

REVIEW

Structural determination of *N*-linked glycans by matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry

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This paper reviews methods for the analysis of *N*-linked glycans by mass spectrometry with emphasis on studies conducted at the Oxford Glycobiology Institute. Topics covered are the release of glycans from sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels, their purification for analysis by mass spectrometry, methods based on matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization for producing fragment ions, and details of their fragmentation. MALDI mass spectrometry provided a rapid method for profiling neutral *N*-linked glycans as their $[M + Na]^+$ ions which could be fragmented by collision-induced decomposition to give spectra containing both glycosidic and cross-ring fragments. Electrospray ionization mass spectrometry was more versatile in that it was relatively easy to change the type of ion that was formed and, furthermore, unlike MALDI, electrospray did not cause extensive loss of sialic acids from sialylated glycans. Negative ions formed by addition of anions such as chloride and, particularly, nitrate, to the electrospray solvent were stable and enabled singly charged ions to be obtained from larger glycans than was possible in positive ion mode. Fragmentation of negative ions followed specific pathways that defined structural details of the glycans that were difficult to obtain by classical methods such as exoglycosidase digestion.

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Abbreviations: 2-AA, 2-aminobenzoic acid; 2-AB, 2-aminobenzoamide; DHB, dihydroxybenzoic acid

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1 Introduction

The function and physicochemical properties of many proteins are determined by post-translational events of which glycosylation is among the most important [1, 2]. Two main types of attached glycan are recognised; O-linked and N-linked. O-linked glycans are attached to either serine or threonine but with no directing consensus sequence whereas N-linked glycans are attached to asparagine in an Asp-Xxx-Ser (Thr or occasionally Cys [3]) motif where Xxx is any amino acid except proline. O-linked glycans from mammalian systems tend to be relatively small diverse structures whereas the N-glycans, although larger, contain a trimannosyl chitobiose pentasaccharide core that is attached to the protein by a GlcNAc link and have a well-defined overall structure.

Because of the branched nature of these carbohydrates, their structural characterisation has not yet achieved the sophistication of that of the protein moiety of glycoproteins whose primary structure consists only of a linear chain of a limited number of amino acids. However, if meaningful biological information is to be obtained from proteomic studies it is essential that the structures of these attached carbohydrates are determined with methods that are sensitive enough to provide information from glycoproteins extracted from the 2-D gels commonly used to separate them.

Structural determination of N-linked glycans has classically been performed by monitoring the products of exoglycosidase digestion by gel filtration chromatography [4] or, more recently, by HPLC [5–7] or MALDI-MS [8, 9]. Determination of the number of monosaccharide units removed during each incubation step gives both the nature of the monosaccharide removed and its linkage. Recent developments have extended the technique to O-linked glycans [10]. Although many investigators use exoglycosidase sequencing, mass spectral fragmentation is becoming more generally accepted as there are problems in obtaining exoglycosidases in a sufficiently pure state and mass spectrometers are now available that can produce high quality MS/MS spectra from small amounts of material. Although fast atom bombardment (FAB) mass spectrometry has been used in this context for many years [11–14] it is now being replaced by the more sensitive techniques of electrospray or MALDI ionization.

This paper, which was originally presented at the inaugural meeting of the British Society for Proteome Research (BSPR), at Hinxton Hall, near Cambridge in July 2004 outlines some of these methods but concentrates on mass spectrometric techniques that are used for the analysis of N-linked carbohydrates at the Oxford Glycobiology Institute. Additional, more comprehensive reviews, give details of similar work that has been performed by other investigators [15–18].

2 Materials and methods

2.1 Materials

N-linked glycans were released from well-characterised glycoproteins (Sigma, Poole, Dorset, UK) with hydrazine [19, 20]. High mannose glycans were from ribonuclease B [21] and porcine thyroglobulin [22, 23], truncated complex and hybrid glycans were from hen ovalbumin [24, 25], sialylated glycans were from bovine fetuin [26], thyroglobulin and α 1-acid glycoprotein [27]. Bi- and tri-antennary complex N-linked glycans were also obtained from Oxford Glyco Sciences (Abingdon, UK) or were derived from the sialylated glycans by desialylation with 10% acetic acid for 30 min at 80°C. Glycan release from other glycoproteins separated by SDS-PAGE was performed by the in-gel method described by Küster *et al.* [28]. Ammonium nitrate was from Aldrich (Poole, UK). Methanol was obtained from BDH (Poole, UK). Water was distilled before use.

2.2 Preparation of reducing-terminal derivatives

Reducing-terminal derivatives were prepared by reductive amination by a modification [29] of the method described by Bigge *et al.* [30]. Briefly, the glycans, in dimethylsulphoxide (20 μ L) and acetic acid (2 μ L) were heated with excess of 2-aminobenzamide (2-AB), N-[2-diethylamino]ethyl-4-aminobenzamide (procainamide, Proc) or 2-aminobenzoic acid (2-AA) for 5 min at 80°C. An excess of sodium cyanoborohydride was added and heating was continued for a further 2 h. Clean-up was by ascending paper chromatography on Whatman (Maidstone, Kent, UK) 3MM Chr paper followed by desalting with a small column of AG50 resin (Bio-Rad, Henel Hempstead, Herts, UK) (100–200 mesh).

2.3 ESI-MS

ESI-MS was performed with a Waters-Micromass Q-ToF Ultima Global instrument (Waters/Micromass, Manchester, UK). Samples (about 50 pm/ μ L) in 1:1 v/v methanol:water containing 0.1 M ammonium hydroxide, or ammonium nitrate (negative ion) or sodium acetate (positive ion), were infused at 5 μ L/min. The ion source was maintained at 120°C, the nebulizer gas at 100°C and the cone and desolvation nitrogen flows were 50 and 450 L/h respectively. The infusion needle potential was 3.0 kV, the cone was at 100 V and the RF-1 voltage, which has an effect similar to that of the cone on older instruments, was normally in the range 180–250 V or 80 V for singly or doubly charged ions respectively. Spectra (2 s scans) were acquired with a digitization rate of 4 GHz and accumulated until a satisfactory signal:noise ratio had been obtained. For MS/MS data acquisition, the parent ion was selected at low resolution (about 5 *m/z* mass window) to allow transmission of isotope peaks and fragmented with argon at a pressure (recorded on the instrument's pressure gauge) of 0.5 Bar. The voltage on the

collision cell was adjusted with mass and charge to give an even distribution of fragment ions across the mass scale or as described below. Typical values were 80–120 V for the singly charged ions and 30–50 V for the doubly charged ions. Other voltages were as recommended by the manufacturer. Instrument control, data acquisition and processing were performed with a MassLynx data system (Version 4.0) running on a Pentium IV PC with the Microsoft Windows XP operating system.

