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Negative ion graphitised carbon nano-liquid chromatography/mass spectrometry increases sensitivity for glycoprotein oligosaccharide analysis

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Negative ion nano-liquid chromatography/mass spectrometry (nano-LC/MS) and tandem mass spectrometry (nano-LC/MS^2), using graphitised carbon as separating medium, were explored for analysing neutral and acidic O-linked and N-linked oligosaccharide alditols. Compared to the sensitivity of capillary LC/MS (flow rate of 6 μL/min) coupled with a conventional electrospray ionisation source, the nano-LC/MS (flow rate of 0.6 μL/min) with a nanoflow ion source was shown to increase the sensitivity ten-fold with a detection limit in the low-femtomole range. The absolute signals for the $[M-nH]^{n-}$ ions of the oligosaccharides were increased 100-fold, enabling accumulation of high-quality fragmentation data in MS^2 mode, in which detection of low abundant sequence ions is necessary for characterisation of highly sialylated N-linked oligosaccharides. Oligosaccharides with high numbers of sialic acid residues gave dominant fragments arising from the loss of sialic acid, and less abundant fragments from cleavage of other glycosidic bonds. Enzymatic off-line desialylation of oligosaccharides in the low-femtomole range prior to MS^2 analysis was shown to increase the quality of the spectra. Automated glycofragment mass fingerprinting using the GlycosidIQ software confirmed the oligosaccharide sequence for both neutral desialylated as well as sialylated structures. Furthermore, the use of graphitised carbon nano-LC/MS enabled the detection of four sialylated O-linked oligosaccharides on membrane proteins from ovarian tissue (5 μg of total amount of protein).

Glycosylation is one of the major post-translational modifications of secreted and membrane-bound proteins. The current understanding of the glycosylation process and its impact on the function of proteins is still in many cases unknown and the relationship between oligosaccharide structure and its biology is not straightforward. Some of the keywords that have emerged as common themes in glycobiology research are interaction and regulation.[1,2] There are examples where interaction is mediated by only a particular oligosaccharide epitope attached to a protein backbone. This, for instance, is the case with the lutropin hormone receptor binding to HSO_4(-4)GalNAc(β1-4)GlcNAc(β1-2Man(α1-),[3] while, in other cases, the interaction specificity is more forgiving and a range of oligosaccharide structures can fulfil a certain task. This is the case, for example, in the interaction between the selectins and their ligands, where structures such as sialyl Lewis x and sialyl Lewis a or its sulphated analogues are key interaction epitopes, although it is unknown whether the rest of the interacting oligosaccharide is of importance, or whether it is the cooperative interaction between diffuse oligosaccharide patches that mediates the interaction.[4,5] Other important interacting oligosaccharide epitopes, such as blood group related epitopes, often manifest themselves on glycoprotein oligosaccharides,[6,7] both from the ABO system and the Lewis system.

The belief that structure can relate to function has encouraged researchers to develop techniques for detailed oligosaccharide structural characterisation. These techniques have comprised a mixture of chemical and enzymatic methods in combination with different levels of technical instrumentation such as high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), capillary electrophoresis (CE) and gel electrophoresis, and have provided enormous amounts of data. Currently, there are efforts worldwide to assemble these data into databases (e.g. GlycosuiteDB[8] and SweetDB[9]).

Mass spectrometry has emerged as a key instrumentation to enable accurate qualitative glycosylation analysis. The reasons behind its success are the sensitivity of detection and the quality of the data. Both matrix-assisted laser desorption/ionisation (MALDI)-MS and electrospray ionisation (ESI)-MS have successfully been employed for oligosaccharide analysis.[10–12] In combination with on-line separation techniques

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such as LC and CE it enables the separation and characterisation of oligosaccharide isomers.[13,14] While normal-phase chromatography using amine- and amide-type stationary phases have traditionally been the most popular for separating oligosaccharides,[15] graphitised carbon is gaining popularity due to its ease of use, capacity, and high resolution.[16] Miniaturisation of HPLC systems has shown that oligosaccharide ESI LC/MS analysis can be performed with sensitivities down to low-femtomole levels in positive ion mode.[17] Negative ion mode provides the additional benefit of allowing highly sensitive detection of both neutral and negatively oligosaccharides in the one separation. The likely increase in sensitivity on going from negative ion capillary mode LC/MS[18–21] to nanoflow LC/MS should allow oligosaccharide analysis to be performed on even low abundance proteins.

Two-dimensional sodium dodecyl sulphate/polyacrylamide (SDS-PAGE) enables isolation of many glycoproteins in one experiment. Identification of the glycosylation of these separated proteins requires not only sensitive detection of ionised molecules of oligosaccharides and their MS^2 fragments, but also intelligent software for oligosaccharide fragmentation analysis in order to cope with the amount of data. This report describes the use of negative ion nanoflow LC/MS of oligosaccharides using graphitised carbon as stationary phase, and with interpretation of the fragmentation data by the glycofragment mass fingerprinting software GlycosidIQ, by matching the 'in-silico' fragmented oligosaccharide structures from the oligosaccharide database GlycosuiteDB.[22] The sensitivity of the technique is compared to that of negative ion capillary LC/MS using neutral O-linked oligosaccharides, and is then evaluated using sialylated reduced N-linked oligosaccharides. Its applicability for N-linked and O-linked oligosaccharide analysis was tested using commercial standards as well as oligosaccharide mixtures isolated from mucosal surfaces and ovarian tissues. In order to gain additional information on highly sialylated oligosaccharide structures, in which the dominating MS^2 fragmentation was the loss of sialic acid, an enzymatic desialylation procedure was developed for sub-picomole analysis of oligosaccharides of these types prior to analysis by negative ion LC/MS^2.

EXPERIMENTAL

Preparation of ovarian tissue

Ovarian tissue was collected immediately following surgery from a patient diagnosed with multiple cancer including ovarian cancer. The tissues were aliquoted into 2 mL capacity cryovials and stored at −80°C. Tissue samples (approximately 0.2 mg) were freeze-fractured and ground into a powder. Proteins were extracted by sonication in 2 mL of 100 mM sodium carbonate using an ultrasonic probe (Branson, Digital Sonifier, Branson, Danbury, CT, USA) (total of 1 min in 15 s intervals at 70% amplitude). The samples were then centrifuged at 14 000 *g* for 45 min at 4°C. The pellet, enriched with membrane proteins, was re-extracted once according to the above protocol before being reduced and alkylated in sample buffer (7.0 M urea, 2.0 M thiourea, 40 mM Tris base, and 4.0% (w/v) CHAPS) using 5.0 mM tributylphosphine and 10 mM acrylamide for approximately 1 h. This membrane fraction of the tissue was dotblotted onto PVDF P^{SQ} (Millipore, Bedford, MA, USA) membrane for global oligosaccharide analysis (50 μL, approximately 5 μg of protein). The protein concentration in the solution was determined with the Bradford reagent (Sigma-Aldrich, St. Louis, MN, USA) using a bovine serum albumin standard curve.

