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Review

Matrix-assisted laser desorption/ionisation mass spectrometry of oligosaccharides and glycoconjugates

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Abstract

The technique of matrix-assisted laser desorption/ionization mass spectrometry (MALDI) is described and examples are given of its use for the examination of glycoproteins, glycopeptides, glycolipids and oligosaccharides. Abundant $[M+H]^+$ ions are produced by the glycoproteins and glycopeptides, whereas glycolipids and oligosaccharides give mainly $[M+Na]^+$ ions. Resolution on time-of-flight (TOF) instruments is poor but improved resolution can be obtained by use of ion cyclotron resonance or magnetic sector instruments. Although the technique gives mainly $[M+Na]^+$ ions from neutral, underivatised oligosaccharides, with little fragmentation when implemented on TOF systems, the use of a reflectron enables fragment ions produced by post-source decay to be obtained. Acidic sugars give less satisfactory positive ion spectra with TOF analysers. but generally produce abundant negative ions. Extensive fragmentation is observed with these compounds when the spectra are recorded with magnetic sector instruments. Neutral glycolipids produce strong spectra from several matrices but acidic glycolipids show extensive fragmentation as the result of sialic acid loss.

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1. Introduction

Mass spectrometry, and in particular combined gas chromatography–mass spectrometry (GC–MS), have been used for monosaccharide analysis for several decades [1,2]. However, it is only in the last few years that ionization techniques capable of handling complex glycopeptides and oligosaccharides have been developed. Monosaccharide analysis requires derivatization and much work has been reported on the preparation and properties of methyl, acetyl and trimethylsilyl derivatives in particular [3]. Today, GC–MS is used extensively for composition analysis of oligosaccharides following hydrolysis or methanolysis to monosaccharides [4] and for the so-called "methylation analysis" for linkage determination [5,6]. In the most commonly used version of the latter technique, the oligosaccharide is permethylated, hydrolysed, and the resulting monosaccharides are acetylated prior to GC–MS analysis. The positions of the acetyl groups in the resulting permethylated alditol acetates identify those hydroxy groups involved in linkage of the parent oligosaccharide.

Fast atom bombardment (FAB) mass spectrometry is the most well known of the desorption techniques for analysis of intact carbohydrates [7,8]. Although not essential, derivatization, usually in the form of permethylation or peracetylation, provides great increases in sensitivity over the use of underivatised molecules and simplifies interpretation of fragmentation. Reducing-terminal derivatization has also given many advantages in both the positive and negative ion modes [9]. FAB spectra are generally characterised by the presence of an abundant molecular ion and many fragments that carry significant sequence information. Additional linkage information may be obtained by MS–MS [10].

The more recent desorption techniques, plasma desorption [11], and matrix-assisted laser desorption/ionization (MALDI) on time-of-flight (TOF) mass spectrometers [12] have, so far, been less widely used for oligosaccharide analysis but show promise in their ability to handle underivatized carbohydrates. Plasma desorption on TOF mass spectrometers has been used for examination of charged sugars such as heparin fragments [13] but, with the advent of MALDI, has received less attention in recent years. MALDI offers good sensitivity and the ability to be made compatible with several other types of mass spectrometric analyser which offer higher resolution than TOF instruments.

Although MALDI is a comparatively new innovation, laser ionization of carbohydrates without the use of a matrix has a longer history. For example, in 1984, Seydel et al. [14] developed a method for ionising glycolipids with the quadrupled frequency from a Nd–YAG laser after mixing the sample with either a sodium or caesium salt. The method has been in use for many years by this group, particularly for studies of dephosphorylated lipid-A [15–22] and other glycolipids [23,24]. Infra red lasers have also been used to ionise sugars [25] and considerable fragmentation has been found in the resulting spectra. Analysis of the mechanisms producing the fragment ions has revealed a considerable contribution from cross-ring cleavages in addition to the more common glycosidic cleavages [26]. All of the above work has been performed with comparatively low resolution TOF instruments as these instruments are compatible with the pulsed nature of the laser ion source. Much higher resolutions have been obtained, however, by use of ion cyclotron resonance (ICR) instruments. Thus, Lam et al. [27–29] have achieved mass accuracies in the order of 15–200 ppm with negative ions obtained from a pentasaccharide obtained from *Klebsiella* capsular polysaccharides. Few instruments equipped for laser ionization existed before 1990, but with the introduction of MALDI, laser instruments are now relatively common, easy to use and can produce spectra from most types of oligosaccharide and glycoconjugate.

2. General aspects of matrix-assisted laser desorption/ionisation

Matrix-assisted laser desorption/ionisation mass spectrometry was originally introduced [12]

and developed [30–35] for the ionisation of large peptides and proteins. However, it soon became apparent that many other types of compound, including glycolipids [36,37] and oligosaccharides, could be made to give strong signals. To obtain a spectrum, the sample is first mixed, in solution, with a large excess (about 5000 fold) of a suitable matrix, as described below, and introduced into the mass spectrometer. Sample sizes are usually in the low pmol range and sample:matrix volumes typically do not exceed 1–2 μl. Ionization is by irradiation with a laser, usually a nitrogen laser operating at 337 nm, although 266 nm has also been used. The technique is very sensitive (upper fmol range for small peptides) and capable of ionising very large molecules (several hundred thousand dalton).

