acidic glycan alditols. Oligosaccharides were chromatographed with an acetonitrile gradient containing 0.04% ammonium hydroxide and analyzed with a linear ion trap mass spectrometer (LTQ) equipped with a modified nanospray interface. Analysis of acidic *N*- and *O*-glycan standards revealed that good quality MS/MS spectra could be obtained when loading 1−3 fmol, a 10-fold increase in sensitivity compared to capillary-LC/MS at pH 8. Analysis of a complex mixture of *O*-glycans from porcine colonic mucins with nano-LC/MS and MS/MS at high pH revealed 170 oligosaccharides in one analysis, predominantly corresponding to sulfated glycans with up to 11 residues. Analysis of the same sample with capillary-LC/MS showed a lower sensitivity for multiply sulfated glycans. Nano-LC/MS of *O*-linked oligosaccharides on MUC2 from a human colon biopsy also illustrated that the ionization of oligosaccharides with multiple sialic acid groups was increased compared to those with only one sialic acid residue. Nano-LC/MS at high pH is, thus, a highly sensitive approach for the analysis of acidic oligosaccharides.

Mucins constitute a subgroup of large glycoproteins and are characterized by dense *O*-glycosylation on serine and threonine in repeated amino acid sequences called PTS domains. Secreted mucins are major protein components in the mucosal secretions of the body, protecting and lubricating the underlying epithelia, whereas membrane bound mucins are expressed on epithelial surfaces, involved in intracellular signaling.[1,2] The mucin *O*-glycans can constitute 50−90 wt % of the mass of the glycoprotein and can be highly diverse, with hundreds of different glycans on a single mucin. Branches protrude from the core GalNAc residues, consisting of repetitive Galβ1−3/4GlcNAc units with fucose, sialic acid, and sulfate with different linkages and often terminated with blood group epitopes of the ABO and Lewis system. Due to the large heterogeneity and technical difficulties to analyze these molecules, the full repertoire and upper size limits of mucin oligosaccharides are not known. Mucins are overexpressed in many adenocarcinomas, and documented glycosylation changes accompany the shift to the cancer associated form of the protein, such as for the breast cancer associated MUC1.[2] Altered mucin *O*-glycosylation has also been associated with infection and inflammation in intestine and lungs.[3−5] Consequently, methodology for analyzing mucins and mucin glycosylation, and performing comparative studies between the health and disease state are, therefore, of medical importance.

In our aim to simultaneously characterize more complex mixtures of *O*-glycans with varying number of acidic residues from mucins and other *O*-glycosylated proteins and also from limited sources, we have turned to negative ion mode capillary-LC/MS with porous graphitized (PGC) carbon columns, which has been used for the analysis of glycans since the beginning of the 1990s.[6] The chromatography step is essential in order to separate glycan isoforms with the same mass, before these are fragmented with CID. The occurrence of isoforms with the same mass in many mucin-derived glycan samples also makes the use of sensitive approaches such as, for example, MALDI-MS of permethylated sulfated glycans[7] less attractive. Several groups have demonstrated the use of nano-LC/MS for *N*- and *O*-glycans using reversed and normal phase HPLC and in conjunction with both positive and negative ion mode mass spectrometry with detection limits around 10−20 fmol[8−10] and down to 1−7 fmol.[11] In our nanospray system, we have used an interface constructed in-house, which

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is a simplified version of the one described by Shen and collegues.[12] Our approach has been used for the analyses of samples of different biological origin.[13−15] We have used ammonium bicarbonate at pH 8 as mobile phase for capillary-LC/MS,[16] but using this for nano-LC/MS was unsuccessful due to the fact that the emitter tip is situated close to the heated capillary inlet and buffer salt precipitation on the tip inhibited the formation of a Taylor cone. In order to achieve a strong basic environment for ESI, we then explored nano-LC/MS at high pH using 0.04% ammonium hydroxide as additive and found it to be compatible with the nanospray conditions. Here, we have compared capillary-LC/MS at pH 8 with nano-LC/MS at pH 11 for the analysis of *O*- and *N*-linked oligosaccharide standards as well as complex mixtures of *O*-glycans from porcine and human colonic mucins. This shows the advantage of negative ion mode nano-LC/MS at high pH for the characterization of large acidic glycans.

EXPERIMENTAL SECTION

Materials and Methods. Deionized water (18.2 MΩ) was obtained from a Milli-Q water system (Millipore). HPLC grade acetonitrile and analytical grade acetic acid, methanol, and ammonium hydroxide (25% solution) were from Merck. All chemicals, unless otherwise stated, were purchased from Sigma. *O*-glycan standards T-antigen (Galβ1-3GalNAcol, Mr = 385), sialyl-T antigen (NeuAcα2-3Galβ1-3GalNAcol, Mr = 676), and disialyl-T antigen (NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAcol, Mr = 967) were purified as described.[17] Su-Le^a (Sulfo Lewis a, SO_3^--3Galβ1-3(Fucα1-4)GlcNAc, Mr = 611), A1 (monosialo(2,6) biantennary *N*-glycan, Mr = 1933), and A2 (disialo(2,6) biantennary *N*-glycan, Mr = 2224) were purchased from Dextra Laboratories (Reading, UK). LNF1 (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-3Glcol Mr = 855) from milk was a gift from David Zoft. Oligosaccharide standards were reduced to their alditol form with 0.5 M $NaBH_4$ in 50 mM KOH, 50 °C, 2 h, followed by desalting as described elsewhere.[17] Complete sialylation of the *N*-glycans standards was confirmed by analysis with capillary LC/MS and MS/MS.