Release of oligosaccharides

Neutral O-linked oligosaccharides from rat small intestine were prepared as described.[21] N-Linked oligosaccharides were released from dotblotted membrane proteins using PNGase F (Roche, Mannheim, Germany)[19] followed by the release of O-linked oligosaccharide by reductive β-elimination.[18] N-Linked oligosaccharides, either from tissue or as standards (Dextra, Reading, UK), were converted into alditols in 20 μL of 20 mM potassium hydroxide/0.5 M sodium borohydride. (2 h at 50°C). The resulting solutions were neutralised by addition of 1 μL of glacial acetic acid, before being desalted with 25 μL of AG50WX8 cation-exchange resin (BioRad, Hercules, CA, USA) laid on top of a reverse-phase μ-C18 ZipTip (Millipore), and dried by vacuum centrifugation. Borate was removed by repeated addition and evaporation of 50 μL of 1% acetic acid in methanol. The N-linked oligosaccharides from tissue samples were further desalted on home-made micro-desalting columns (~2 μL) containing Carbograph material (Alltech, Deerfield, IL, USA) laid on top of a reverse-phase μ-C18 ZipTip (Millipore).[19]

Enzymatic desialylation of N-linked oligosaccharides

A mixture of oligosaccharide alditols from Dextra, described in Figs. 2(b), 2(d), 2(e) and 2(f) (1–70 fmol of each), was dissolved in 18 μL of 25 mM sodium phosphate buffer pH 6.0 and 2 μL (approximately 10 mU) of *Arthrobacter ureafaciens* sialidase (Glyko, Novato, CA, USA) was added and incubated at 37°C for 16 h. Samples were desalted on the Carbograph micro-desalting columns described above.

Capillary LC/MS and nano-LC/MS of oligosaccharides

Oligosaccharide samples (10 μL) were dissolved in water and injected via a Surveyor autosampler and HPLC system (Thermo Finnigan, San Jose, CA, USA). Solvent A was 10 mM ammonium bicarbonate and solvent B was 10 mM ammonium bicarbonate in 80% acetonitrile. Flow rate was 110–120 μL/min, split down before the autosampler into approximately 6 μL/min (capillary LC) or 0.5 μL/min (nano-LC). A 0.5 μm Rep frit (Upchurch Scientific, Oak Harbor, WA, USA) was in-line with the ProteCol Hypercarb (5 μm particles) column (from SGE, Ringwood, Australia), i.e., a 100 mm × 0.3 mm column for capillary LC, or a 100 mm × 0.15 mm column for nano-LC. The gradient was developed from 0–37.5% B over 27.5 min for capillary LC and from 2–30% B for nano-LC. Both columns were then washed with 100% B for 4–7 min and equilibrated with the starting ratio of solvents for at least 12 min.

The ion trap mass spectrometer (LCQ-XP+, Thermo Finnigan) was tuned with reduced maltoligosaccharides

(10 μg/μL) infused at 3 μL/min in 40% acetonitrile/10 mM ammonium bicarbonate using the standard ESI source. The capillary voltage was reduced from 3.5 to 1.1 kV for nano-LC applications using the nano-ESI source. For nano-LC, the ProteCol column was connected via a nanoprobe (SGE) to a distal coated TaperTip emitter (20 μm i.d.; New Objectives, Woburn, MA, USA). The mass spectrometer was scanned between *m/z* 350–2000 followed by a data-dependent MS^2 scan of the most abundant signal from the MS^1 scan. Sensitivity testing for sialylated N-linked oligosaccharides was done using oligosaccharide alditols in the range 1–70 fmol, using linear regression analysis for evaluation of data and calculation of detection limit (2× noise estimated from the zero concentration intercept).

Fragmentation analysis of oligosaccharides was performed using the GlycosidIQ glycofragment mass fingerprinting software matching with 'in-silico' fragmented oligosaccharides from the GlycosuiteDB[8] oligosaccharide database.[22] The precursor ion was searched within ±1 Da and the fragments within ±0.7 Da. Structures were evaluated using the segmentation score (number of oligosaccharide sequences that could be created from the identified glycosidic fragments) and glycosidic correspondence score (sum of the intensities of all glycosidic fragments found in the spectrum).[22]

RESULTS AND DISCUSSION

We have previously described the release and sample preparation of N- and O-linked oligosaccharides released from glycoproteins blotted onto PVDF membranes, either from 1D or 2D SDS-PAGE.[18–21] The sample preparation involved isolating N-linked oligosaccharides using graphitised carbon microcolumns after PNGase F treatment of gel-separated glycoproteins, and microcolumn anion-exchange desalting after reductive β-elimination of O-linked oligosaccharides. Both methods are compatible with negative ion graphitised carbon LC/MS separation analysis of the released oligosaccharides. In order to gain increased sensitivity for oligosaccharide detection and characterisation we have now developed a robust nanoflow LC/MS system and tested it for its capability of identifying and characterising both N- and O-linked oligosaccharides. In contrast with positive ion mode MS, the technique has the capacity to detect both highly sialylated oligosaccharides as well as neutral oligosaccharides in the same experiment. MS^2 fragmentation can be performed both before and after exoglycosidase treatment (sialidase) at the low-femtomole level.