2.1. Instrumentation

Because of the high mass capability and pulsed nature of MALDI, the technique is ideally coupled with a TOF analyser. The major disadvantage of linear TOF instruments is that they have low resolution, normally only a few hundred. Because of this, isotope peaks, alkali metal adducts and photochemical adducts are often not resolved. Additional peak broadening is seen at high laser powers as the result of an increased energy spread of the desorbed ions. Under these conditions, ions with reduced energy become relatively more abundant and, because they travel more slowly than the other ions, arrive at the detector later. Consequently, they appear to have higher masses than ions of the same m/z value having higher velocities. At high laser powers, therefore, peaks broaden to the high mass side and an increased mass is recorded. To obtain the best mass accuracy and resolution on a TOF instrument, the laser power needs to be kept as low as possible and is ideally set at just above the point at which the ions appear. It should be remembered that, because the TOF instruments have insufficient resolution to resolve the stable isotope peaks, the masses obtained will be the chemical or average masses unlike the monoisotopic masses usually encountered in other forms of mass spectrometry In spite of these difficulties, however, mass accuracies for oligosaccharides obtained with a linear TOF mass spectrometer have been reported to be as high as 0.1% under favourable conditions. Calibration may be external or internal, but the best results are obtained if an internal calibration compound is used. This compound should be chemically similar to the analyte and added at a concentration which gives an equivalent peak height.

Several methods are available to improve the resolution over that which can be obtained from linear TOF instruments. The first is to include a reflectron consisting of a reflecting electric field arranged so that the more energetic ions penetrate further into the field to such an extent that their increased path length just compensates for their increased velocity. The second method is to use a magnetic sector instrument fitted with an array detector [38] to capture the signal from the pulsed ion source. Normally sector instruments are scanned, but at rates that are much too slow to be compatible with the pulsed nature of the laser-generated ion beam. With an array detector, ions with differing m/z ratios can be accumulated from many shots of the laser and then read out into a data system. Resolutions in the order of 1000–3000 have been obtained. The main disadvantage of sector instruments is that the upper mass limit is restricted by the magnet, usually to only a few thousand dalton. However, this range is suitable for most oligosaccharide work. A third method for improving resolution would be to use an ICR instrument of the type mentioned above in connection with the direct laser ionization techniques [27–29].

2.2. Matrices

The function of the matrix is to dilute the sample, absorb the laser energy and ionise the sample. Each pulse from the laser vaporises both sample and matrix from the surface of the target and catalyses a chemical reaction which, in the positive ion mode, results in a proton or alkali metal atom being attached to the sample molecules. In the negative ion mode, the ionic species produced is usually $[M-H]^-$. The exact nature

of the ionization mechanism is unclear but it would appear that, at least in some cases, photochemical products from the matrix are responsible for the ionization. Vertes et al. [39] have proposed a hydrodynamic model of the MALDI process in which a plume of matrix and sample molecules is generated from the target surface after heating by the laser beam. Subsequent rapid expansive cooling of the plume prevents thermal degradation of the sample.

Most matrices are crystalline compounds, although a few liquids such as *m*-nitrobenzyl alcohol are effective. For the crystalline matrices, a solution of the matrix and the sample is allowed to crystallise on the mass spectrometric target as the solvent evaporates. Intimate contact between the matrix and the sample is essential and it is believed that the sample must be entrained within the crystal lattice for successful ionization to occur. Satisfactory matrices for positive ion work are usually low molecular weight organic acids with low volatility and the ability to absorb at the laser frequency (Fig. 1).

The first matrix to be reported for MALDI was nicotinic acid (I, Fig. 1) [12] but this compound, in addition to ionising the sample, produces abundant ions due to addition of photochemical degradation products to the sample molecules. These photochemical adducts are difficult to resolve with TOF instruments at higher masses, thus giving inaccurate mass measurements. A large number of other matrices have been investigated by Beavis and Chait and sinapinic acid (II) [40] has been found to be the best. This compound is still preferred for the ionization of larger proteins and glycoproteins. For smaller proteins, α-cyano-4-hydroxy-cinnamic acid (III) [41] is now preferred, although 2,5-dihydroxybenzoic acid (2,5-DHB, IV) [42] has also been found to give strong signals. In the negative ion mode, compounds that abstract a proton, such as 3-hydroxypicolinic acid (V), are preferred [43].

The first matrix to be used for oligosaccharides, 3-amino-4-hydroxybenzoic acid (VI), was reported by Mock et al. in 1991 [44]. However, this matrix has now been superseded by 2,5-DHB [45,46] which gives stronger signals. Typically, this matrix will be dissolved in a mixture of aqueous acetonitrile or aqueous ethanol, and the sugar in water. Unfortunately, when such mixtures evaporate, 2,5-DHB tends to crystallise from the periphery of the target spot in the form of long needles that print towards the centre of the target. In many cases the centre is devoid of crystals. To overcome this problem, we [46] have found that recrystallization of the dried target from ethanol leads to a much more even dis-