2.4 MALDI mass spectra

MALDI mass spectra were recorded either with the above Q-ToF instrument or with a Waters-Micromass ToFSpec 2E reflectron-TOF instrument. The sample (about 10 pM) in water (0.5 µL) was mixed with the matrix (0.3 µL of a saturated solution of 2,5-dihydroxybenzoic acid (DHB) in acetonitrile) and allowed to dry under ambient conditions on the MALDI target. The sample:matrix mixture was then recrystallised from ethanol (0.1 µL). MS³ and MS⁴ spectra were obtained with a Shimadzu-Kratos (Manchester, UK) Axima QIT tandem ion-trap/TOF mass spectrometer [31] and high energy MS² spectra were acquired with a Micromass AutoSpec QFPD instrument as described earlier [32].

3 Results and discussion

3.1 Intact glycoproteins

Spectra of intact glycoproteins can be obtained either by MALDI or ESI but individual glycoforms can only be resolved from small proteins (up to about 20–40 kDa) containing a limited number of glycans, preferably attached to only one site [33–36], as shown for glycoforms of soluble CD-59 [34] in Fig. 1. If the mass of the protein is known, the gly-

can masses can be obtained by difference. Glycan compositions, in terms of the constituent isobaric monosaccharides, can then be calculated from these masses because *N*-linked glycans contain only a limited number of monosaccharide types (hexose HexNAc *etc.*) and combinations of the masses of these monosaccharides give unique masses for most combinations in the mass range of the common *N*-linked glycans. Table 1 lists the masses of the common monosaccharide constituents.

Larger glycoproteins and those with several glycosylation sites usually give unresolved mass peaks, particularly by MALDI and, in these cases, the protein needs to be digested with a protease, usually trypsin, to produce a mixture of low molecular weight peptides and glycopeptides. If the glycosylation sites are now separated to specific glycoproteins, the glycosylation profile at each individual site can be determined. However, glycopeptides often give very weak spectra in the presence of peptides and some form of fractionation is usually necessary. Several methods for identifying glycopeptides in peptide/glycopeptide mixtures separated by HPLC have been published [37, 38]. Another approach is to digest the protein with a nonspecific protease, such as pronase, to leave either Asp or a small peptide attached to the glycan [39, 40]. The sequence of the peptide can sometimes be used to determine the glycosylation site.

3.2 Glycan release

Although structural determination of the glycans can be performed with the glycopeptides, this is more commonly achieved following their release from the glycoprotein either chemically, usually with hydrazine [19, 20, 41] or enzymatically. Common enzymes are protein *N*-glycosidase F (PNGase F) [42, 43] which releases most glycans, except those containing a 3-linked fucose attached to the reducing terminal GlcNAc residue [44], PNGase A which will release

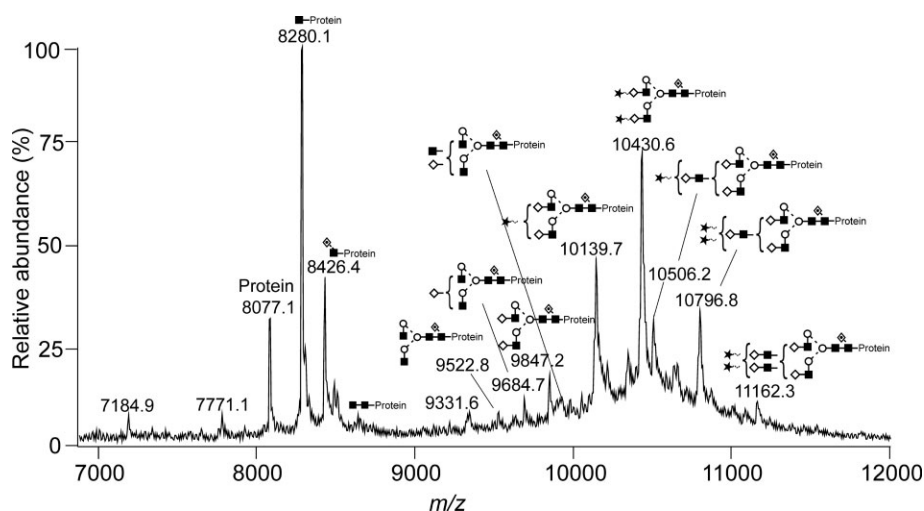


Figure 1. Positive ion MALDI mass spectrum of glycoforms of soluble CD59. Key to symbols used for the constituent monosaccharides in this and subsequent figures: $\diamond$ = galactose, $\blacksquare$ = GlcNAc, $\circ$ = mannose, $\diamond$ = fucose, $\star$ = *N*-acetylneuraminic (sialic) acid.

Table 1. Residue masses of common monosaccharides

Monosaccharide	Residue formula	Residue mass
Pentose	C ₅ H ₈ O ₄	132.042 ^{a)} 132.116 ^{b)}
Deoxy-hexose	C ₆ H ₁₀ O ₄	146.078 146.143
Hexose	C ₆ H ₁₀ O ₅	162.053 162.142
Hexosamine	C ₆ H ₁₁ NO ₄	161.069 161.157
HexNAc	C ₈ H ₁₃ NO ₅	203.079 203.179
Hexuronic acid	C ₆ H ₈ O ₆	176.032 176.126
<i>N</i> -acetyl-neuraminic acid	C ₁₁ H ₁₇ NO ₈	291.095 291.258
<i>N</i> -glycoyl-neuraminic acid	C ₁₁ H ₁₇ NO ₉	307.090 307.257

a) Top figure = monoisotopic mass (based on C = 12.000000, H = 1.007825, N = 14.003074, O = 15.994915)

b) Lower figure = average mass (based on C = 12.011, H = 1.00794, N = 14.0067, O = 15.9994)

The masses of the intact glycans can be obtained by addition of the residue masses given above, plus the mass of the terminal group (H₂O for an unmodified glycan) 18.011 (monoisotopic) and 18.152 (average) and the mass of any reducing-terminal or other derivative.

these glycans, and endoglycosidase H (endo H) which releases high mannose and hybrid glycans. The PNGases are amidases that release the glycans as the intact glycosylamine whereas endo H cleaves between the GlcNAc residues of the chitobiose core. Reducing termini are left intact with all of these release methods allowing the glycans to be derivatised with fluorescent or other reagents to aid detection by various techniques. PNGase F, in fact, releases the carbohydrate as the glycosylamine but this rapidly converts to the native sugar, particularly at low pH. The Asn residue to which it was attached is converted into Asp producing a mass difference of one mass unit and enabling the attachment site and degree of glycosylation to be determined by mass spectrometry. Performing the reaction in H¹⁸O introduces an ¹⁸O label to aid detection [45, 46]

For proteomic work, several methods are available for releasing the glycans within the separating gel. The method that we use was developed by Küster *et al.* [28] and involves the diffusion of PNGase F into the gel after protein separation, followed by extraction of the glycans with water and acetonitrile. Clean-up of neutral glycans is with a three-bed microcolumn containing AG50, C18 and AG3 to remove cations, organics and anions respectively. For sialylated glycans it is necessary to neutralise the acid groups by formation of methyl esters [47] to avoid their removal by the AG3 resin. Several other methods have been developed elsewhere;

of particular interest are those that involve the release of the glycan from 2-D gels [48, 49] and those that involve release from glycoproteins directly on the MALDI target [50, 51].