Purification of Mucin Oligosaccharides. Mucin oligosaccharides from porcine colonic mucins were purified as described elsewhere.[18] Briefly, oligosaccharides from 0.5 mg of mucin were chemically released by β-elimination and reduced into alditols with $NaBH_4$/KOH, followed by desalting on cation exchange media, and elimination of borate by repetitive evaporation with 1% acetic acid in methanol. MUC2 derived oligosaccharides from a human biopsy (sigmoid colon) from a healthy individual were purified by extraction with 4 M guanidinium hydrochloride containing protease inhibitors, followed by reduction and alkylation of the insoluble mucin-containing fraction. The detailed protocol is published elsewhere.[15] The oligosaccharides were released as alditols as described above.

HPLC Columns. Porous graphitic carbon HPLC columns were packed in-house at 500 bar pressure in fused silica capillaries (nano-LC/MS: 25 cm × 75 μm i.d. or 30 cm × 100 μm i.d.; capillary-LC/MS: 20 cm × 180 μm i.d.; o.d. 375 μm; Polymicro Technologies, Phoenix, AZ). The columns were packed with 5 μm graphite particles (Hypercarb, Thermo Hypersil-Keystone, Bellefonte, PA) using tetrahydrofurane/isopropanol (9:1) as slurry solvent and methanol as pusher. For connection of the fused silica columns, sleeves were made of PEEK tubing, 1/16 in. o.d., 400 μm i.d. (Upchurch Scientific, Oak Harbor, WA).

Nano-LC/MS Interface. From the packed fused silica capillary column, a nanoESI-emitter tip was connected via a through-bore 1/16 in. union (ZU1T, Vici AG, Switzerland). Two steel screens with pore sizes of 1 and 0.5 μm and thicknesses of 50 and 40 μm, respectively (Vici AG International), were placed between the column and the emitter. Double steel screens are needed in order to prevent graphite particles to penetrate into the emitter. The tapered nanoESI-emitter tips (around 30 mm length) were made from 20 μm i.d. and 150 μm o.d. fused silica capillary and held in position by 20 mm PEEK tubing (150 μm i.d.) as a sleeve. This connection produces a negligible dead volume for nanocolumn flow rates. The high voltage (−1.6 kV) was connected via the bore union. The interface holder was made in-house of a nonconductive polymer (Delrin, Dupont, DE).

Nano-LC/MS and MS/MS. The HPLC pump (binary pump 1100 series, Agilent Technologies, Palo Alto, CA) delivered a flow of 200 μL/min which was split down in a 1/16 in. microvolume connector T with a 0.15 mm bore, by a 50 cm × 50 μm i.d. fused silica capillary before the injector, allowing approximately 400 nL/min through the analytical column. The sample (1 μL) was injected using an HTC-PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). A fused silica capillary (30 cm × 75 μm i.d.) was used as transfer line from the injector to the column connected via a 1/16 in. through-bore union with a steel screen (1 μm pore size). As graphite is electrically conductive, unions on both sides of the column will be at high voltage. The voltages at either end were measured to be −1.600 and −1.608 kV (probe union), respectively. The union at the column inlet was electrically isolated with silicone rubber tubing. The gradient [5−50% B; A = 0.04% NH_3 (equivalent to 21.4 mM, ionic strength of approximately 2 to 3 mM) in mpH_2O; B = 100% acetonitrile] was started 5 min after injection and eluted for 43 min with a subsequent washing step of 4 min in 80% B and equilibrated for 16 min. The mass spectrometer was a LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron, San José, CA). The heated capillary was kept at 200 °C; the capillary voltage was −50 V, and the electrospray voltage was −1.6 kV. Full-scan (m/z 380−2000, 2 microscans, maximum 100 ms, target value of 30 000) was performed, followed by data dependent MS^2 scans of the three most abundant ions in each scan (2 microscans, maximum 100 ms, target value of 10 000). Signal threshold for MS^2 was set to 500 counts. Normalized collision energy was 35%; an isolation window of 3 u, an activation q = 0.25, and an activation time of 30 ms were used. The samples were evaluated and integrated manually using the Xcalibur version 2.0,

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	O-GLYCANS	*N*-GLYCANS
NEUTRAL	**T-antigen** Galβ1-3GalNAcol	
	LNF1 Fucα1-2Galβ1–3GlcNAcβ1-3Galβ1-3Glcol	
ACIDIC	**Sialyl-T** NeuAcα2-3Galβ1–3GalNAcol	NeuAcα2-6{Galβ1-4GlcNAcβ1-2Manα1 (6/3) Galβ1-4GlcNAcβ1-2Manα1} Manβ1-4GlcNAcβ1-4GlcNAcol **A1**
	Disialyl-T NeuAcα2-3Galβ1 (3/6) NeuAcα2 GalNAcol	NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1 (6/3) NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1 Manβ1-4GlcNAcβ1-4GlcNAcol **A2**
	Su-Le^a SO_3^--3Galβ1 (3/4) Fucα1 GlcNAcol	

Figure 1. Structures of oligosaccharides analyzed with capillary and nano-LC/MS and MS/MS.