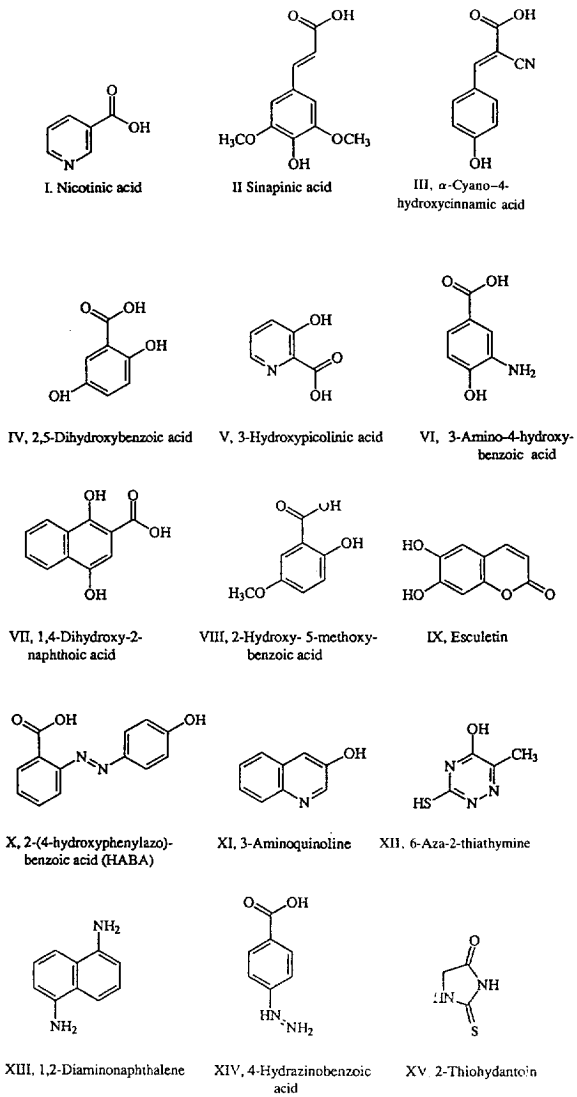

Fig. 1. Structures of common matrices used in matrix-assisted laser desorption mass spectrometry.

tribution of crystals and a stronger signal. In addition to aiding crystallisation, it is thought that this solvent also enables more of the sample molecules to become trapped into the crystal lattice. This is because, when the original aqueous:organic solvent is evaporating, the residual liquid becomes enriched in the aqueous phase, thus keeping the sugars in solution until late into the evaporation process. With only ethanol present, the lower solubility of the sugars probably aids uptake by the crystal. A modified form of 2,5-DHB, namely 1,4-dihydroxy-2-naphthoic acid (VII) [46] has also been found to have matrix properties and to crystallise in a much more even layer from aqueous solvents than 2,5-DHB. However, it does not produce such a strong signal. Karas et al. [47] have achieved some 2–3 fold improvements in the signal obtained from dextrans with molecular weights between 500 and 2000 dalton by incorporating 2-hydroxy-5-methoxybenzoic acid (VIII) into the 2,5-DHB matrix. The rationale behind this experiment was that the methoxy analogue caused disruption of the crystal lattice, thus facilitating the release of the sample ions.

Several other compounds have been investigated as suitable matrices for oligosaccharides. Most isomeric dihydroxybenzoic acids produce signals, but of considerably reduced intensity to that produced by the 2,5-dihydroxy isomer [46]. The reason for this is not clear although it may be speculated that, because the 2,5-isomer is the only isomer capable of decarboxylation to give a stable product, *para*-benzoquinone, then this photochemical reaction is responsible for the ionization. Other matrices such as α-cyano-4-hydroxycinnamic acid, esculetin (IX) (6,7-dihydroxycoumarin) and 2-(4-hydroxyphenylazo)benzoic acid (HABA, X) produce weaker signals from oligosaccharides on TOF instruments but are generally more effective for ionising glycolipids. 3-Aminoquinoline (XI) has recently been reported to give better results than 2,5-DHB for the ionization of plant inulins [48]. In particular, the spectral peaks produced from this matrix appeared sharper and the base line was lower. However, to date, the general applicability of this matrix for oligosaccharide analysis has not been determined.

2.3. General characteristics of MALDI spectra

Proteins and glycoproteins generally give abundant $[M + H]^+$ ions accompanied by lower amounts of dimeric and doubly-charged ions. Tri- and tetra-meric ions are occasionally seen. Multiply-charged ions are particularly abundant with α-cyano-4-hydroxy-cinnamic acid as the matrix. Oligosaccharides, on the other hand, give only single molecular peaks produced by the $[M + Na]^+$ species (Fig. 2). No multimeric ions are present. Weak fragment ions are occasionally seen in strong spectra recorded with linear TOF instruments but with reflectron instruments, considerable fragmentation is apparent. This is because, with linear TOF spectrometers, no electric or magnetic fields affect the ions after acceleration and, consequently, any fragment ions that are formed during the ion's path down the flight tube are not separated. The imposition of a reflectron, on the other hand, provides such a field, and the fragments may be focused by suitable adjustment of its voltage relative to that of the acceleration potential. Such fragmentation is known as post-source decay and has been used

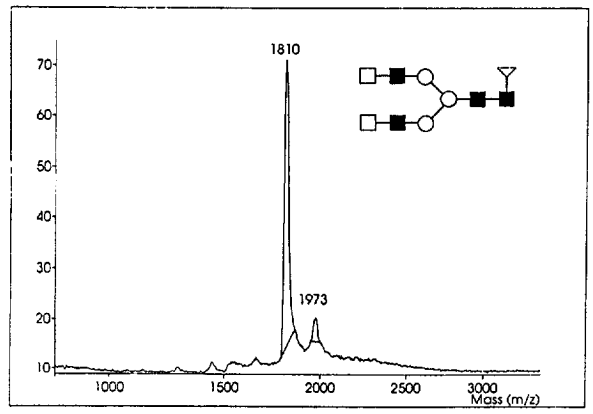

Fig. 2. MALDI mass spectrum of a typical underivatised sugar on a TOF instrument. Symbols used to identify the monosaccharides in this and other figures are: D-Galactose (□), D-mannose (○), L-fucose (6-deoxy-β-L-galactopyranose) (▽), 2-acetamido-2-deoxy-D-glucose (■), *N*-acetylneuraminic acid (sialic acid, ◆). Reprinted from Ref. [46].

by several investigators to obtain sequence information from oligosaccharides [49,50]. For example, in the study by Huberty et al. [49] on sugars obtained from recombinant human macrophage colony stimulating factor (rhM-CSF), cleavages were observed to occur at each glycosidic linkage in a biantennary oligosaccharide. Fragmentation seen in the spectra obtained with magnetic sector instruments (Fig. 3) is greater than that seen on linear TOF instruments, probably because the lower acceleration potential increases the ion lifetime before acceleration, thus allowing more ions to decay.