3.3 Structural determination of released glycans

Released glycans can be profiled by techniques such as gel filtration chromatography (see [4], by normal-phase HPLC [52] after derivatisation with a fluorescent dye such as 2-AB [6, 10, 30], 2-aminopyridine (2-AP) [53, 54], 2-AA [55], trimethyl-(4-aminophenyl)ammonium (TMAPA) chloride [54] or 2-aminoacridone (AMAC) [56], or by MALDI-MS [8, 9]. Structural information can be obtained directly by normal-phase HPLC because retention time is directly proportional to composition [10]. MALDI mass measurements yield composition information by matching experimental masses with various compositions of monosaccharides using the data in Table 1. Structural details may be obtained by sequential exoglycosidase digestions (see Dwek *et al.* [4] for suitable enzymes) with profiling by the above methods. With respect to profiling by MALDI-MS, several investigators have developed micro-methods whereby the exoglycosidase digest can be performed directly on the MALDI target [50, 57, 58]. An alternative approach to structural determination that we [29, 31, 32, 59–68] and others [e.g. 13, 69–82] have been developing, is by MS fragmentation.

3.4 Ionization

3.4.1 MALDI ionization

The use of MALDI to obtain ions from carbohydrates was first reported by Karas and Hillenkamp [83] in one of their early papers on the technique and it was first applied to *N*-linked glycans by Mock *et al.* in 1991 [33]. Many matrices have been developed [15], but one of the earliest, DHB [84] is still the most popular. Although the dried droplet method of sample preparation works reasonably well, more homogeneous targets and, consequently, better performance has been achieved by techniques such as recrystallisation of the initially dried sample spot from ethanol [85], or by mixing the DHB with various additives such as 2-hydroxy-5-methoxybenzoic acid [86], 1-amino-*iso*-quinoline (HIQ) [87], spermine [88] or fucose [89]. 2,4,6-Trihydroxyacetophenone (THAP) is more appropriate for sialylated glycans, particularly when mixed with ammonium citrate [90].

MALDI is comparatively tolerant to the presence of contaminants but it is important to desalt samples before analysis. Suitable methods include the use of resins, such as those described above, or graphitised carbon [91] usually packed into microcolumns or added directly to the MALDI target [92]. Alternatively, clean-up can be achieved by use of membranes such as low molecular cut-off dialysis membranes or Nafion 117 (Aldrich, Poole, Dorset, UK) [93]. We prefer the latter technique; a small square of membrane is floated on the surface of water and about 1 µL of the aqueous glycan

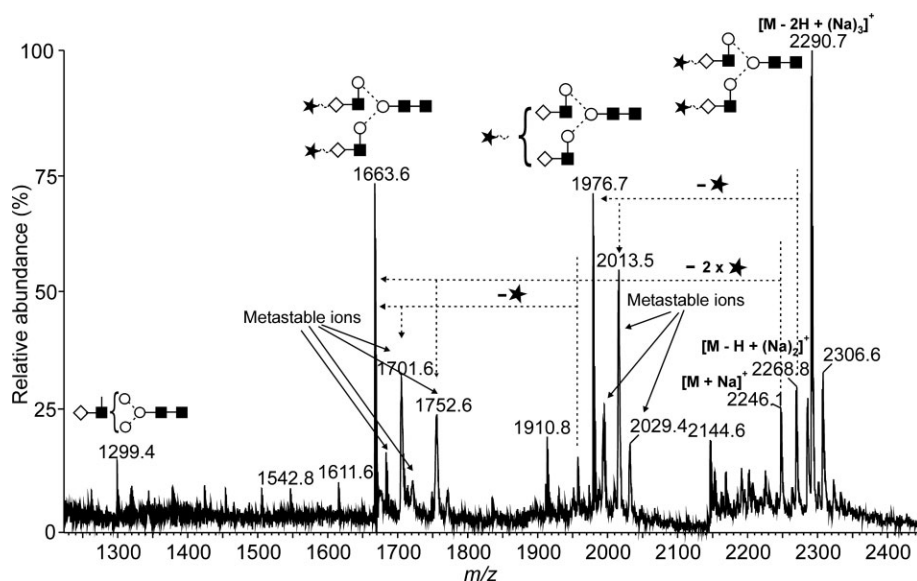


Figure 2. Positive ion MALDI mass spectrum of a disialylated biantennary *N*-linked glycan recorded with a reflectron-TOF mass spectrometer. Symbols as in Fig. 1.

solution is placed onto the surface. After 15–30 min, the solution is transferred to the MALDI target and mixed with the matrix.

MALDI ionization produces predominantly $[M + Na]^+$ ions from neutral glycans [15, 33, 94] although other cations can be introduced by doping the matrix with an appropriate salt [93]. Neutral glycans do not usually form negative ions. Sialylated glycans produce a mixture of $[M - H]^-$, $[M + Na]^+$ and $[M - nH + (n+1)Na]^+$ ions, the latter being partially the result of salt formation. However, there is considerable in-source and post-source fragmentation by loss of sialic acid. When recorded with reflectron-TOF instruments, the post-source fragments produce prominent broad metastable peaks (Fig. 2) whose apparent mass can be related to the parent and fragment ion masses by means of the relationship published by Harvey *et al.* [60]. In order to minimise the effects of fragmentation, some investigators examine sialylated glycans with TOF instruments operated in linear mode [36]. However, although this method only removes the post-source component, Mortz *et al.* [95] have reported that there is only a small amount of in-source fragmentation from sialylated glycans if conditions are optimised. We prefer to form methyl esters of the sialic acids by reaction of their sodium salts with methyl iodide [47]. This technique not only stabilises the sialic acids but also prevents negative ion formation and the production of positive ions produced by the sodium salts, thus allowing quantitative glycan profiling to be made in the positive ion mode.