Detection of neutral O-linked oligosaccharides

Neutral oligosaccharides released from small intestinal mucosal proteins from six rats were isolated in 1995[23] in a project where identification of acidic oligosaccharides was the main focus. This sample has since become a standard in our laboratory for the development and testing of the performance and sensitivity of our LC/MS separations. We have previously shown that negative ion capillary LC/MS can give sensitivity of detection of individual oligosaccharides in the pmol range or lower.[18] We tested the effect of using nanoflow LC/MS instead of capillary LC/MS by dropping the flow rate from around 6–7 μL/min down to 0.5–0.6 μL/min and by changing from the traditional ESI-MS source to a nano-ESI-MS source. This should, in principle, reduce the chemical background by at least ten-fold. Using the same tune file for detecting oligosaccharides by negative ion capillary LC/MS and nano-LC/MS (and lowering the spray voltage from 3.5 to 1.1 kV for nano-LC/MS) showed that, despite an actual increase in the absolute background noise, the signal-to-noise ratios for detecting $[M-nH]^{n-}$ ions were increased by five- to ten-fold for most components (Fig. 1, Table 1). This indicated that it is the more efficient ion transfer in the ion source, rather than a lowering of the chemical background, that contributes to increased sensitivity by nano-LC, and is thus the main reason behind the increase in sensitivity.

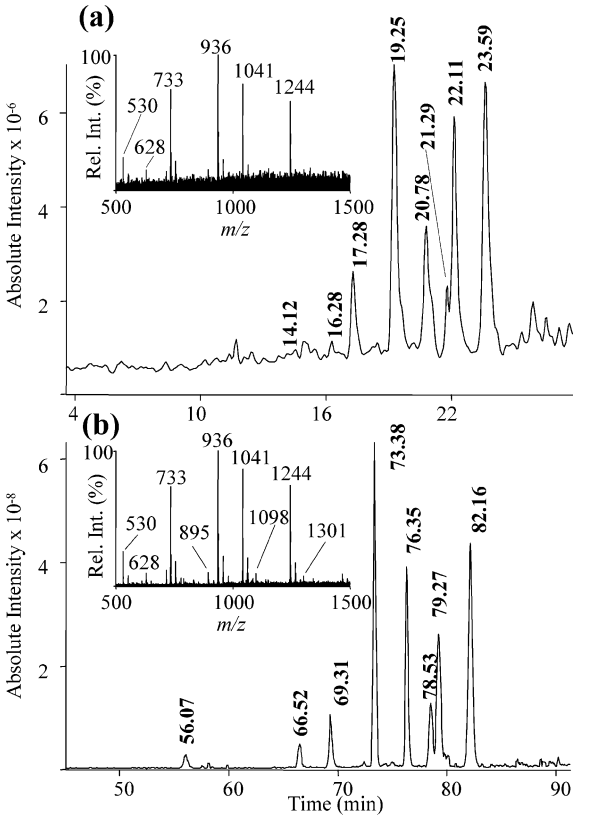
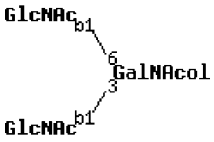
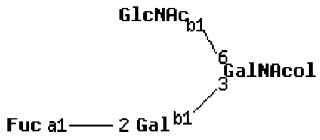
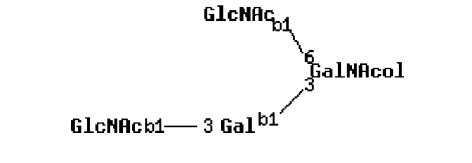
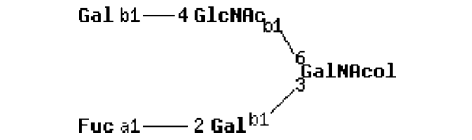
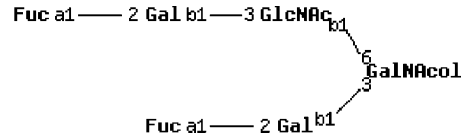


Figure 1. Comparison of negative ion (a) capillary LC/MS versus (b) nano-LC/MS analysis of neutral O-linked oligosaccharides (5.5 ng) using graphitised carbon chromatography (base peak chromatograms). Combined MS^1 mass spectra of the region where oligosaccharides were eluted are shown as inserts. Retention times and detected molecular ions relate to Table 1.

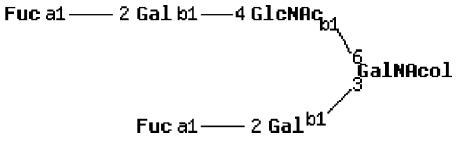
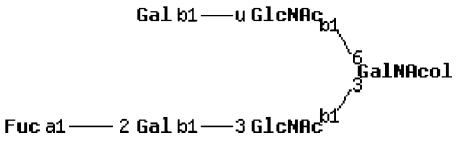
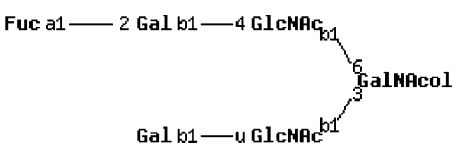
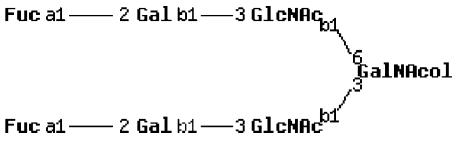
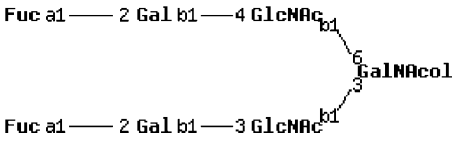
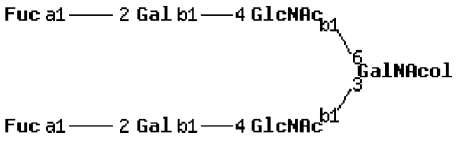
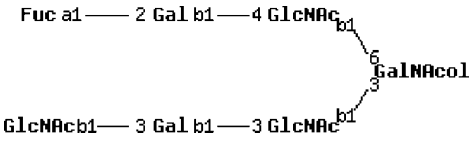
For each mass (MS^1) spectrum produced there was also an MS^2 experiment on the most intense precursor ion recorded. The increase in the absolute ion intensity of the $[M-H]^-$ precursor ion in the chromatogram by at least 100 times (compare absolute signal in Fig. 1(a) with 1(b)) meant that high-quality fragment spectra could be produced and interpreted according to the rules of negative ion fragmentation of oligosaccharide alditols[24] (data not shown). The higher sensitivity allowed us to identify oligosaccharides previously unidentified from rat intestine[25] such as the $[M-H]^-$ ion at *m/z* 1098 corresponding to the sequence Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)[Gal(β1-u)GlcNAc(β1-6)]GalNAcol,