2.4. Quantitative aspects

Sample consumption with MALDI is minimal, but amounts in the fmole to low pmole range are currently required for successful sample handling. Only a small fraction of this amount is actually ionised. It has been reported that MALDI is 10–100 times as sensitive as FAB mass spectrometry for detection of glycopeptides [49] and, unlike FAB, the technique is reasonably tolerant to the presence of buffer salts and other additives [35], although excessive amounts cause signal suppression.

The original carbohydrate matrix, 3-amino-4-hydroxybenzoic acid, does not appear to give a quantitative response with oligosaccharides as a saturation effect occurs [51]. However, if a chemically related internal standard is included in the sample mixture and peak ratios are measured, oligosaccharides may be quantified over a large concentration range with detection limits in the low pmole range. Saturation does not occur with 2,5-DHB and the signal produced from oligosaccharides reflects the sample amount over several decades of concentration [51]. It would appear from the limited studies that have appeared that, at least with sugars of similar structure, there is a good correlation between signal strength and signal intensity. Thus, with high mannose oligosaccharides, the response appears to be well correlated with amount, although a slight drop in signal intensity has been found with the larger sugars [51]. $\beta(1,2)$-cyclic glucans have also been reported to give a quantitative response and to give profiles from both 2,5-DHB and α-cyano-4-hydroxycinnamic acid which closely match profiles obtained by techniques such as HPLC [52]. The relative abundances of signals given by different types of sugar has not yet been determined. With mixtures, the maximum signal which can be recorded from an individual sugar is lower than that from the sugar in isolation because the ion current produced with each laser shot is distributed over each of the components of the mixture.

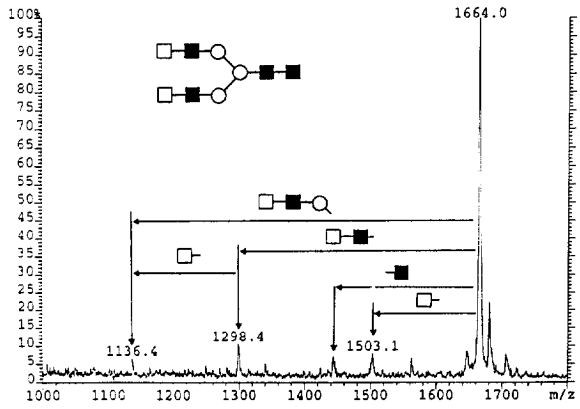

Fig. 3. MALDI mass spectrum of a typical underivatised sugar on a magnetic sector instrument. Fragment ions are indicated by the arrows. Reprinted from Ref. [46].

3. Use of MALDI-TOF for analysis of glycoproteins and their constituent oligosaccharides

3.1. Intact glycoproteins

MALDI analysis has been used in several laboratories to examine both intact glycoproteins and the oligosaccharides present at different glycosylation sites. Three types of glycoprotein structure are recognised, those containing the so-called *N*-linked oligosaccharides which contain a trimannosyl-chitobiose core attached through an amide bound to asparagine, those containing the *O*-linked oligosaccharides whose

sugars are generally smaller, more structurally diverse and attached to serine, threonine or tyrosine, and glycoproteins containing the complex glycophosphatidylinositol (GPI) membrane anchors.

Poor resolution generally restricts the use of MALDI on TOF instruments to studies of glycoproteins with masses below about 20 000. Above this mass, even structures differing by the mass of a monosaccharide residue are not resolved. Early results from ribonuclease B (around 15 000), a glycoprotein containing five glycoforms each differing by one mannose residue, illustrate the problem [1]. Although the glycoforms could be seen, resolution was very poor. Above about M_r 20 000, only broad peaks are produced with no glycoform resolution. However, MALDI can be used at much higher masses to determine the presence or absence of glycosidation as illustrated by the study of the carbohydrate-deficient glycoprotein syndrome reported by Wada et al. [53]. Human serum transferrin from normal controls was shown to produce an ion at M_r 79 600 with sinapinic acid as the matrix, whereas patients with the syndrome produced transferrins with additional peaks, appearing as shoulders on the main peak, at M_r 77 400 and 75 200. These additional peaks corresponded to species lacking one and two sialylated biantennary oligosaccharides (XVII) respectively.

Another use of MALDI in this area is to determine the extent of non-enzymatic glycation of proteins by monosaccharides such as glucose and fructose in attempts to understand the tissue damage resulting from complications in diabetes mellitus [54,55]. Glycation reactions between glucose and bovine serum albumin were clearly demonstrated in the first of these two publications and the mechanisms investigated with a smaller protein, ribonuclease in the second. Sinapinic acid was used as the matrix Even though the products with ribonuclease were poorly resolved, the results illustrated that, although glucose reacted faster than fructose, rearrangements of the Amadori adducts are favoured with fructose.

3.2. Glycopeptides obtained by enzymatic digestion

Identification of the sites in glycoproteins that are occupied by oligosaccharides generally depends on enzymatic cleavage of the glycoprotein and separation of the resulting peptides and glycopeptides by reversed-phase HPLC. Examination of these compounds can then be achieved with a variety of mass spectrometric techniques, either off-line as in the case of MALDI or on-line with electrospray or flow-FAB. Assuming that the peptide sequence of the glycopeptide is known, the masses of the oligosaccharides can be obtained by differences. Alternatively, the oligosaccharides can be removed from the peptide prior to mass spectrometric examination. Each approach has its advantages. Examination of the intact glycopeptide by MALDI takes advantage of the fact that peptides generally give stronger signals than oligosaccharides because the basic groups can be readily protonated. However, because the masses of the glycopeptides are greater than those of the constituent oligosaccharides, resolution with linear TOF instruments is more difficult. Use of the released oligosaccharide, in addition to reducing the molecular weight, provides a unique site, the reducing terminus, for specific derivatization reactions.