Early studies on the quantitative nature of MALDI-MS showed that there was a linear relationship between the signal produced by the $[M + Na]^+$ ion from neutral glycans and the amount applied to the target [85] and that reasonably accurate quantitative measurements could be made with and without an internal standard if precautions were taken to allow for the inhomogeneous nature of the target surface.

Although accuracy was reasonably good, precision was lower and in the region of $\pm 10\%$. *N*-linked glycans with masses above about 1 kDa were found to give comparable signals, irrespective of structure [96], probably as the result of a similar ionization mechanism in the formation of the $[M + Na]^+$ ion. Peptides and glycopeptides, on the other hand, give signals of very different abundance depending on the proton affinity of their constituent amino acids.

3.4.2 ESI

ESI produces several types of ion, depending on the ion source conditions and additives to the solvent. $[M + Na]^+$ ions can be obtained, as in MALDI, at high cone voltages [63] but are usually accompanied by a considerable amount of in-source fragmentation. Sensitivity, however, generally falls as a function of increasing mass such that it is difficult to record $[M + Na]^+$ ions from the larger glycans. Nevertheless, both the $[M + Na]^+$ ions from underivatised and derivatised glycans and ions formed by adduction with other metals provide informative fragmentation spectra containing both glycosidic and cross-ring fragments (see below) [32, 62–65, 69, 97]. Both $[M + H]^+$ and doubly charged ions produced by protonation are formed under milder ion-source conditions but their MS/MS spectra are comparatively simple and do not generally contain ions produced by cross-ring cleavages. An advantage of ESI over MALDI is that the milder ionization conditions cause little, if any, sialic acid loss.

In negative ion mode, the predominant ion from neutral glycans is $[M - H]^-$ but these ions frequently give rise to in-source fragments. However, stable negative ions of the type $[M + A]^-$, where *A* = an anion such as SO_4^{2-} [98, 99], Cl^- [100–103], Br^- or NO_3^- [67], can be produced by use of the appropriate salt. We have found that nitrate adducts formed by addition of ammonium nitrate to the electrospray solvent

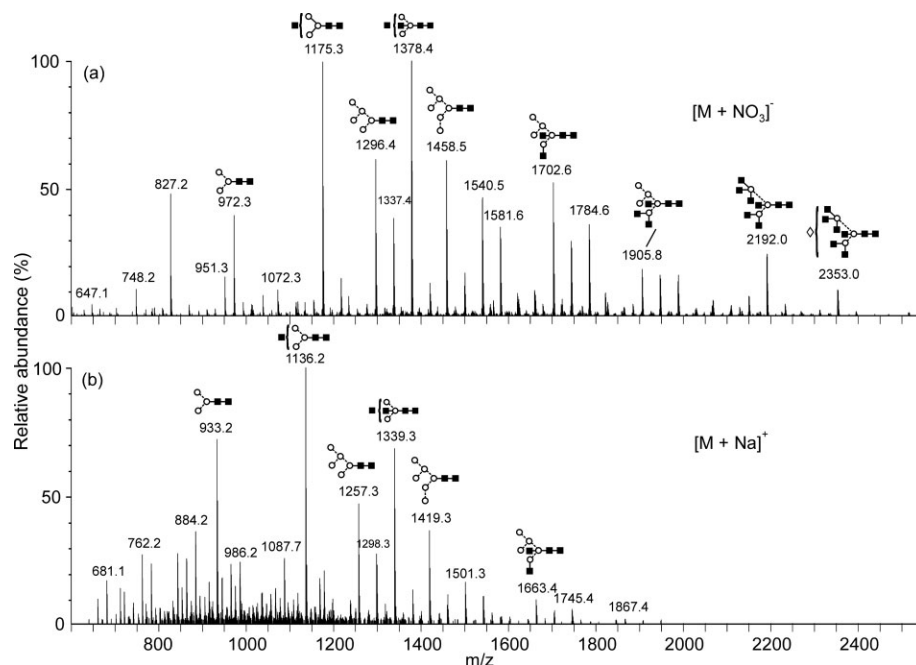


Figure 3. (a) Negative ion spectrum of $[M + \text{NO}_3]^-$ ions from *N*-glycans from chicken egg glycoproteins (mainly ovalbumin). (b) Positive ion electro spray spectrum of the same glycan mixture recorded with a high cone voltage to produce mainly $[M + \text{Na}]^+$ ions. Symbols as in Fig. 1.

is one of the best methods for producing these ions [29]; the nitrate adducts are stable and increase sensitivity by about an order of magnitude over formation of $[M - \text{H}]^-$ ions. Addition of the nitrate in the form of metal salts, such as silver nitrate, produces a higher background as the result of cluster formation, thus limiting the sensitivity. Another advantage of the nitrate adducts is that they enable larger glycans to be seen by electrospray than is possible with other ions such as $[M - \text{H}]^-$ or $[M + \text{Na}]^+$ (Fig. 3). As well as forming $[M + \text{NO}_3]^-$ ions, the larger glycans form $[M + (\text{NO}_3)_2]^{2-}$ ions that fragment with the exclusive production of singly charged ions that are almost exactly the same as those formed from the singly charged $[M + \text{NO}_3]^-$ adducts (see below). Chloride adducts provide comparable spectra but glycan profiles can be distorted by the chlorine isotope pattern.

3.5 Fragmentation

Carbohydrates show two types of fragmentation, cleavages of the bonds between the sugar rings, known as glycosidic cleavages, and cleavages of the rings themselves, known as cross-ring cleavages. The glycosidic cleavage ions provide information on carbohydrate sequence and branching whereas the cross-ring cleavage ions provide information on linkage. Glycosidic cleavages from even-electron ions such as $[M + \text{Na}]^+$ result in the loss of neutral molecules and are, thus, always accompanied by hydrogen migrations.

The nomenclature generally used for describing these ions is that introduced by Domon and Costello in 1988 [104]; ions retaining the charge on the nonreducing terminus are named A (cross-ring) B and C (glycosidic) whereas those ions retaining charge on the reducing terminus are X (cross-ring)

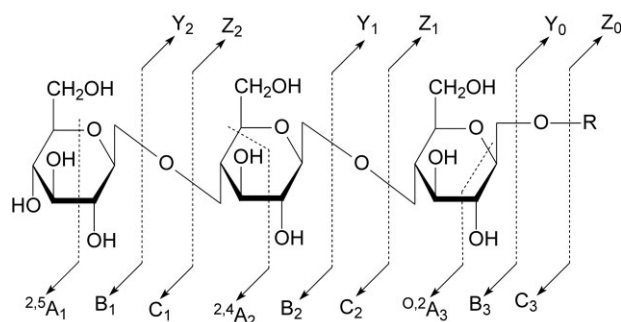


Figure 4. Nomenclature introduced by Domon and Costello [104] for describing the fragmentation of carbohydrates.