Table 1. Sensitivity for oligosaccharides analysed by nano- and capillary-LC/MS

$[M-H]^-$ *m/z*	Retention time (min)		Structure	S/N[a]		Increased sensitivity
	Nanoflow	Capillary		Nanoflow	Capillary	
530	78.53	21.79	Fuc a1—2 Gal b1—3GalNAcol	50	6	9
628	56.07	14.21		28	2	12
733	66.52	16.28	Fuc a1—2 Gab1—3 GlcNAcb1—3GalNAcol	51	3	15
733	72.15	22.15	Fuc a1—2 Gab1—4 GlcNAcb1—3GalNAcol	3	ND[b]	—
733	79.27	23.10		83	11	7
790	66.43	ND		6	ND	—
895	72.43	18.76		6	1	5
895	79.55	22.34		5	2	3
936	69.31	17.28		43	7	6
936	73.38	19.25		176	17	10
936	74.97	ND		6	ND	—
1041	80.04	22.49		17	ND	—

Continues

Table 1. Continued

[M−H]⁻ *m/z*	Retention time (min) Nanoflow	Capillary	Structure	S/N[a] Nanoflow	Capillary	Increased sensitivity
1041	82.16	23.59		226	19	12
1098	71.55	ND		3	ND	—
1098	74.57	ND		4	ND	—
1244	73.38	19.25		32	3	12
1244	76.35	20.74		98	10	10
1244	81.87	23.69		26	2	12
1301	75.02	ND		4	ND	—

[a] Signal measured as the sum of all isotopes. Noise measured between *m/z* 1500–1600.
[b] ND = not detected.

and the $[M-H]^-$ ion at *m/z* 1301 corresponding to the sequence GlcNAc(β1-3)Gal(β1-u)GlcNAc(β1-3)[Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6)]GalNAcol (Table 1).

Detection of sialylated N-linked oligosaccharides

In order to obtain an absolute comparison of the detection limits for individual oligosaccharides, we used commercially available N-linked oligosaccharides. It was found that free oligosaccharides showed anomeric separation on the graphitised carbon column at the high pH used in the system (data not shown). By reducing the oligosaccharides to their alditols this problem was eliminated and a single peak was seen for each structure.

A standard way to determine sensitivity of detection is to create a dilution series of the analyte of interest, and to approximate the noise level in the system as the extrapolated signal for a sample containing a zero amount of analyte. A signal twice the noise is then an approximation to the detection limit. We performed a dilution series experiment on standard sialylated biantennary and tetra-antennary N-linked oligosaccharides (structures described in Fig. 2), using between 1–70 fmol of individual oligosaccharide, and were able to detect the $[M-2H]^{2-}$ ions down to the 1 fmol level. However, the prescribed calculation of the detection limit, using the method based on 2× the intercept from linear regression analysis, gave a detection limit between 0.6–7 fmol of the analysed oligosaccharides (Table 2). We believe that the conditions could be further optimised and an even lower detection limit possibly obtained by careful selection of solvents, pumping system and by reducing the dimensions of the separating column and flow rate.

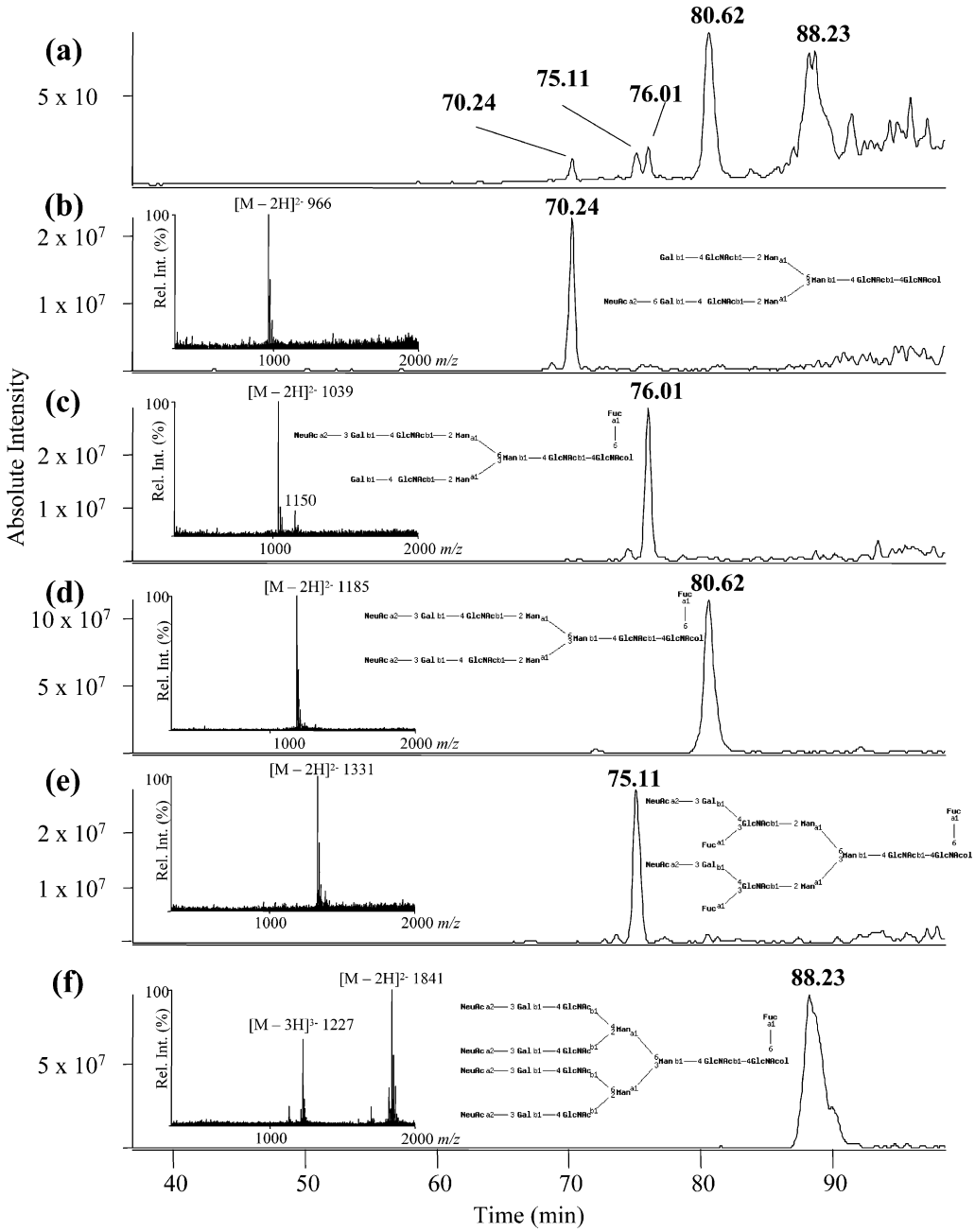


Figure 2. Negative ion nano-LC/MS of sialylated N-linked oligosaccharide alditols (20 fmol), showing base peak chromatogram (a), and selective ion chromatograms of $[M-nH]^{n-}$ ions *m/z* 966 (b), *m/z* 1039 (c), *m/z* 1184 (d), *m/z* 1331 (e), and *m/z* 1841 (f). Combined full scan mass spectra of the corresponding regions where components were detected are inserted.