Because very little, if any, fragmentation of oligosaccharides or glycopeptides is seen with MALDI in linear time-of-flight systems, it can be said with some confidence that each peak in the spectrum is due to a single component or to several isobaric components rather than to a fragment ion. The mass of the oligosaccharide ion, or of the oligosaccharide portion of the molecular ion from a glycopeptide, is unique to a specific combination of isobaric monosaccharides, thus allowing the composition of the oligosaccharide to be determined. Furthermore, because neutral mammalian *N*-linked oligosaccharides generally contain only three types of monosaccharide, namely hexose [mannose (Man), galactose (Gal), glucose (Glc)], deoxyhexose [fucose (Fuc)] and *N*-acetylaminohexose [*N*-acetylgalactosamine (GalNAc), *N*-acetyl-

glucosamine (GlcNAc)], a mass measurement can lead directly to a proposed structure. For example, a neutral mammalian *N*-linked sugar of nominal mass 1665 reduces to a composition of (Hexose)$_5$(*N*-acetylaminohexose)$_4$ for which a biantennary structure is the most likely. Table 1 lists the residue masses of common monosaccharides. It must be emphasised, however, that structures proposed purely on the basis of a molecular weight measurement must be confirmed by additional information such as a composition and linkage analysis obtained by techniques such as GC–MS, MS–MS and NMR.

Treuheit et al. [56] have used the technique of enzymatic digestion and mass profiling for site analysis and to propose structures for *N*-linked oligosaccharides from the β-subunit of (Na, K)-ATPase from lamb and dog kidney. Tryptic digestion and HPLC fractionation were first used to obtain the three glycopeptides containing the *N*-linked glycosylation sites. Masses of the molecular ions obtained by negative ion MALDI-TOF analysis, with 2,5-DHB as the matrix, combined with the results from lectin binding studies, indicated that the major oligosaccharides were tetraantennary with additional lactosamine extensions to some of the antennae. However, no further analysis was made on the glycopeptides to confirm these structures.

Further information on the structure of such glycoproteins can be obtained by monitoring the products of successive exoglycosidase digestion. This technique involves incubating the glycopeptide with an enzyme which cleaves a specific sugar in a specific linkage from the non-reducing end of the oligosaccharide and measuring the mass of the residual glycopeptide by MALDI to determine what, if anything, has been removed. The process is then repeated with further exoglycosidases until the oligosaccharide has been fully digested.

This method was first demonstrated by Sutton et al. [57,58] for recombinant human tissue inhibitor of metalloproteinases (TIMP). The well characterised glycoproteins fetuin, α_1-acid glycoprotein and tissue plasminogen activator were included in the study as controls. The glycoprotein was first hydrolysed with trypsin and the two glycopeptides were identified by further incubation of a sample of the peptide mixture with endoglycosidase-F (PNGase-F). Comparison of the peptide masses before and after PNGase-F digestion revealed the glycopeptides. A 200-pmol amount of each of the two glycopeptides was then incubated with a sialidase from *Arthrobacter ureafaciens* and the resulting glycopeptides containing neutral oligosaccharides were examined by MALDI with α-cyano-4-hydroxy-

Table 1
Residue masses of monosaccharides commonly found in oligosaccharides

Monosaccharide	Formula	Monoisotopic mass	Chemical mass
Pentose	$C_5H_8O_4$	132.0423	132.12
Deoxyhexose	$C_6H_{10}O_4$	146.0579	146.14
Hexose	$C_6H_{10}O_5$	162.0528	162.14
Hexuronic acid	$C_6H_8O_6$	176.0321	176.13
Na salt	$C_6H_7O_6Na$	198.0140	198.11
N-Acetylaminohexose	$C_8H_{13}NO_5$	203.0794	203.19
N-Acetylneuraminic acid	$C_{11}H_{17}NO_8$	291.0954	291.26
Na salt	$C_{11}H_{16}NO_8Na$	313.0773	313.24

The oligosaccharide masses are obtained by addition of the residue masses together with the mass of one molecule of water (18.0106 monoisotopic, 18.02 chemical). [Alternatively the full masses of the monosaccharides less $n-1$ water molecules (where n = the number of monosaccharides) may be used]. For the mass of the $[M+Na]^+$ion, an additional 22.9899 (monoisotopic) or 22.00 (chemical) mass units should be added.

cinnamic acid as the matrix. The measured masses indicated that the oligosaccharides were mainly core-fucosylated bi-, tri- and tetraantennary structures. Successive incubations with galactosidases, hexoseaminidases, mannosidases and fucosidases enabled the structures of these compounds to be established.

Huberty et al. [49] have examined the glycoprotein, recombinant human macrophage colony stimulating factor (rhM-CSF), a 223 amino acid glycoprotein with two *N*- and several *O*-linked carbohydrate attachment sites. The glycoprotein was first digested with the lysine-specific protease, Achro K and the resulting 15 peptides were separated by reverse-phase HPLC using a Vydac C_{18} column. Glycopeptides were examined by both FAB and MALDI mass spectrometry (2,5-DHB matrix). Structural information was obtained using exo- and endo-glycosidase digestions. Thus, incubation with the endoglycosidase PNGase-F released the intact biantennary *N*-linked oligosaccharides whose masses were determined by the difference in glycopeptide mass before and after digestion. Alternative incubations of the intact glycopeptides with exoglycosidases such as neuraminidase, were used to define the structures further. Information on the *O*-linked oligosaccharides was obtained with enzymes such as *O*-glycanase. Similar methods have recently been used to examine the oligosaccharides of baculovirus-expressed mouse interleukin-3 peptides [59].