(Thermo Electron, San José, CA). No smoothing was applied. For the porcine colonic mucin oligosaccharides, one MS/MS spectrum of at least one glycoform of every oligosaccharide was required for positive identification. The MS/MS spectrum should contain characteristic fragment ions that could be assigned as saccharide fragments, even though the full sequence in many cases could not be assigned. Fragmentation annotations applied are based on the nomenclature suggested by Domon and Costello[19,20]

Capillary-LC/MS and MS/MS. The HPLC pump delivered a flow of 250 μL/min and splitted down in a Valco-T by a 50 cm × 50 μm i.d. fused silica capillary before the injector, allowing approximately 3 μL/min through the column. The oligosaccharides (2 μL) were injected onto the column and eluted with an acetonitrile gradient (A: 8 mM ammonium bicarbonate; B: 100% acetonitrile). The gradient (0−45% B) was started 3 min after injection and eluted for 43 min, followed by a wash step with 80% B and equilibration of the column for 14 min. A 30 cm × 50 μm i.d. fused silica capillary was used as transfer line to the ion source. The IonMax standard ESI source on the LTQ mass spectrometer was equipped with a stainless steel needle kept at −3.5 kV. Compressed air was used as nebulizer gas. The heated capillary was kept at 270 °C, and the capillary voltage was −50 V. Instrument scan settings and MS^2 experiment settings were the same as for nano-LC/MS.

RESULTS

Capillary-LC/MS at pH 8 of Oligosaccharide Standards. Seven *O*- and *N*-glycan alditol standards (T-antigen, LNF 1, sialyl-T, disialyl-T, Su-Le^a, A1, and A2, see Figure 1) were analyzed with carbon column capillary-LC/MS with an acetonitrile gradient containing 8 mM ammonium bicarbonate. Increasing amounts (12.5, 25, 50, 100, and 200 fmol, triple analyses) were injected on the HPLC column, and the signal response for each saccharide was plotted against the injected amount (Figure 2). Neutral and acidic glycans consisting of up to five residues (T-antigen, LNF 1, sialyl-T, disialyl-T, Su-Le^a) were detected as singly charged deprotonated molecular ions ($[M - H^+]^-$), whereas the two larger sialylated biantennary *N*-glycans A1 and A2 were detected as doubly charged ions $[M - 2H^+]^{2-}$. The standard

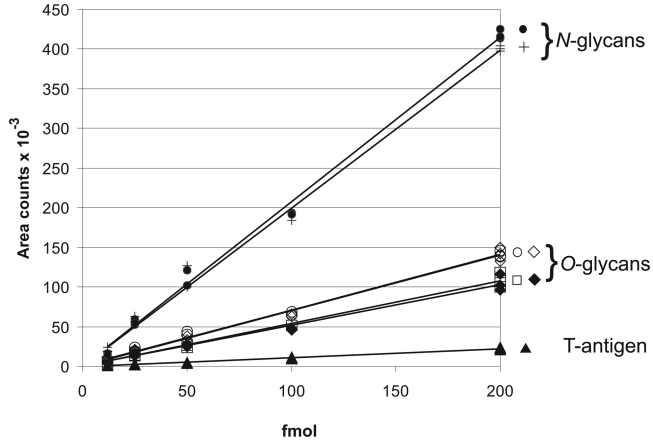

Figure 2. Capillary-LC/MS at pH 8 of standard neutral and acidic *N*- and *O*-glycans. Peak areas of the molecular ions plotted against the amount injected on the column. Each sample was analyzed three times. Symbols: ●,(A1); +, (A2); ◇, (sialyl-T antigen); ○, (disialyl-T antigen); □, (LNF1); ♦, (Su-Le^a); ▲,(T-antigen). The vertical order of individual lines is marked by symbols adjacent to the plot. R^2 values for the standard curves were between 0.98 and 0.99.

curves for the seven saccharides were linear with R^2 values between 0.98 and 0.99. The lowest amounts required to generate good quality MS/MS spectra of the different compounds was 25 fmol, except for T-antigen, where 50 fmol was required. The signal responses were different for the individual glycans, with the large acidic *N*-glycans ionized most efficiently, followed by the acidic *O*-glycans, by the neutral pentasaccharide LNF1 displaying responses in the range of 25−40% of that of the *N*-glycans, and finally by the neutral disaccharide T-antigen with approximately 5%. The relative responses for the three sugars T-antigen, sialyl-T, disialyl-T presented here are in line with the response factors that we previously have published (0.4:1:1.3, respectively).[17] It is not completely clear why the *N*-glycans were ionized much more efficiently than the *O*-glycans. The large *N*-glycans have many more hydroxyl groups which are sites for deprotonation and, therefore, are probably easier ionized in negative mode MS as compared to the smaller *O*-glycans. The presence of one or two acidic residues (sulfate or carboxyl groups) did not improve ionization significantly (compare signal response for A1 and A2 or sialyl-T and disialyl-T), which was expected as these groups are fully deprotonated at pH 8.

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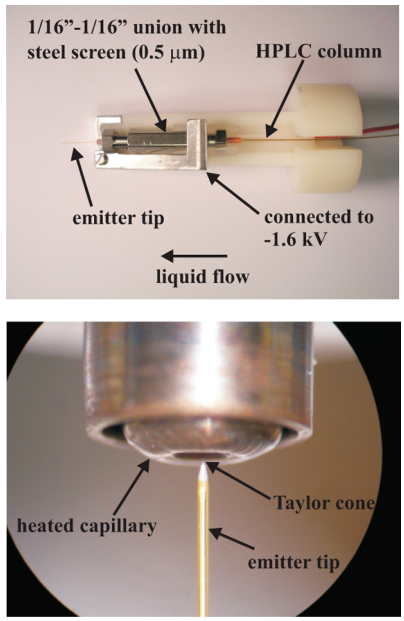

Figure 3. Nano-LC/MS interface constructed in-house, modified from the interface described by Shen et al.[12] The fused silica HPLC column is connected via a steel screen to the emitter tip in a Valco union (upper panel). The emitter tip is situated less than 1 mm from the heated capillary (lower panel).