Table 2. Detection limits for sialylated N-linked oligosaccharide alditols with nano-LC/MS

Monosaccharide composition[a]	$[M-2H]^{2-}$ *m/z*	Limit of detection (fmol)[b]	r^2 linear regression value
[5,4,0,1]	966	7	0.96
[5,4,1,1]	1039	0.6	0.97
[5,4,1.2]	1184	2	0.96
[5,4,3,2]	1331	5	0.99
[7,6,1,4]	1841	4	0.97

[a] [Hex, HexNAc, dHex, NeuAc] including reducing end GlcNAcol as HexNAc.
[b] 2 × background noise.

Fragmentation analysis of sialylated N-linked oligosaccharides

Automated MS^2 experiments were performed in each of the analyses described in this report. This allowed interpretation of fragment spectra for further characterisation of oligosaccharide structures. The combination of LC with data-dependent MS^2 is a powerful approach to allow characterisation of oligosaccharide isomers in mixtures. Interpretation of oligosaccharide fragment spectra is usually quite labour intensive and demands high-level skills to make sense of the fragmentation pattern. To aid in the interpretation we used the GlycosidIQ glycofragment mass fingerprinting software tool (matching MS^2 spectra with theoretical

spectra generated from oligosaccharides in GlycosuiteDB) and the GlycoFrag tool (generating theoretical fragments of submitted oligosaccharides). Figure 3 shows the fragmentation of the $[M-2H]^{2-}$ ion of the N-linked oligosaccharides from 20 fmol of sample loaded onto the column. Major fragment ions corresponding to glycosidic fragmentation are labelled, but additional abundant fragments, including internal fragments from more than two separate glycosidic and cross ring cleavages, were also detected. The carboxyl group introduced by sialic acid was the site for at least one of the charges of the doubly charged ions detected by MS on all the N-linked oligosaccharides tested here. In the case of the tetrasialylated tetraantennary structure there were fewer charges ($[M-2H]^{2-}$ and $[M-3H]^{3-}$ ions in Fig. 2(f)) on the molecule than the number of sialic acid residues. Tuning the mass spectrometric setting in order to introduce more charges onto the molecule (each sialic acid potentially contributing one negative charge), and thus detecting a quadruply charged precursor ion, were largely unsuccessful.

The analysis of the fragmentation pattern of all oligosaccharides indicated that charge-remote fragmentation was the main mechanism. For singly sialylated structures, the localisation of the sialic acid at the non-reducing end of the biantennary structure gave predominantly fragmentation