Oligosaccharide profiles from glycoproteins, with no regard for site specificity, can be obtained either by releasing the sugars or by complete digestion of the protein with pronase. The latter approach leaves an amino acid at the reducing terminus of the oligosaccharide whose basic amino group can be protonated efficiently. This approach was used by Bock et al. [60] in their study of the oligosaccharides from the crystalline surface layer glycoprotein from *Thermoanaerobacter thermohydrosulfuricus* L111-69. Much of the structural analysis was performed by NMR and GC–MS, but MALDI was used to obtain the oligosaccharide profile. Twelve linear oligosaccharides averaging 60 monosaccharide residues were found (mean mass around 9500 dalton) with the linkage being between galactose and tyrosine. This was the first report of such a linkage in glycoproteins. 2.5-DHB was used as the matrix and ions of the type $[M - H + 2Na]^+$ were formed as the result of salt formation at the tyrosine carboxylic acid group.

3.3. Examination of released oligosaccharides

N-Linked oligosaccharides from glycoproteins can be released by hydrazinolysis [61,62] or by the action of enzymes such as PNGase-F. Hydrazinolysis, although generally achieving a more quantitative release of oligosaccharides than PNGase-F, can present some problems. Perhaps the most serious of these results from the fact that this reagent cleaves amide bonds reasonably unspecifically. Thus, in addition to cleavage of the bonds between amino acids and the amide bond linking the asparagine-linked oligosaccharides, the reagent also cleaves the acyl groups from the acylamino sugars. Following hydrazinolysis, therefore, amino sugars are reacetylated with the assumption that all acylamino sugars originally contained acetyl groups. However, this is not always the case. For example, some constituent sugars of GPI anchors contain unacylated sugars and the sialic acids from human a1-acid glycoprotein contain about 50% glycolylamino rather than acetylamino groups. In addition, hydrazinolysis can give rise to by-products which appear in the MALDI spectra and which may be mistaken for additional sugars. Thus, in Fig. 4, the ion at m/z 1526 is thought to be from an oligosaccharide that has retained a nitrogen at the reducing terminus and undergone additional acetylation in the reacetylation process. The incorporation of an additional acetylamino group by chemical means produces an oligosaccharide whose mass is indistinguishable from that of a natural oligosaccharide. Further problems can occur through over-reaction with hydrazine and cleavage of the HexNAc from the reducing terminus of the released sugar. Although these problems can be overcome by using PNGase-F release, the enzyme is not totally effective and some sugars may not be released.

Endoglycosidase H has also been used to release *N*-linked oligosaccharides but, because it cleaves the sugar between the core GlcNAc residues, it results in loss of information on the reducing-terminal GlcNAc and its possible substituents. Nevertheless, this approach has been used by Ziegler et al. [63] to record the molecular ions of $(Hex)_{10}GlcNAc$ and $(Hex)_{13}GlcNAc$ from the yeast *Schizosaccharomyces pombe* in a study of general glycosidation mechanisms in this species. Only sugars at the non-reducing termini were important in this study.

Detection of the products of oligosaccharide release has traditionally been carried out using Bio-Gel P4 gel filtration columns to measure the hydrodynamic volume of the oligosaccharide. However, this process is time-consuming (typically several hours), has relatively poor resolution, and requires that the oligosaccharide is radio- or fluorescently-labelled at the reducing terminus. In addition, these gel filtration columns are only effective with neutral sugars and, thus, the sialic acids, sulphate and phosphate groups normally present on *N*-linked structures must first be removed. Detection by MALDI analysis, however, is rapid, accurate, requires no labelling and can be used with both neutral and acidic oligosaccharides.

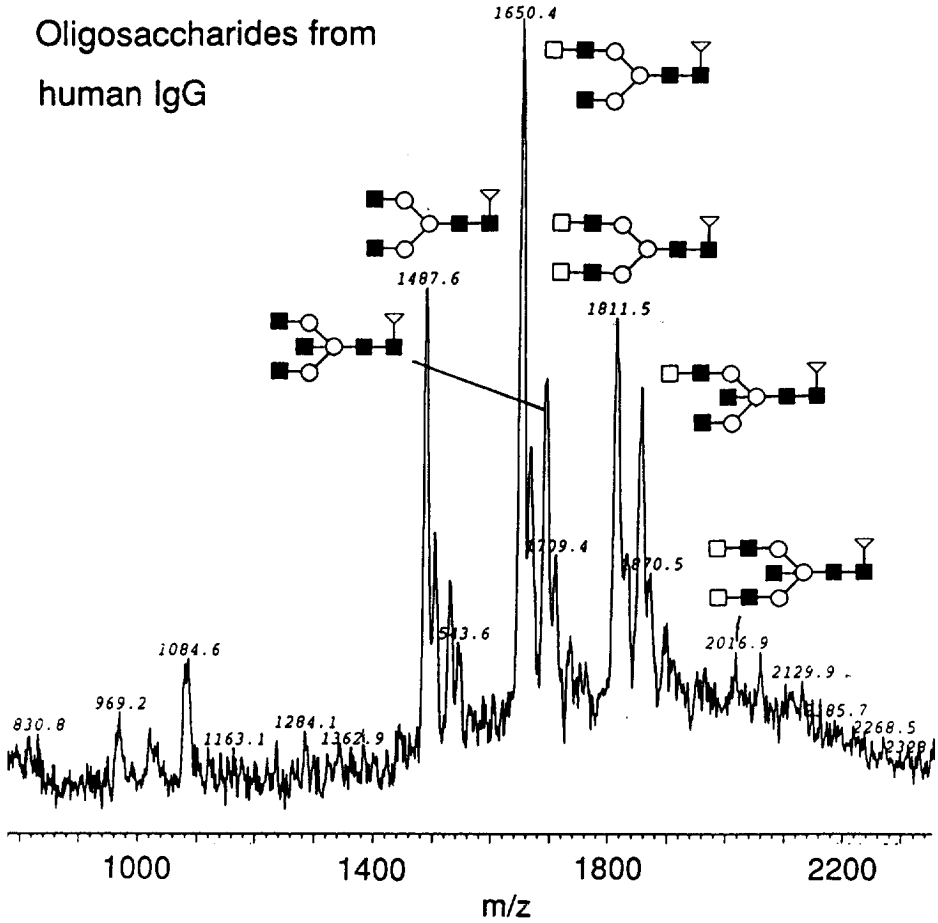

Fig. 4. Positive ion MALDI-TOF spectrum of the oligosaccharides released by hydrazinolysis from human IgG and ionised with 2,5-DHB. Reprinted from Ref. [46].