Nano-LC/MS at pH 11 of Oligosaccharide Standards. To optimize the nanospray interface, we designed a new nanospray probe for the LTQ mass spectrometer (Figure 3), inspired by the work of Shen et al.[12] Dead volumes are kept low, and the emitter tip is positioned less than 1 mm from the heated capillary, which enables almost all of the analyte to enter the mass spectrometer compared to the commercially available interfaces. The shape of the Taylor cone is monitored by a stereo microscope mounted above the nanospray housing. This interface is currently also used on a routine basis for positive ion mode nano-LC/FTICR MS analyses for proteomics peptide analyses.[21,22]

Dilution series of oligosaccharide standards were analyzed with nano-LC/MS with an acetonitrile gradient containing 0.04% ammonium hydroxide generating an approximate pH of 11. The T-antigen standard was not analyzed, as this is sometimes not retarded on the carbon column at this pH. Su-Le^a and sialyl-T were observed as singly charged molecular ions ($[M - H^+]^-$), and disialyl-T, A1, and A2 were predominantly observed as doubly charged ions ($[M - 2H^+]^{2-}$) and with a minor amount of the singly charged form of disialyl-T. Combined full scan MS and MS/MS spectra from the analysis of disialyl-T (3 fmol) and Su-Le^a (1.5 fmol) are shown in Figure 4. Despite low amounts, there are sufficient fragment ions to confirm the monosaccharide units and partial sequence information. The MS/MS spectrum of the $[M - 2H^+]^{2-}$ precursor ion of disialyl-T (Figure 4B) revealed B-, Y-, and Z-ions, generated by the loss of NeuAc monosaccharide units, as well as a doubly charged cross ring fragment $^{0,2}X_{1\alpha}$ at m/z 372, diagnostic of NeuAc linked α2-6 to the core GalNAc residue.[23] In the MS/MS spectrum of the trisaccharide Su-Le^a (Figure 4D), two fragment ions were formed by cleavage of the two glycosidic bonds, and of which the fragment at m/z 241 ($B_{1\beta}$) was indicative of sulfate linked to the Gal residue. LNF1 was not consistently detected in all analyses, and an MS/MS spectrum was first obtained when 25 fmol was analyzed (Figure 4F). The mass chromatograms of three saccharides analyzed with nano-LC/MS and capillary-LC/MS are shown in Figure 5. The peak shapes of disialyl-T and Su-Le^a were considerably improved when analyzed with ammonium hydroxide at pH 11 (nano-LC/MS) compared to ammonium bicarbonate at pH 8, although this was not found as a general phenomena during the analysis of larger oligosaccharides.

Increasing amounts (2, 5, 10, and 20 fmol, two analyses) of the acidic glycan standards were injected on the HPLC column, and the signal response for each saccharide was plotted against the injected amount (Figure 6). The standard curves were not as linear as those obtained with capillary-LC/MS (Figure 2), and consequently, the R^2 values were lower (0.76−0.96). These results highlight that the signal response with the nano-LC/MS setup is not as stable as for capillary LC/MS. The lowest amounts required to generate MS/MS spectra of the different compounds was in the range of 1−3 fmol for the acidic glycans, which is approximately 10 times more sensitive than with capillary LC-MS/MS, and the improvements are in the same range as previously reported when negative mode capillary- and nano-LC/MS of glycans at pH 8 was compared.[11] A rough estimation of the signal response based on the standard curves indicated that sugars with two acidic residues (disialyl-T, A2) and A1 were ionized most efficiently, followed by the smaller glycans with one acidic residue (sialyl-T and Su-Le^a). Compared to capillary LC/MS at pH 8 (Figure 2), the relative ionization of disialyl T was improved, it was mainly detected as a doubly charged precursor ion, and the signal response compared to sialyl-T had nearly doubled. It appeared most likely that the increase of pH from 8 to 11 in the mobile phase supported the deprotonation of the acidic groups on disialyl-T in the ionization process and, thus, subsequently improved its ionization. It is, therefore, hypothesized that the high pH could be most favorable when analyzing oligosaccharides with multiple acidic residues. This idea was confirmed by the analysis of complex mixtures of multiply sialylated and sulfated mucin *O*-glycans from porcine colon and from human biopsies.

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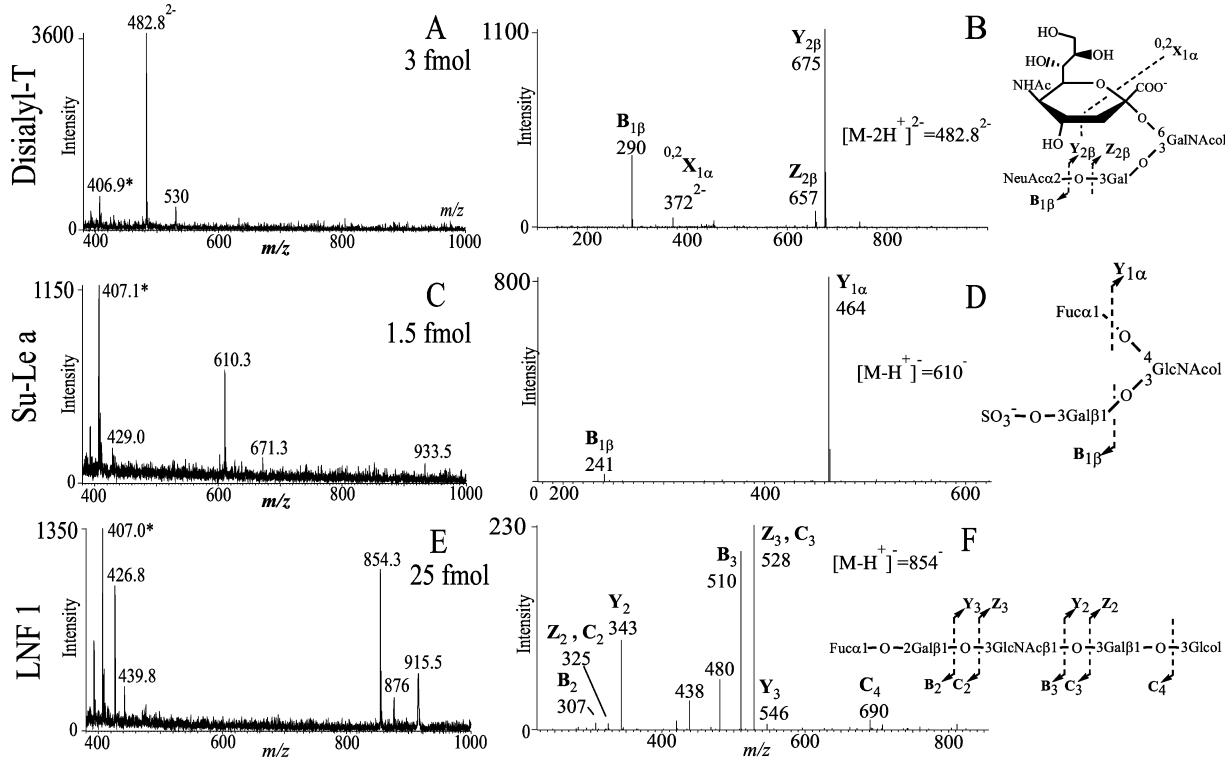