Y and Z as outlined in Fig. 4. Numbering (as subscripts) is from the reducing terminus for the X, Y and Z ions and from the nonreducing end for the others. Cross-ring cleavage ions additionally have a superscript number defining the bonds that are broken. In order to avoid the problem of the subscript number changing for similar fragmentations as the result of different chain lengths, we use a modification of this system in which B-ions produced by cleavage of the reducing-terminal GlcNAc are termed B_R ions and Y-ions from the nonreducing terminus are Y_{NR} ions. By extension, cleavages at the next glycosidic bonds are B_{R-1} and Y_{NR-1} respectively.

High quality fragmentation spectra can be obtained from electrosprayed ions by use of Q-ToF-type instruments [72] but, until recently, recording of the fragment ions from MALDI-generated ions was restricted mainly to post-source decay (PSD) technology [77] with its inherent problems of

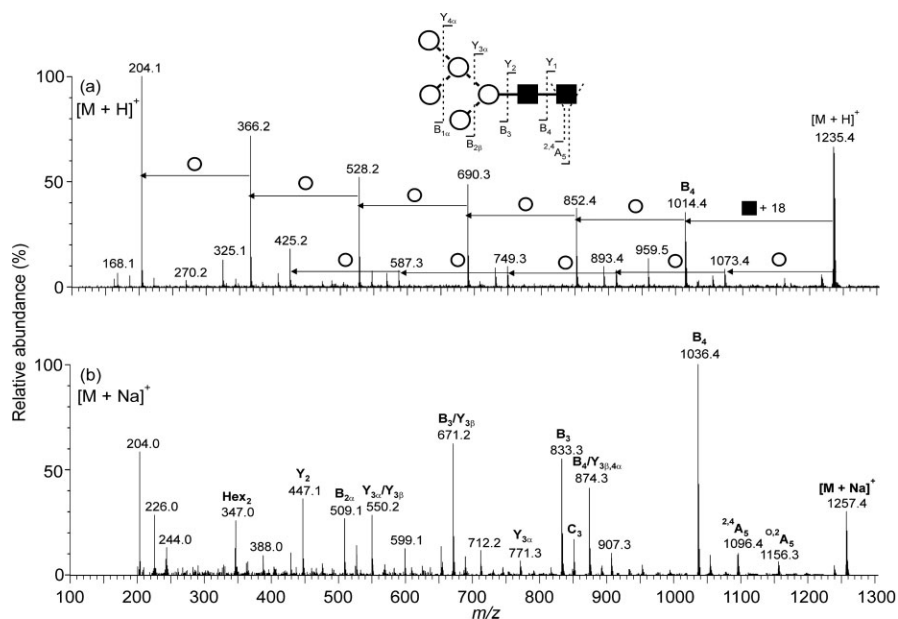


Figure 5. Positive ion MS² spectra of (a) the $[M + H]^+$ and (b) the $[M + Na]^+$ ions from the high-mannose glycan $(Man)_5(GlcNAc)_2$. Symbols as in Fig. 1.

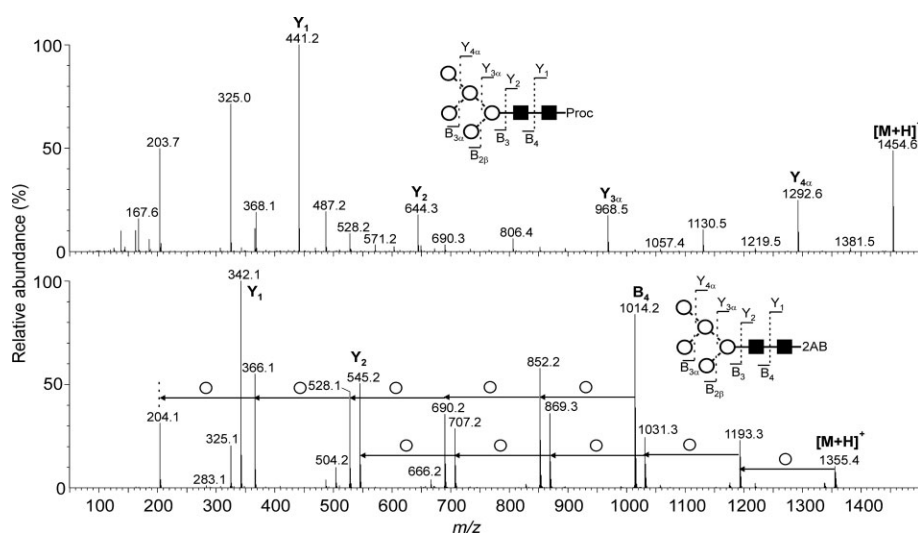


Figure 6. Positive ion MS² spectra of (a) the procainamide (Proc) [65] and (b) the 2-AB derivatives of the high-mannose *N*-glycan $(Man)_5(GlcNAc)_2$. Symbols as in Fig. 1.

low resolution and the necessity to record spectra in segments with the majority of instruments. However, MALDI ion sources have now been interfaced to Q-ToF instruments [97, 105–107] allowing CID to be used as with electrospray. Spectra recorded from each ionization method are identical [97].

3.5.1 Fragmentation of $[M + H]^+$ ions

Fragmentation of $[M + H]^+$ ions from underivatized *N*-linked glycans are relatively simple and not particularly informative as they contain mainly B-, Y- and B/Y internal cleavage ions (Fig. 5a) [63]. Cross-ring cleavage ions are generally absent. An important complicating factor is the appearance of rearrangement ions [108–111], particularly from glycans derivatised at the reducing terminus. The

presence of such ions can considerably complicate the interpretation of the spectra of unknown glycans. An example from the *N*-linked glycans is the migration of fucose towards the derivatised reducing terminus and expulsion of sugar residues from the internal region of the structure [112, 113].