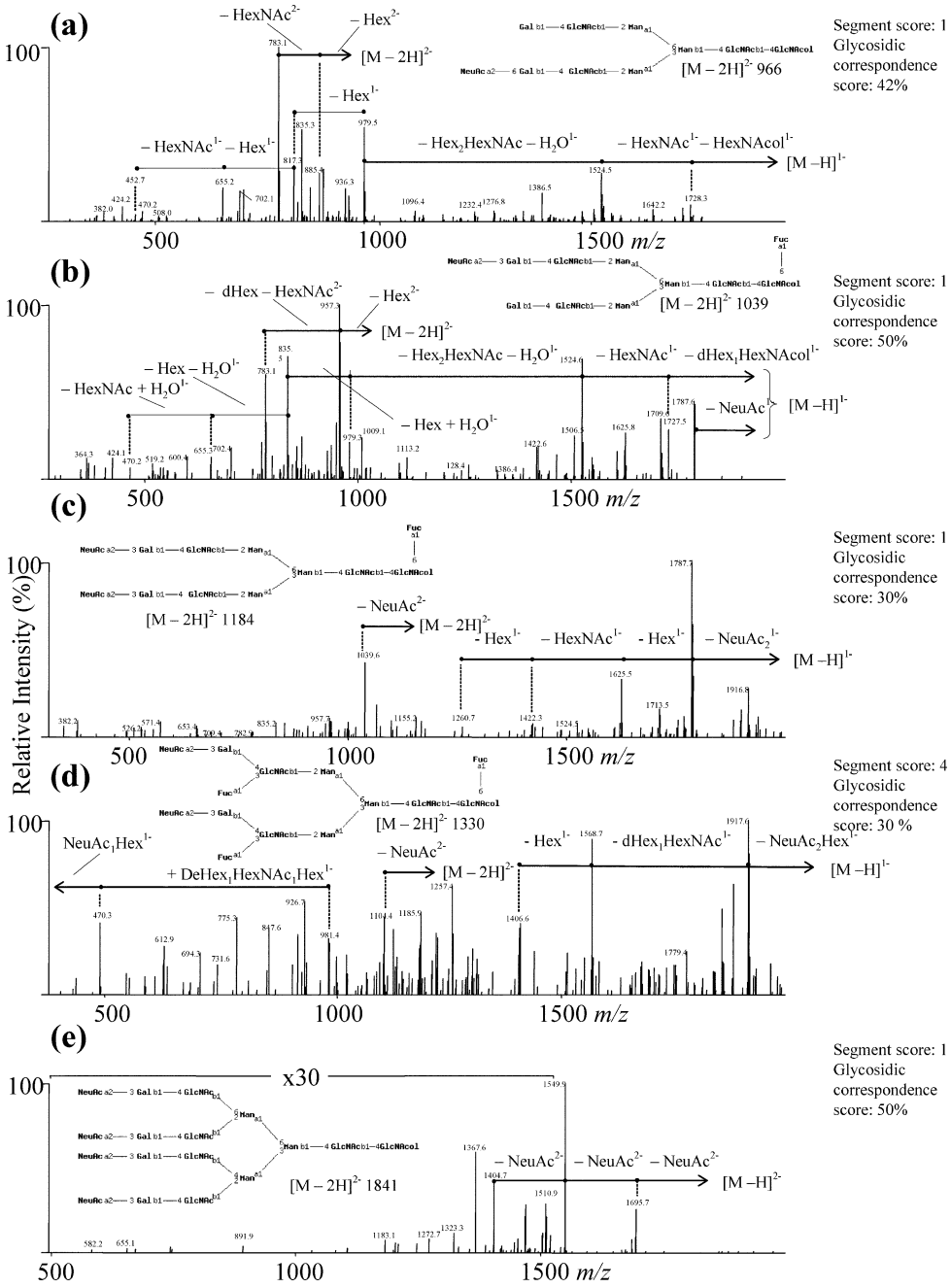


Figure 3. Negative ion nano-LC/MS^2 fragmentation of sialylated N-linked oligosaccharide alditols (20 fmol) of $[M-nH]^{n-}$ ions *m/z* 966 (a), *m/z* 1039 (b), *m/z* 1184 (c), *m/z* 1330 (d), and *m/z* 1841 (e). Results from the GlycosidIQ software are included on the right-hand side to illustrate the quality of the spectral matching.

patterns directed from the reducing end (Figs. 3(a) and 3(b)). The second charge of the doubly charged precursor ion was probably located mostly on the reducing end. This assignment is supported by the fact that the only doubly charged fragment ions detected contained both the reducing end GlcNAcol and the sialylated antennary. The GlycosidIQ glycofragment mass-fingerprinting tool[22] gave high confidence to the sequencing of the monosialylated biantennary structures (Figs. 3(a) and 3(b)). This is shown by a segmentation score of 1 (non-ambiguity of assigned glycosidic fragments), and the high proportion of glycosidic fragments matched (>40% of the fragment intensities corresponding to glycosidic fragmentation). Sequence ions were mainly detected as B_i and C_i fragments (Domon and Costello nomenclature[26]).

Disialylated structures were also detected as doubly charged ions in the negative ion mode MS, and it is most likely that the two sialic acid residues held both the charges. A consequence of this would be that charge-remote fragmentation could be directed towards the chemically almost equivalent sites (the sialic acid residues) (exemplified in Figs. 3(c) and 3(d)). This resulted in two dominant pathways for the fragmentation, making the interpretation of the mass spectra more complicated. The dominant sequence ions in the two cases presented here are the Y_i and Z_i ions (Figs. 3(c) and 3(d)). Since it is less likely that a charge will be localised on the reducing end of these structures, fewer fragments will be detected containing the reducing end core. The properties of negative ion mode fragmentation therefore make manual interpretation of the spectra difficult. However, this is less of a problem when using the glycofragment mass-fingerprinting tool, GlycosidIQ, since its primary scoring is based around glycosidic fragmentation from the reducing and the non-reducing end without any preferences.[22] Figure 3(c) shows the fragmentation spectrum of a disialylated core fucosylated biantennary stucture, with the assignment relying on low intensity fragment ions in the spectrum. These fragments would be difficult to detect with the less efficient ion transfer provided by the capillary LC/MS approach. The biantennary core fucosylated structure with two terminating sialyl Lewis x epitopes on each antenna (Fig. 3(d)) displayed a significantly more complex spectrum compared to that for the structure with only sialyl core 2 epitopes on each antenna (Fig. 3(c)). The increased number of branches made this structure more prone to cross-ring fragmentation and generation of internal fragments from more than one glycosidic cleavage. This latter phenomenon has already been observed[24] for neutral oligosaccharides in negative mode containing unsialylated Lewis x. The high number of internal fragments from glycosidic cleavages will still give a reasonably high number for the glycosidic correspondence score, but it will become increasingly difficult to predict the structure from these fragments, resulting in a worse segmentation score. The segmentation score of 4 shows that there are three additional possible oligosaccharide sequences that could be assembled from the assigned glycosidic fragments in the spectrum.

Oligosaccharides with more sialic acid residues than the number of charges usually gave intense fragment ions arising from the loss of each individual sialic acid residue (Fig. 3(e)). The dominant counter ions on the non-charge-bearing sialic acid residues were usually found to be protons. The presence of acidic protons in close proximity to the sialic acid glycosidic bond is probably the reason for the labile sialic acid, in this case working as a catalyst for the cleavage of that bond. We found that inclusion of sodium salts in the mobile phase stabilised the sialic acid glycosidic bond when a doubly charged sodiated precursor ion of the tetrasialylated tetraantennary oligosaccharide was subjected to fragmentation (data not shown). However, this approach was abandoned since the inclusion of the sodium salt also reduced the sensitivity of oligosaccharide detection. Despite poor fragmentation, the tetraantennary tetrasialylated structure gave low intensity fragment ions that still enabled additional features of the molecule to be determined in addition to the fact that it contained sialic acid residues. For instance, the low intensity ion at m/z 655.1 is the B_3 ion of a NeuAc-Gal-GlcNAc sequence (Fig. 3(e)), and this together with other low intensity ions were included in the scoring of the correct sequence by the software. The feature that was observed in this and other spectra of multisialylated species was that sialic acid is extremely labile in the MS^2 fragmentation mode. This results in the production of a high number of uncharged fragments since the sialic acid fragments from the core oligosaccharide chain. Thus, for sialylated oligosaccharides the total efficiency of generating fragments containing structural information is low, and the detection of the low-intensity charge-bearing fragments is enhanced by the efficient ion transfer of the precursor ion into the ion trap by nano-LC. A higher number of precursor ions will then compensate for the inefficient fragment generation, still enabling efficient oligosaccharide characterisation. Despite the high glycosidic correspondence score of the tetrasialylated structure described in Fig. 3(d), the assignment of this structure is less reliable since the sequence was deduced using the low intensity fragment ions in the presence of pronounced sialic acid fragmentation.

Fragmentation analysis of desialylated N-linked oligosaccharides

In order to gain more information about highly sialylated structures, several strategies can be used. One includes the unsatisfactory use of metal ion adducts, such as sodium, to prevent the catalytic cleavage of sialic acid from the oligosaccharide chain as described above. An alternative that has been used for stabilising sialic acid residues is the covalent derivatisation of the carboxyl group of the sialic acid by methyl ester or amide formation.[27–30] As in all chemical derivatisation there is the probability of loss and incomplete derivatisation. There is also the possibility of enzymatically removing sialic acid with specific sialidases. This provides information on the sialic acid linkage but will use more of the sample, since, in order to determine the degree of sialylation, each sample has to be run both with and without sialidase treatment. The advantage of sensitive mass spectrometric detection in this case cannot be emphasised enough.