3.3.1. Neutral sugars

The results of an analysis by MALDI-TOF of the neutral sugars obtained from human IgG by hydrazinolysis are shown in Fig. 4 [46] as an example of the results that can be obtained. The matrix was 2,5-DHB. These oligosaccharides are typical of those found as *N*-linked sugars. Fig. 5 shows the same mixture of oligosaccharides recorded with a higher resolution magnetic sector instrument for comparison. All of the structures have been determined by other techniques but the mass measurement accuracy is sufficient to confirm the monosaccharide compositions.

Molecular weights of *N*-linked oligosaccharides are typically in the 900–3000 dalton region and most encountered compounds can be resolved with TOF instruments. If the molecular weights of possible *N*-linked oligosaccharides are calculated, it is found that the mass differences between oligosaccharides with less than about 10 monosaccharide residues is large enough for resolution by even the smallest TOF instrument. With larger oligosaccharides, however, the mass difference between possible structures becomes too small and instruments of higher resolution have to be used. Resolutions in the 3000 range are sufficient to handle most commonly occurring structures in *N*- and *O*-linked glycoproteins and to resolve closely similar structures such as $(Man)_9(GlcNAc)_2$ (M_r 1905.7) from $(Hex)_4(GlcNAc)_6$ (M_r 1907.7). The latter structure is a major constituent of ovalbumin oligosaccharides.

As with the glycopeptides discussed above, further structural information can be obtained by successive exoglycosidase digestions with MALDI being used to analyse the results. This is illustrated in Fig. 6 which shows a study of the glycosylation of human IgG. The oligosaccharides from this glycoprotein are mainly galactosylated analogues of the core-fucosylated biantennary sugars shown in the Figure. Incubation

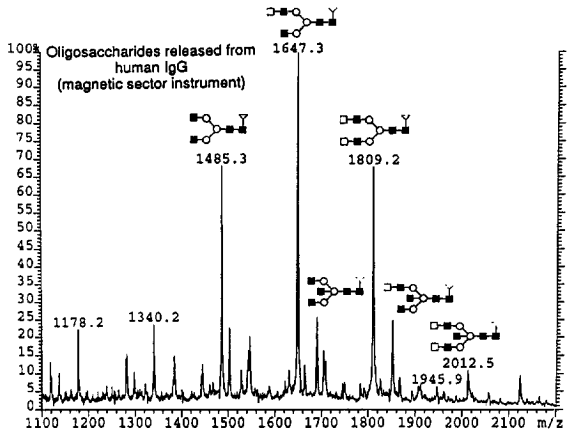

Fig. 5. Positive ion MALDI spectrum of the oligosaccharides released by hydrazinolysis from human IgG and recorded on a magnetic sector instrument with 2,5-DHB as the matrix. Reprinted from Ref. [46].

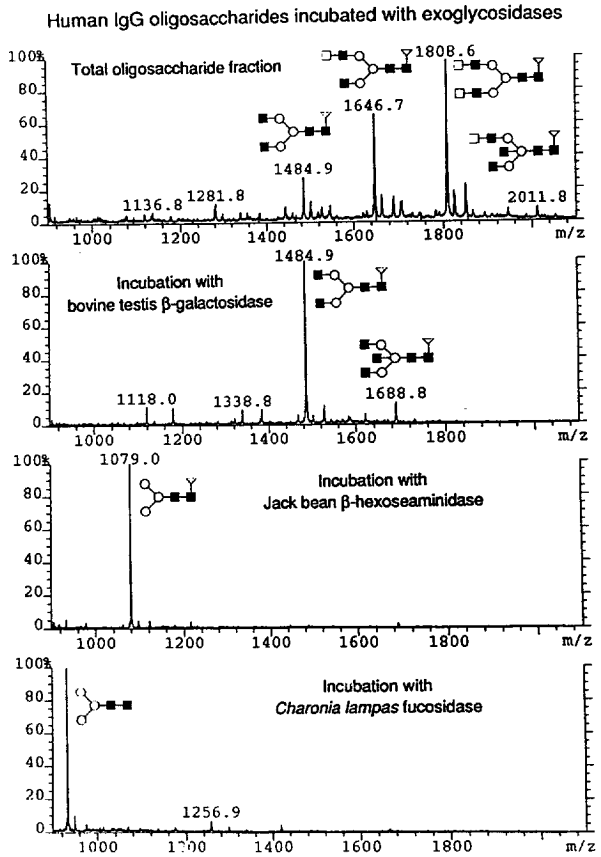

Fig. 6. MALDI spectra of oligosaccharides from human IgG successively digested with exoglycosidases. Reprinted from Ref. [46].