Glycans that are derivatised at the reducing terminus by reductive amination such that the derivative contains a quaternary ammonium group [114, 115] or an amine that can be protonated [65, 70, 116], retain the charge on the derivative and, consequently, their fragmentation spectra are dominated by Y-type cleavages that provide predominantly sequence information (Fig. 6a). Glycans derivatised with neutral fluorophores such as 2-AB and ionized as $[M + H]^+$ ions, fragment in a similar manner to the underivatized glycans (Fig. 6b). When examined by LC/MS using normal-

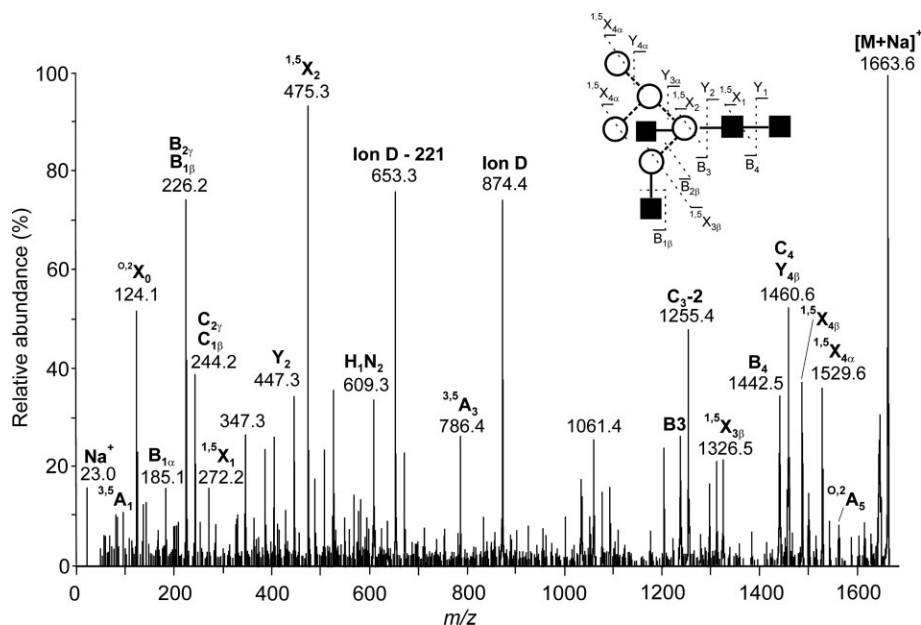


Figure 7. High (800 eV) energy CID spectrum of the $[M + Na]^+$ ion from a hybrid *N*-linked glycan. H = hexose, N = GlcNAc. Symbols as in Fig. 1.

phase HPLC columns and an ammonium formate buffer, a range of $[M + 2X]^{2+}$ ions where $X = H^+$, NH_4^+ or Na^+ can be produced. If one of the X atoms is hydrogen, fragmentation is normally similar to that of the $[M + H]^+$ ion with the production of abundant singly charged Y ions providing linkage information.

3.5.2 Fragmentation of $[M + \text{alkali metal}]^+$ ions

Fragmentation of $[M + Li]^+$ and $[M + Na]^+$ (Fig. 5b) ions from *N*-linked glycans are more complicated than fragmentation of the $[M + H]^+$ ions. Internal fragments (losses from two or more terminal sites) are more common and, in addition, A-type cross-ring fragments are present [63, 73] although generally in fairly low abundance from the low energy spectra that are typical of spectra produced by Q-ToF instruments. The most significant of these ions are the $^{0,2}A_R$ and $^{2,4}A_R$ ions from the reducing terminus and $^{0,3}A$, $^{0,4}A$ and $^{3,5}A$ ions from the branching mannose residue; the latter two ions can be used to define the composition of the individual antennae attached to the core mannose. An additional advantage of the fragmentation of the $[M + \text{metal}]^+$ ions is that the rearrangement ions reported from the $[M + H]^+$ ions are absent [117]. It is increasingly difficult to fragment adducts of the higher alkali metals to the extent that $[M + Cs]^+$ ions yield practically no fragments other than Cs^+ [63, 118, 119]. Under high energy conditions, such as with spectra recorded with magnetic sector (Fig. 7) [32] or ToF/ToF instruments [78, 79], additional abundant X-type fragment ions are present. In addition, in the spectra of *N*-glycans containing a bisecting GlcNAc residue, such as that shown in Fig. 7, a prominent diagnostic ion is produced by loss of the bisecting

GlcNAc residue as 221 mass units (C-cleavage) from the internal cleavage ion, labelled "Ion D", that is formed by loss of the chitobiose core and 3-antenna [32].

Instruments such as the ion-trap are able to perform successive stages of fragmentation allowing MS to be used to probe more deeply into the molecular structures and to investigate fragmentation mechanisms leading to diagnostic ions. We have recently used the Shimadzu-Kratos MALDI-QIT tandem ion-trap/TOF instrument to define fragmentation pathways in *N*-linked glycans and have confirmed that many fragment ions in the positive ion spectra are the result of several mechanisms [31] with the result that structural conclusions can be ambiguous. For example, in the high mannose series, an ion containing the constituents of the 6-antenna and the branching mannose (e.g., B_{R-1}/Y_{NR-2} ion ($B_4/Y_{3\beta}$) from $(Man)_9(GlcNAc)_2$, Fig. 8) is more abundant than other ions containing only mannose and is indicative of the 6-antenna composition. However, in the spectra of complex glycans such as the common biantennary glycan $((Gal)_2(Man)_3(GlcNAc)_4)$, the abundant ion at m/z 712 with a mass indicating a similar structure is formed mainly by a $B_R/Y_{NR-1}/Y_{NR-1}$ mechanism (Fig. 9).

3.5.3 Fragmentation of negative ions

Fragmentation of negative ions has received less attention than that of positive ions but recent work has shown considerable differences in the mechanisms that produce the various fragments. Whereas fragmentation of positive ions yields mainly B- and Y-type glycosidic cleavage ions (Fig. 5b), negative ions of the type $[M - H]^-$ and $[M + X]^-$, where X = an anion such as Cl^- or NO_3^- , and which fragment to give

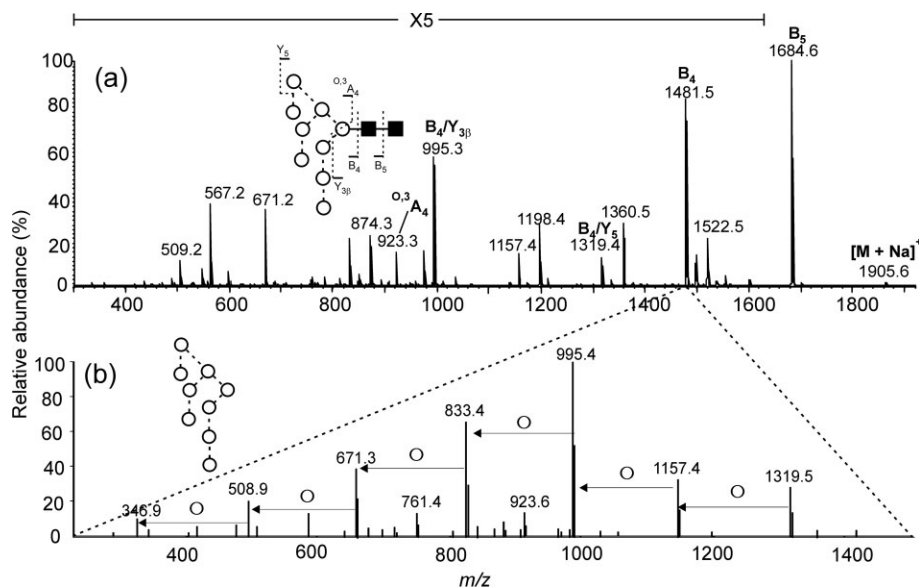


Figure 8. (a) Positive ion MALDI MS² spectrum of the $[M + Na]^+$ ion from high mannose glycan $(Man)_5-(GlcNAc)_2$, (b) MS³ spectrum of the B_4 ion ion at m/z 1481.5. Symbols as in Fig. 1.