Figure 4. Nano-LC/MS and MS/MS of oligosaccharide standards. Combined full scan mass spectra of disialyl-T (3 fmol) detected as a doubly charged precursor ion ($[M - 2H^+]^{2-}$) at *m/z* 482.8 eluting at 17.3 min (A), Su-Le^a (1.5 fmol) detected as a singly charged precursor ion ($[M - H^+]^-$) at *m/z* 610.3 eluting at 16.6 min (B), and LNF1 (25 fmol) detected as a singly charged precursor ion ($[M - H^+]^-$) at *m/z* 854.3 eluting at 21.8 min (C). The corresponding MS/MS spectra of the parent ions are shown in (B), (D), and (F), respectively. *: Background ion.

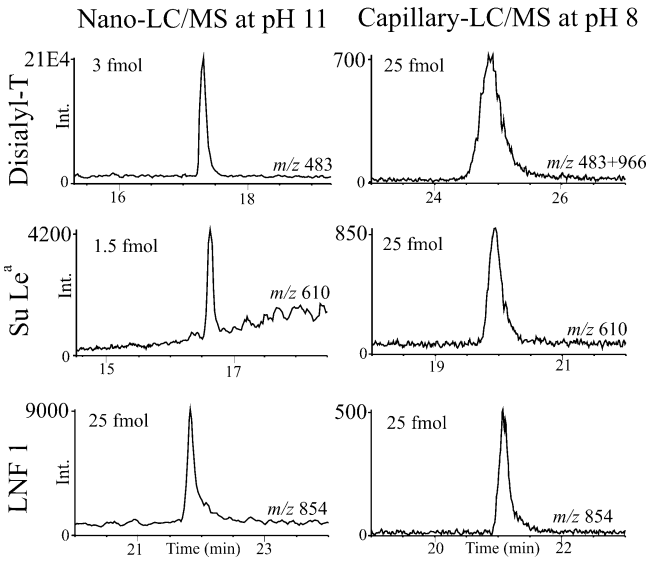

Figure 5. Mass chromatograms of standard oligosaccharides analyzed with nano-LC/MS at pH 11 and capillary-LC/MS at pH 8. Details are described in the Experimental Section.

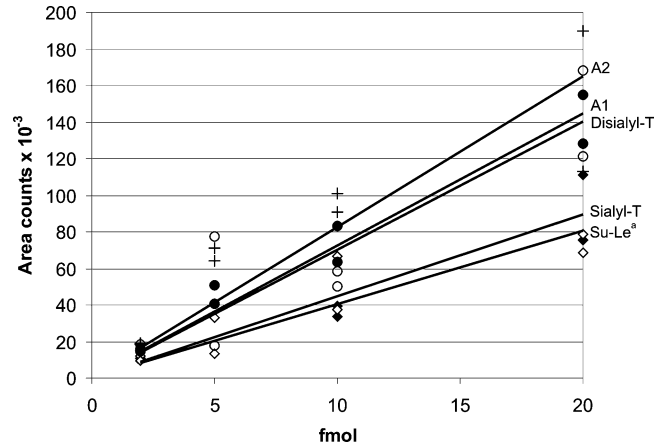

Figure 6. Nano-LC/MS at pH 11 of standard acidic *N*- and *O*-glycans. Peak areas of the molecular ions plotted against the amount injected on the column. Each sample was analyzed twice. Symbols: ◇, (sialyl-T antigen); ♦, (Su-Le ^{a}); ●, (A1); +, (A2); ○, (disialyl-T antigen). R^2 values for the standard curves were 0.76−0.96.

Nano- and Capillary-LC/MS and MS/MS of Porcine Colonic Mucin Oligosaccharides. Oligosaccharides from approximately 1.25 μg of purified porcine colonic mucin were analyzed with capillary- and nano-LC/MS in ammonium bicarbonate at pH 8 and ammonium hydroxide at pH 11, respectively. The samples were analyzed twice and at different occasions, giving reproducible results. The base peak chromatograms of the *O*-glycans analyzed with the two methods were found to be dominated by different oligosaccharides (Figure 7). The two largest peaks in the nano-LC/MS chromatogram in the upper panel corresponded to two monosulfated oligosaccharides at *m/z* 708 and 813, followed by a number of peaks corresponding to mono-, di-, and trisulfated oligosaccharides.