Four of the standard N-linked oligosaccharide alditols were treated with *A. ureafaciens* sialidase and were submitted to LC/MS^2 analysis. We found that neutral N-linked

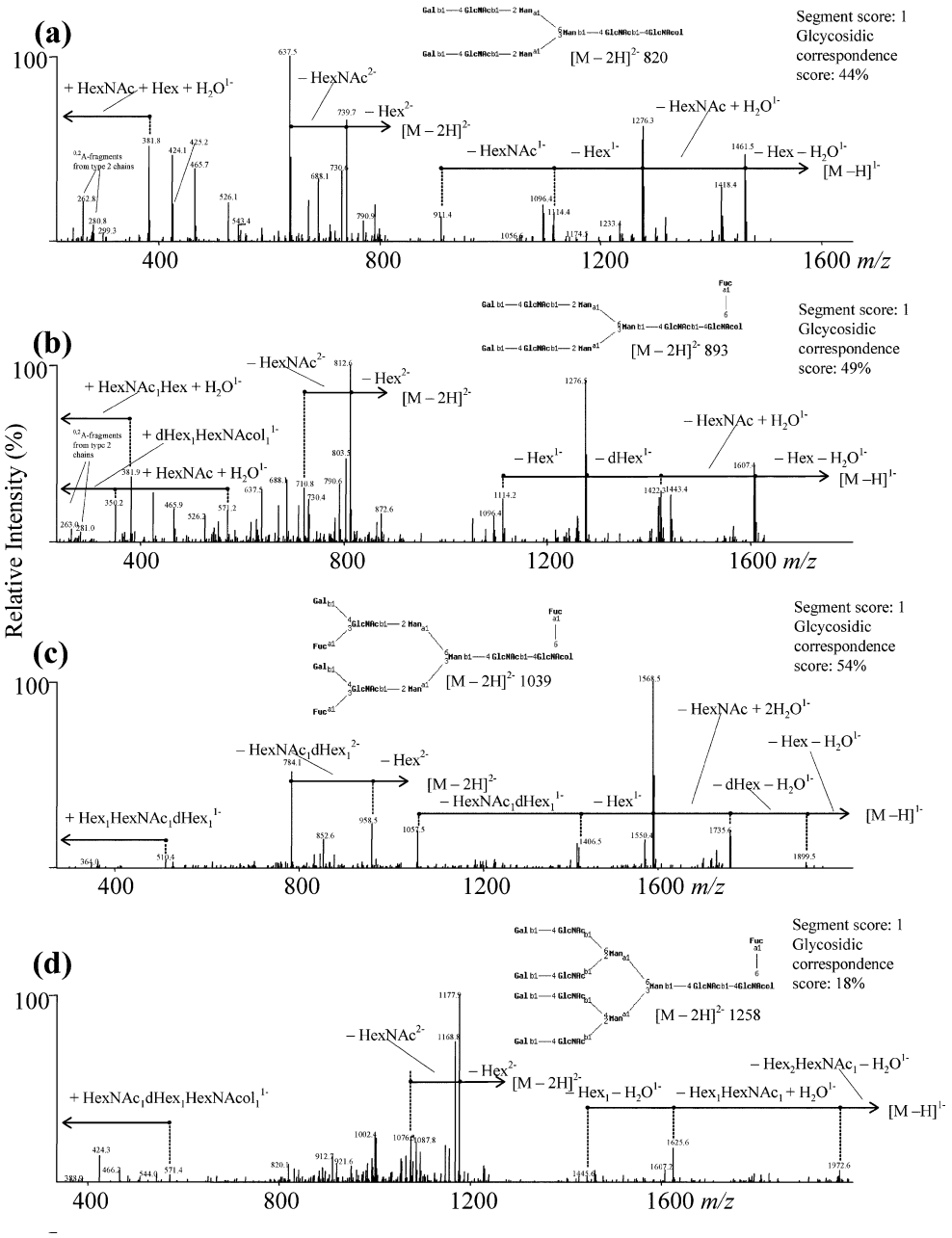


Figure 4. Negative ion nano-LC/MS^2 fragmentation of sialidase-treated N-linked oligosaccharide alditols (20 fmol) of $[M-nH]^{n-}$ ions *m/z* 820 (a), *m/z* 893 (b), *m/z* 1039 (c), and *m/z* 1258 (d). Results from the GlycosidIQ software are included on the right-hand side to illustrate the quality of the spectral matching.

oligosaccharide alditols displayed many of the features previously described for O-linked oligosaccharide alditols in negative ion mode.[24,31] This includes a high proportion of reducing end Y_i and Z_i fragments, $^{0,2}A_i$ fragmentation of 4-linked GlcNAc, and Z_i/Z_i fragmentation of 3/4-disubstituted GlcNAc. Oligosaccharides were desialylated and detected in LC/MS full scan MS^1 mode down to the 20 fmol level with a signal-to-noise greater than 5. The MS^2 spectra of 20 fmol of each oligosaccharide are shown in Fig. 4, together with the structures predicted by the GlycosidIQ fragmentation tool. All showed excellent matches to the correct structures, with segmentation scoring of 1 and high sequence coverage as measured by the high glycosidic correspondence score. The quality of the spectra is also characterised by an even distribution of fragment ions over the mass range.

Identification of oligosaccharides from ovarian tissue

Glycosylation has been reported to be altered in cancer progression.[32] Cancers such as colon and breast cancer are accompanied by an altered O-linked glycosylation. Some of the most used markers for ovarian cancer are CA 19-9 and CA 125. CA 19-9 is reported to be analogous to the sialyl Lewis a epitope, while CA 125 is part of the highly glycosylated mucin MUC16.[33] Both of these markers indicate that glycosylation plays an important role in understanding

ovarian cancer. A protocol was developed to enrich for ovarian cancer tissue cell membrane proteins to investigate their glycosylation profiles. PVDF dot blots with ovarian tissue membrane proteins were treated with PNGase F to remove the N-linked oligosaccharides followed by reductive β-elimination to release O-linked oligosaccharides.[19] When equivalent oligosaccharide samples from this tissue were analysed by capillary LC/MS, no oligosaccharides could be detected. By using nano-LC/MS small amounts of N-linked oligosaccharides were found on the ovarian tissue membrane proteins (data not shown), but the dominant glycosylation was found to be O-linked. Four main $[M-H]^-$ ions with corresponding MS^2 spectra were detected (Fig. 5). The fragment spectra were used to assign the structures as singly and doubly sialylated Gal(β1-3)GalNAcol and Gal(β1-3)[Gal(β1-)GlcNAc(β1-6)] GalNAcol. The most dominant structure was the disialylated structure with the $[M-H]^-$ ion at *m/z* 966 and retention time 67.09 min. The high abundance of this structure in the sample resulted in observation of a small amount of in-source desialylation, as indicated by the