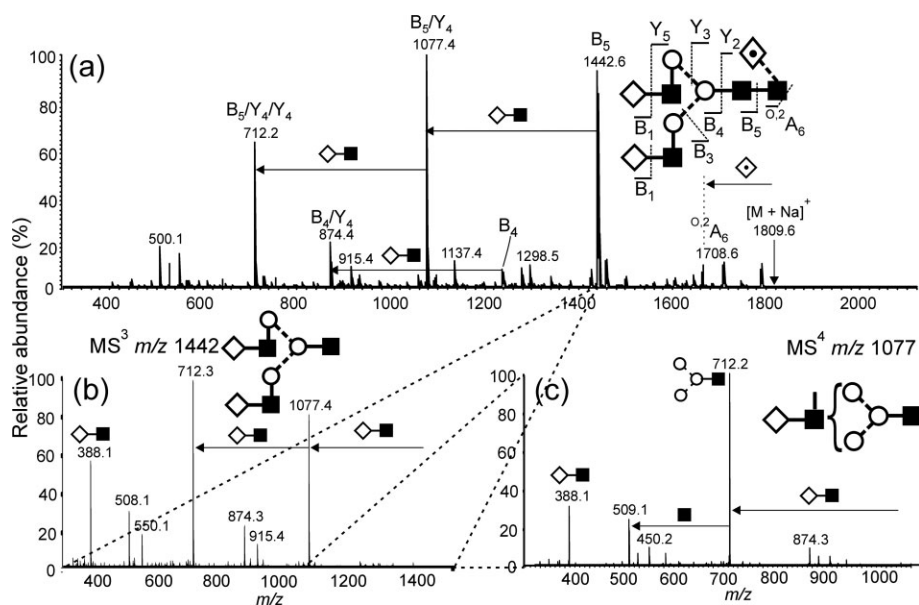


Figure 9. (a) MALDI mass spectrum $[M + Na]^+$ ion of the biantennary *N*-linked glycan $(Gal)_2(Man)_3(GlcNAc)_4(Fuc)_1$, (b) MS³ CID spectrum of the B_5 ion (m/z 1442.6), (c) MS⁴ spectrum $[M + Na]^+ \rightarrow 1442.6 (B_5) \rightarrow 1077.4 (B_5/Y_3) \rightarrow$. Symbols as in Fig. 1.

$[M - H]^-$, tend to form A-type cross-ring cleavage ions and C-type fragments (Fig. 10) [72, 120–125]. This difference is the result of the $[M - H]^-$ ions being formed by loss of a proton from various hydroxy groups to leave an electron-rich centre that catalyses ring cleavage by mechanisms similar to that shown in Fig. 11 [29, 68]. Loss of protons from different positions catalyse specific cleavage reactions with the result that the ions present in the negative ion spectra are much more specific and diagnostic than those found in the positive spectra and which are frequently the result of multiple reactions [31]. For example, the ion containing the 6-antenna and branching mannose residue (Ion D) is specific for all glycans studied and is formed essentially by a C_{R-1}/Z_{NR-X} process. The

ion is accompanied by another 18 mass units lower formed, presumably, by loss of H_2O . When a bisecting GlcNAc residue is present, the D-ion eliminates GlcNAc as a GlcNAc molecule (221 u) rather than water to give what is usually the most abundant ion in the spectrum (Fig. 10b). The mass of an abundant $^{2,4}A_R$ ion indicates the presence or absence of a fucose residue at the 6-position of the reducing-terminal GlcNAc. Specific structural features that are revealed by ions in these negative ion spectra are summarised in Table 2.

Negative ions formed from derivatised glycans, where the derivative is neutral, such as 2-AB, give spectra that are similar to those of the underivatised glycans with the exception that the abundant $^{2,4}A_R$ ion is missing. Acidic deriva-

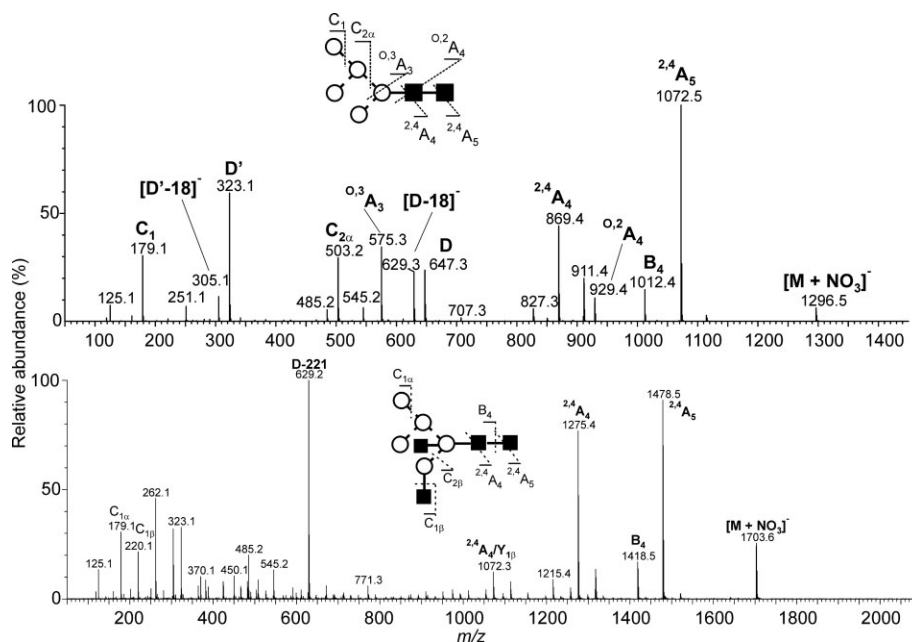


Figure 10. Negative ion CID spectra of the nitrate adducts of (a) the high-mannose *N*-linked glycan (Man)₅-(GlcNAc)₂ and (b) the hybrid glycan (Man)₅(GlcNAc)₄. Symbols as in Fig. 1.