The base peak chromatogram obtained with capillary-LC/MS (lower panel) was on the contrary dominated by neutral and monosulfated saccharides, and di- and trisulfated glycans were observed as minor peaks with intensities less than 10% of the largest glycan in the chromatogram. As the same amount of

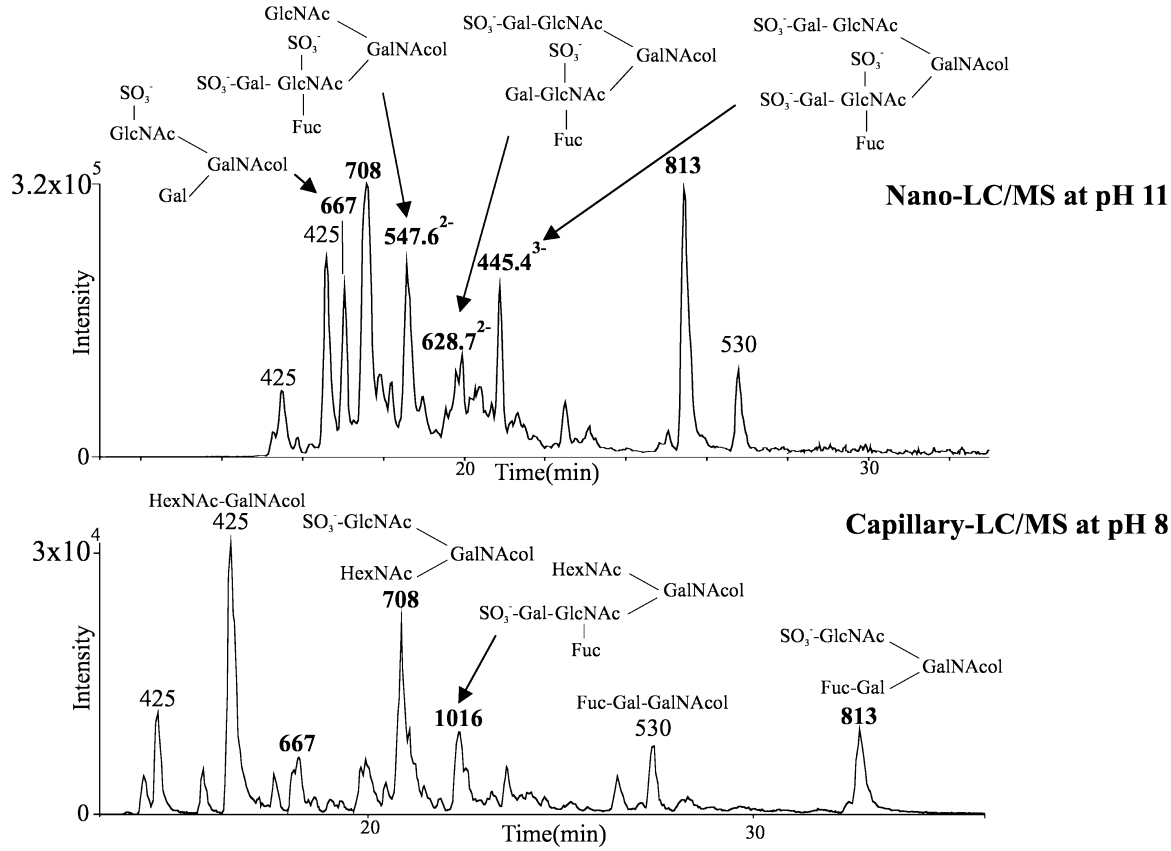

Figure 7. Base peak chromatograms of mucin oligosaccharides from porcine colon mucins analyzed with nano-LC/MS and MS/MS at pH 11 and with capillary-LC/MS at pH 8. The most abundant components in major peaks are annotated, and deduced sequences of major oligosaccharides are displayed. Sulfated $[M - nH^+]^{n-}$ ions are highlighted in bold. Detected oligosaccharides and their monosaccharide composition are summarized in Table 1 and given in detail in Table S-1 in the Supporting Information.

Table 1. Porcine Colonic *O*-linked Mucin Oligosaccharides Analyzed with Nano-LC/MS at pH 11 and Capillary LC/MS at pH 8[a]

	number of sequences[c]	
number of acidic[b] residues	nano-LC/MS	capillary-LC/MS
0	21	45
1	75	77
2	55	48
3	19	5

[a] Number of detected components corresponding to individual oligosaccharide sequences. [b] Sulfate and/or sialic acid residues. [c] Complete list with number of glycan isoforms and their deduced monosaccharide composition is found in Table S-1 in the Supporting Information.

oligosaccharides were injected on the columns, these results supported the hypothesis that multiply charged glycans are ionized more efficiently with nano-LC/MS at high pH. The oligosaccharides detected with the two methods are summarized in Table 1 and given in detail in Table S-1 in the Supporting Information. A total of 45 neutral and 156 mono-, di-, and triply sulfated and/or sialylated oligosaccharides were identified, of which most were sulfated. This is far more than our previously published analysis of the same porcine colonic mucin oligosaccharides using a first generation Q-TOF mass spectrometer when we detected only 38 monosulfated and two disulfated components.[24]