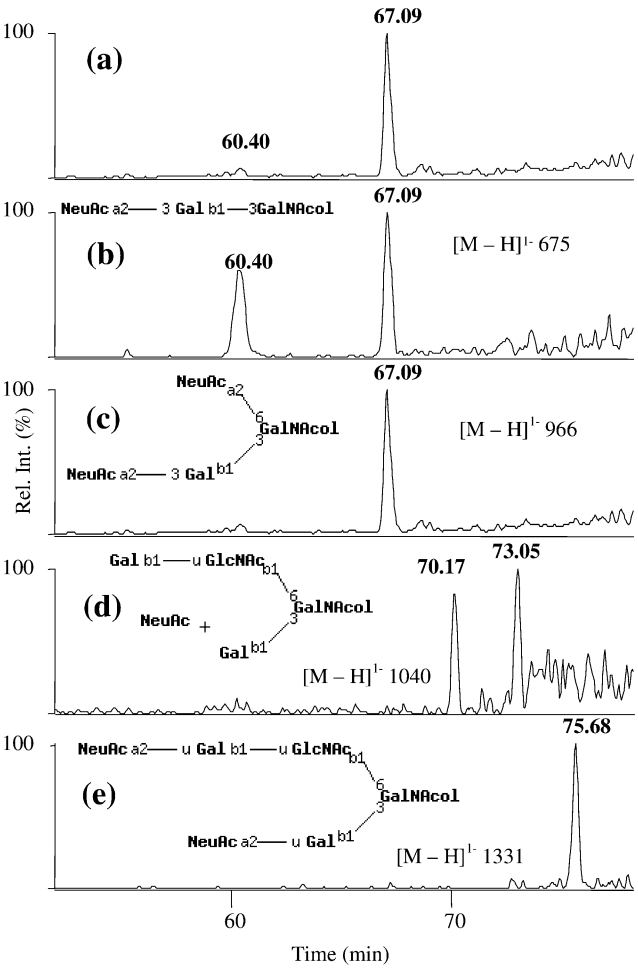


Figure 5. Negative ion nano-LC/MS of O-linked oligosaccharide alditols released from ovarian tissue membrane proteins, showing base peak chromatogram (a), and selective ion chromatograms of $[M-H]^-$ ions *m/z* 675 (b), *m/z* 966 (c), *m/z* 1040 (d), and *m/z* 1331 (e). Included in the figure are GlycosidIQ matched structures based on MS^2 glycofragment mass fingerprinting.

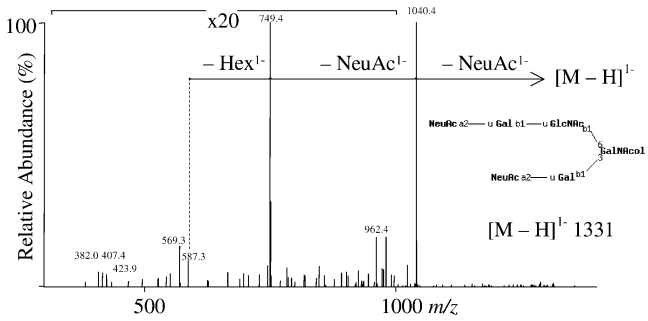


Figure 6. Negative ion nano-LC/MS^2 fragmentation of a disialylated structure with a $[M-H]^-$ ion at *m/z* 1331 from ovarian cell membranes.

in-source formation of the desialylated component *m/z* 675 apparently coeluting with the *m/z* 966 component (Figs. 5(b) and 5(c)). The first component (retention time 60.40 min) corresponded to the structure NeuAc(α2-3)Gal(β1-3)GalNAcol according to glycofragment mass fingerprinting. We have observed that the extent of in-source fragmentation via loss of sialic acid increases with the degree of sialylation and decreases with the molecular mass of the oligosaccharide. The level of in-source fragmentation of the O-linked disilaylated tetrasaccharide was estimated to be <15%, was <5% in the MS^1 spectra of mono- and disilaylated N-linked oligosaccharides (Figs. 2(d) and 2(e)), but increased to about 20% for the tetrasialylated N-linked structure (Fig. 2(f)).

Fragmentation of sialylated O-linked oligosaccharides presented problems similar to those for sialylated N-linked oligosaccharides. Since the O-linked oligosaccharides are detected as singly charged species, oligosaccharides with more than one sialic acid will produce high intensity fragment ions from the loss of sialic acid. This is illustrated in Fig. 6 by the disialylated structure with the $[M-H]^-$ ion at *m/z* 1331, where only low intensity glycosidic fragment ions were detected after the precursor ion lost both sialic acid residues. Again, with a less intense precursor ion, these lower intensity fragments would have been undetected, making the sequence assignment difficult.

CONCLUSIONS

Analysis of released oligosaccharides by negative ion graphitised carbon nano-LC/MS provides sufficient sensitivity of detection for analysis of fmol amounts of oligosaccharides. Together with appropriate sample preparation, as described previously,[19] this technique will provide extensive information in cases where only limited amounts of oligosaccharides are available. Acidic and neutral oligosaccharides can be detected by a single method. However, sialylation often makes the assignment of oligosaccharide sequence difficult by MS^2 fragmentation, either producing complex fragmentation patterns or producing fragmentation that is not conclusive for assigning a full sequence. Problems arising from complex fragmentations can be overcome by using, for example, the GlycosidIQ glycofragment mass-fingerprinting tool which searches for glycosidic fragmentation, cross ring cleavages and internal fragments by matching to the theoretical fragmentation of structures present in the GlycoSuiteDB

database. Desialylation of sialylated structures prior to fragmentation analysis was shown to be a method for increasing the MS^2 spectral quality, and a procedure was devised by which this could be performed at a low-femtomole scale. Nano-LC/MS and LC/MS^2 were used for characterisation of oligosaccharides from ovarian tissue samples illustrating the power of the system for investigating biologically interesting questions.

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