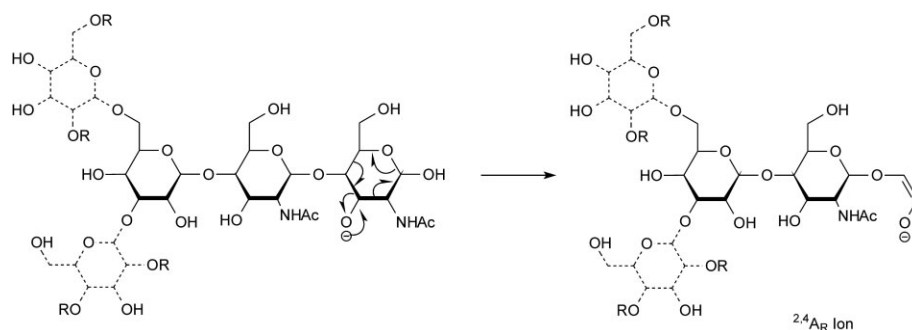


Figure 11. Proposed mechanism for the formation of $^{2,4}A_R$ ions in the negative ion CID spectra of *N*-linked glycans.

tives, such as those prepared from 2-AA yield mainly C-type glycosidic fragments that provide sequence information as the result of the localised negative charge (Fig. 12) [126]. Similarly, fragmentation of sialylated glycans is mainly directed by the localised charge on the acid group resulting in the hydroxy-derived fragments appearing in only low abundance.

Negative ion formation by use of ammonium nitrate, therefore, not only provides one of the most sensitive and versatile electrospray techniques available but also gives ions that fragment by specific pathways to provide structural information that is not readily available by other techniques. Since this work was presented at the BSPR meeting, high quality fragmentation data have been obtained at the low fm level from glycoproteins extracted from serum and introduced into the electrospray ion source of a Q-ToF mass spectrometer using nanospray capillaries. Sialylated glycans, in particular, give strong multiply charged ion peaks whose height can provide quantitative information on the glycan content. Zamfir *et al.* [127] have already demonstrated that

nanospray sample introduction can be automated with equipment such as the Advion Nanomate™ (Norwich, UK). Consequently, this technique appears to offer great promise for high-throughput proteomic work where the structures of attached glycans need to be determined.

4 Concluding remarks

MALDI-MS provides a rapid method for profiling neutral *N*-linked glycans and obtaining information on their constituent monosaccharides. However, sialylated glycans are unstable and require special treatment such as derivatisation of the acid groups or the use of particular matrices. Positive ion spectra are dominated by $[M + Na]^+$ ions which can be fragmented by CID to give informative spectra containing both glycosidic and cross-ring fragments. A disadvantage of these spectra, however, is the presence of many internal fragments and ions produced by multiple pathways that complicate their interpretation.

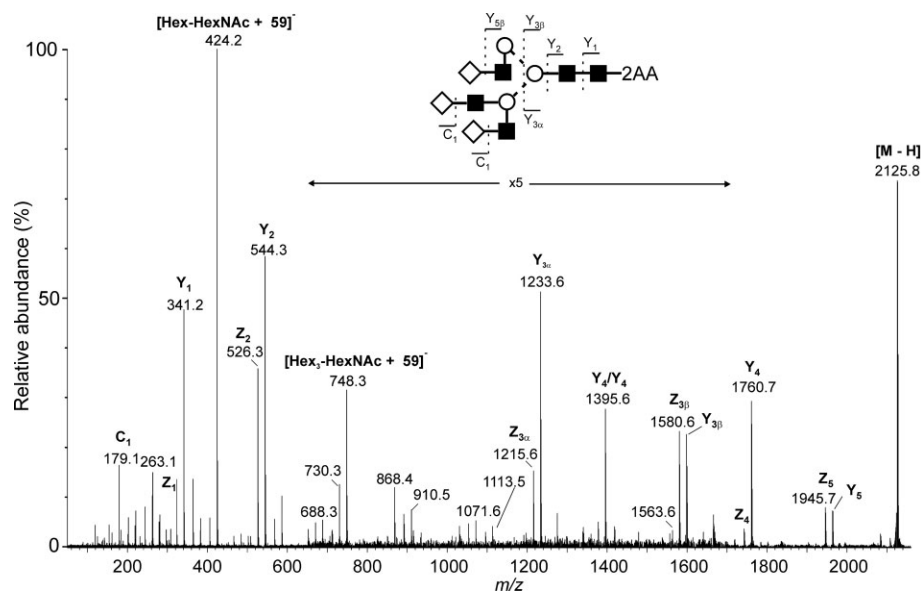


Figure 12. Negative ion CID spectrum of the 2-AA derivative of the complex triantennary glycan (Gal)₃-(Man)₃(GlcNAc)₅. Symbols as in Fig. 1.

Table 2. Ions defining structural features in the negative ion spectra of *N*-linked glycans

Structural feature	Ion
Composition	Molecular ion (data from Table 1)
Antenna sequence	C ions
Fucose at 6-position of reducing terminus	[M – X – 307] [–] (^{2,4} A _R ion) ¹
No fucose at 6-position of reducing terminus	[M – X – 161] [–] (^{2,4} A _R ion)
Composition of 6-antenna	D and [D-18] [–] ions (e.g. <i>m/z</i> 688, 670 = Gal-GlcNAc-Man; 647, 629 = Man ₃) ^{0,3} A _{R-2} ^{0,4} A _{R-2}
Presence of bisect	No or weak D ion, strong D-221
Composition of antennae with 2- or 4-linked substituents	Substituents plus 101 u from mannose (E ion)
Presence of sialic acid	<i>m/z</i> 290.1
Presence of α2→6-linked sialic acid	<i>m/z</i> 306.1
Gal-GlcNAc in antenna	<i>m/z</i> 424.1 (Gal-GlcNAc + 59)
Presence of α-galactose on antenna	<i>m/z</i> 586

1. X = Anionic adduct or H.

ESI-MS, although more sensitive to the presence of contaminants, is more versatile in that it is easy to change the type of ion that is initially formed by suitable manipulation of additives to the electrospray solvent. The problem of sialic acid instability is also minimised. Negative ion formation by the use of anion addition is particularly advantageous as it is possible to form singly charged ions of larger glycans that do not give abundant ions under positive ion conditions. Fur-

thermore, these negative ions fragment by specific pathways to give definitive structural information from a single spectrum, some of which is difficult to obtain by classical methods such as exoglycosidase digestion. Consequently, these negative ion spectra offer considerable advantages for the identification of the glycans attached to glycoproteins in proteomic and glycoproteomic studies.

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