Capillary-LC/MS at pH 8 revealed 45 neutral oligosaccharides consisting of 2−7 residues, compared to only 21 detected with nano-LC/MS at pH 11. These results are consistent with the analyses of the neutral oligosaccharide LNF1 (Figure 5), suggesting that there is no improved sensitivity when analyzing neutral glycans with nano-LC/MS at high pH, instead it is rather the opposite. Approximately the same number of oligosaccharides with one or two acidic residues were detected with the two methods (75/77 with one acidic residue and 55/48 with two acidic residues with capillary and nano-LC/MS, respectively). However, the MS/MS spectra coverage with capillary-LC/MS of these glycans was not as complete as with nano-LC/MS (not shown). Only five oligosaccharides with three acidic residues were detected with capillary-LC/MS, whereas 19 were observed with nano-LC/MS. The triply sulfated oligosaccharide at m/z 445 (M_r 1339) was observed as a prominent component with nano-LC/MS but was not detected at all with capillary-LC/MS (Figure 7). The MS/MS spectrum of the hexasaccharide at m/z 445 is presented in Figure 8. The three sulfate groups, which strongly promoted charge remote fragmentation, gave rise to singly, doubly, and triply charged fragment ions. The doubly charged B-ions at m/z 261.4^{2-} ($B_{2\beta}/Y_{3\beta'}$) and m/z 334.4^{2-} ($B_{2\beta}$) confirmed the presence of a disulfated Hex-HexNAc branch with an additional fucose, an interpretation also supported by a promi-

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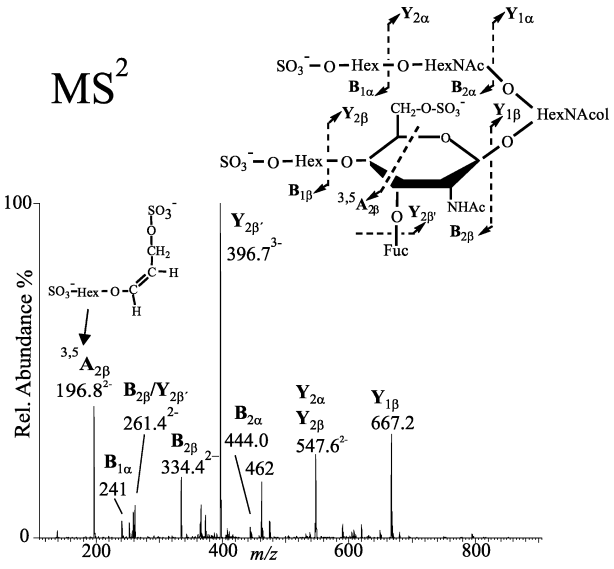

Figure 8. CID spectrum from nano-LC/MS/MS of a major trisulfated oligosaccharide from porcine colonic mucins (Figure 7, upper panel) and with the molecular mass of 1339.3 Da. CID was performed on the triply charged parent ion at *m/z* 445.6, and the sequence was deduced to SO_3^--Hex-4(Fuc-3)(SO_3^--6)HexNAc-(SO_3^--Hex-HexNAc-)HexNAcol.

nent ion at m/z 196.8^{2-}, formed by a cleavage in the sulfated HexNAc residue ($^{3,5}A_{2\beta}$). This fragment ion was found to be diagnostic for disulfated Hex-HexNAc residues and was also indicative of a type 2 configuration (Galβ1−4GlcNAc), as well as for assigning the sulfate to C-6 of the HexNAc residue. Finally, the ions at m/z 241 ($B_{1\alpha}$ or $B_{1\beta}$) and m/z 547.6^{2-} ($Y_{2\alpha}$/$Y_{2\beta}$) supported a doubly branched oligosaccharide with the sequence: SO_3^--Hex-4(Fuc-3)(SO_3^--6)HexNAc-(SO_3^--Hex-HexNAc)HexNAcol.

Nano- and Capillary-LC/MS and MS/MS of the MUC2 Mucin Oligosaccharides from a Single Human Colon Millimeter Sized Biopsy. The MUC2 mucin oligosaccharides from a human colon biopsy were analyzed with capillary and nano-LC/MS and MS/MS as shown in Figure 9. The mucin *O*-glycans were highly sialylated in contrast to the highly sulfated porcine colon glycans. The base peak chromatogram from capillary-LC/MS at pH 8 (Figure 9, lower panel) was dominated by two isoforms of HexNAc-3**(NeuAc-6)**GalNAcol at m/z 716, and oligosaccharides with two or more acidic residues were small components. In contrast, the largest peaks in the nano-LC/MS base peak chromatogram were the doubly charged molecular ions at m/z 551.8^{2-} and m/z 685.8^{2-}, interpreted as glycans with two sialic acid residues or one sialic acid and one sulfate, respectively. In addition, numerous minor components corresponding to glycans with multiple acidic residues were detected. Many of these glycans have not been observed before in a human colon as recently described.[15] A total of 17 triply and one tetra sialylated and sulfated glycan were found, of which a majority could be sequenced by MS^n experiments. The formation of multiply charged molecular ions was more prominent with nano-LC/MS at pH 11 than with capillary LC/MS at pH 8. An example

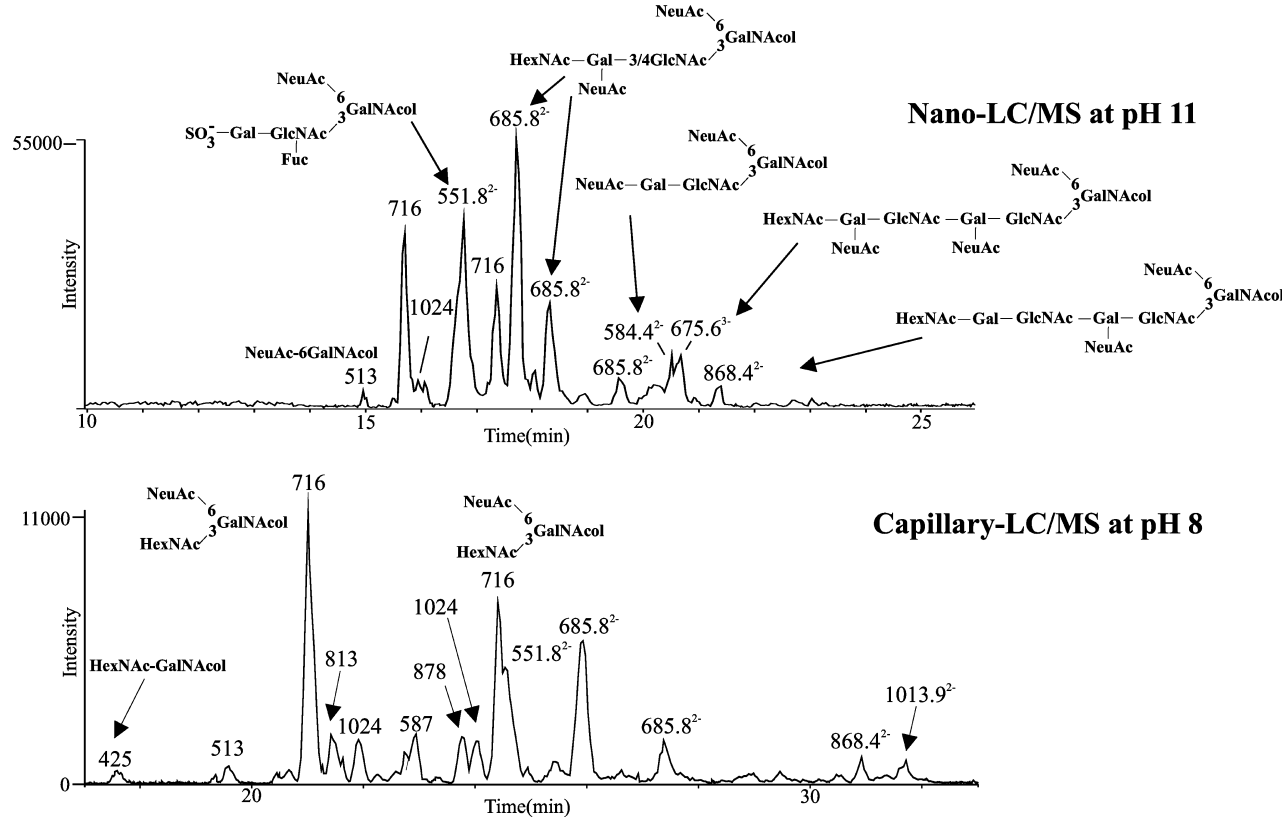

Figure 9. Base peak chromatograms of mucin oligosaccharides from the MUC2 mucin from a millimeter sized human colon biopsy analyzed with nano-LC/MS at pH 11 and with capillary-LC/MS at pH 8. Major components are annotated with the observed molecular ions ($[M - nH^+]^{n-}$), and some deduced oligosaccharide sequences are shown. For all details, see Larsson et al.[15] Some of the deduced glycan structures have also been found by others in human colon.[27]

of this is a nonasaccharide containing three acidic residues (3 HexNAc, 2 Gal, 3 NeuAc, GalNAcol) with the mass of 2029.7 Da. This was detected as a triply charged ion at m/z 675.6^{3-} ([M − 3H$^+$]$^{3-}$) with nano-LC/MS and as a doubly charged ion at m/z 1013.9^{2-} ([M − 2H$^+$]$^{2-}$ with capillary LC/MS (Figure 9). In nano-LC/MS, some oligosaccharides with three acidic residues were observed both as doubly and triply charged precursor ions, something that could be a disadvantage as it will slightly increase the detection limit of an individual oligosaccharide. However, we have also found this useful, as the MS/MS spectra of the two parent ions can be quite different and consequently aid in the sequence deduction (not shown).

DISCUSSION AND CONCLUSIONS

Porous graphitic carbon (PGC) chromatography of *O*-glycans using negative ion mode nano-LC/MS with a mobile phase at high pH was studied and compared to capillary LC/MS at pH 8. The results highlight that the signal response of individual glycans do vary with size and number of acidic residues when analyzed with both methods. Acidic oligosaccharides were ionized more efficiently than neutral species with both methods. This should be taken into account when profiling complex oligosaccharide mixtures and makes it necessary to use correction factors to obtain a better estimation of relative amounts, as we have done before.[17] Nano-LC/MS at high pH was even more selective toward the detection of glycans with multiple acidic residues. This was demonstrated by the analysis of oligosaccharide standards, as well as complex mixtures of mucin oligosaccharides from porcine and human colonic mucins.

The graphitized carbon nanocolumn is in electrical contact with the emitter tip in our interface design and is floating at the nanospray voltage (−1.6 kV) between the two unions. Despite this, we have not observed technical difficulties with this design as been discussed by others.[25,26] This may be due to a combination of several factors, the low ionic strength of the mobile phase, the high pH, the use of negative mode MS, and also the relatively low voltage used in nanospray.

The enhanced detection of the acidic glycans by nano-LC/MS allows for detailed structural characterization of these components with MS^n experiments.[15] For multiply sialylated oligosaccharides, sequential MS^n experiments are often required to eliminate the sialic acid residues before the remaining part of the saccharide is fragmented. This is often not possible to achieve with capillary-LC/MS but is easily obtained with nano-LC/MS. Our nano-LC/MS approach is a valuable alternative to negative ion mode capillary LC/MS for highly sensitive analyses of complex negatively charged oligosaccharide mixtures.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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