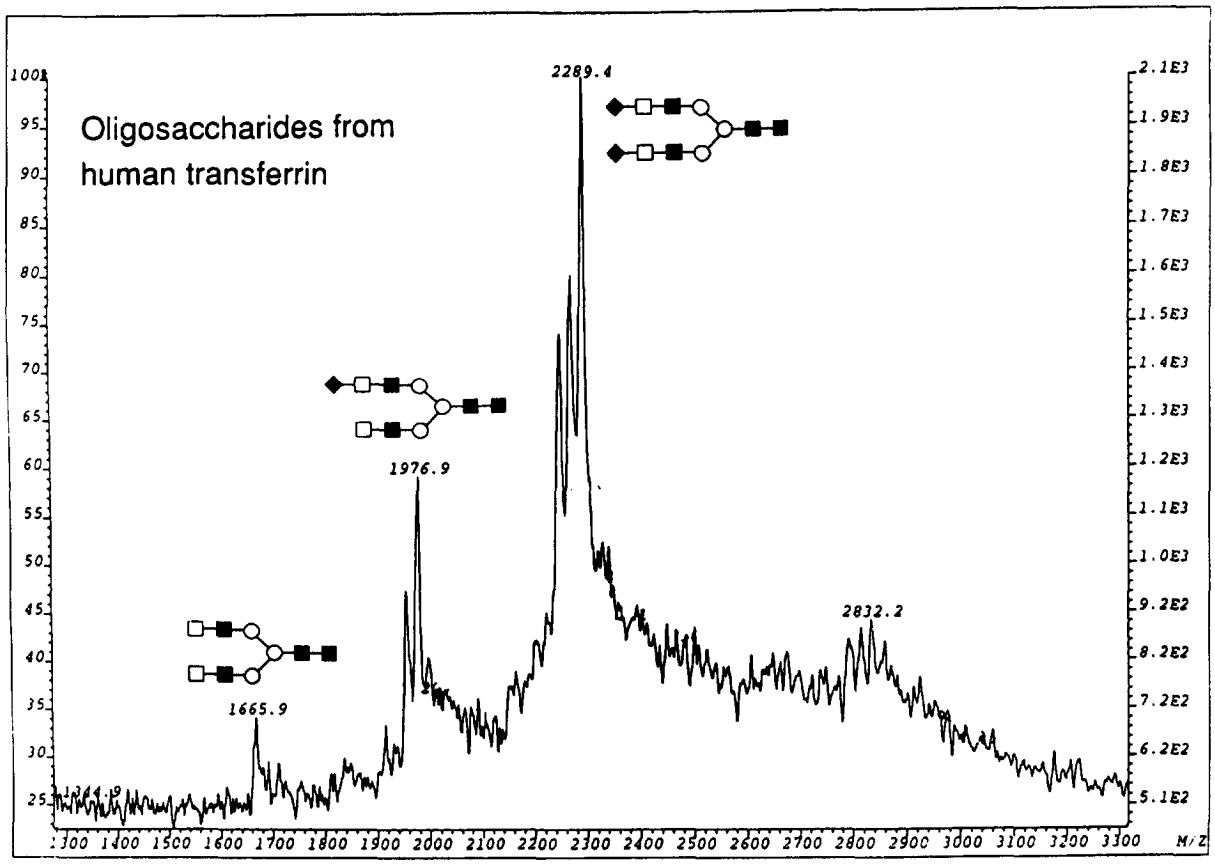

Fig. 7. Positive ion MALDI-TOF spectrum of the oligosaccharides released by hydrazinolysis from human transferrin and ionised with 2,5-DHB. Reprinted from Ref. [46].

with β-galactosidase derived from bovine testis reduced the profile to essentially two oligosaccharides (Fig. 6b) and shows that each exists in the mixture as its mono- and di-galactosylated derivative. Removal of the GlcNAc with Jack bean β-hexoseaminidase reduces the profile to one oligosaccharide (Fig. 6c) and, finally, incubation with α-fucosidase from *Charonia lampas* can be used to demonstrate the presence of the core fucose moiety (Fig. 6d). An interesting feature of this analytical sequence is that, because the number of oligosaccharides usually reduces after each incubation stage, the signal:noise ratio of the spectrum increases even though some material will inevitably have been lost during the extraction of the oligosaccharides from the incubation medium.

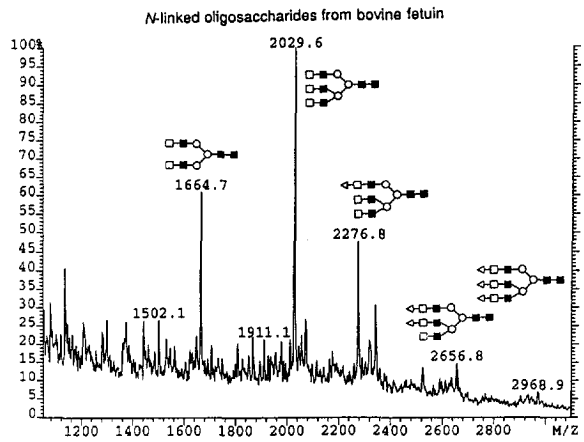

Fig. 8. Positive ion MALDI spectrum of the oligosaccharides released by hydrazinolysis from bovine fetuin and recorded on a magnetic sector instrument with 2,5-DHB as the matrix.

3.3.2. Anionic sugars

Negatively charged oligosaccharides, such as those containing sialic acid, give weak signals in the positive ion mode but frequently produce more abundant ions ($[M-H]^-$) in the negative mode. In the positive ion mode these compounds usually appear as the sodium salt although peaks corresponding to the free acid can sometimes be seen. Fig. 7 shows the MALDI-TOF spectrum of oligosaccharides from transferrin where the disialylated biantennary oligosaccharide is the major constituent. This peak can clearly be seen to be composed of the free acid and both mono- and di-sodium salts. The ability of sialic acids to form sodium salts under these conditions is fortuitous as the residue mass difference between sialic acid (M_r 291) and two deoxyhexose residues (M_r 292) is only one mass unit, an interval that is difficult to determine with a TOF mass spectrometer. The presence if sodium adds a further 22 mass units to the mass of the sialic acid.

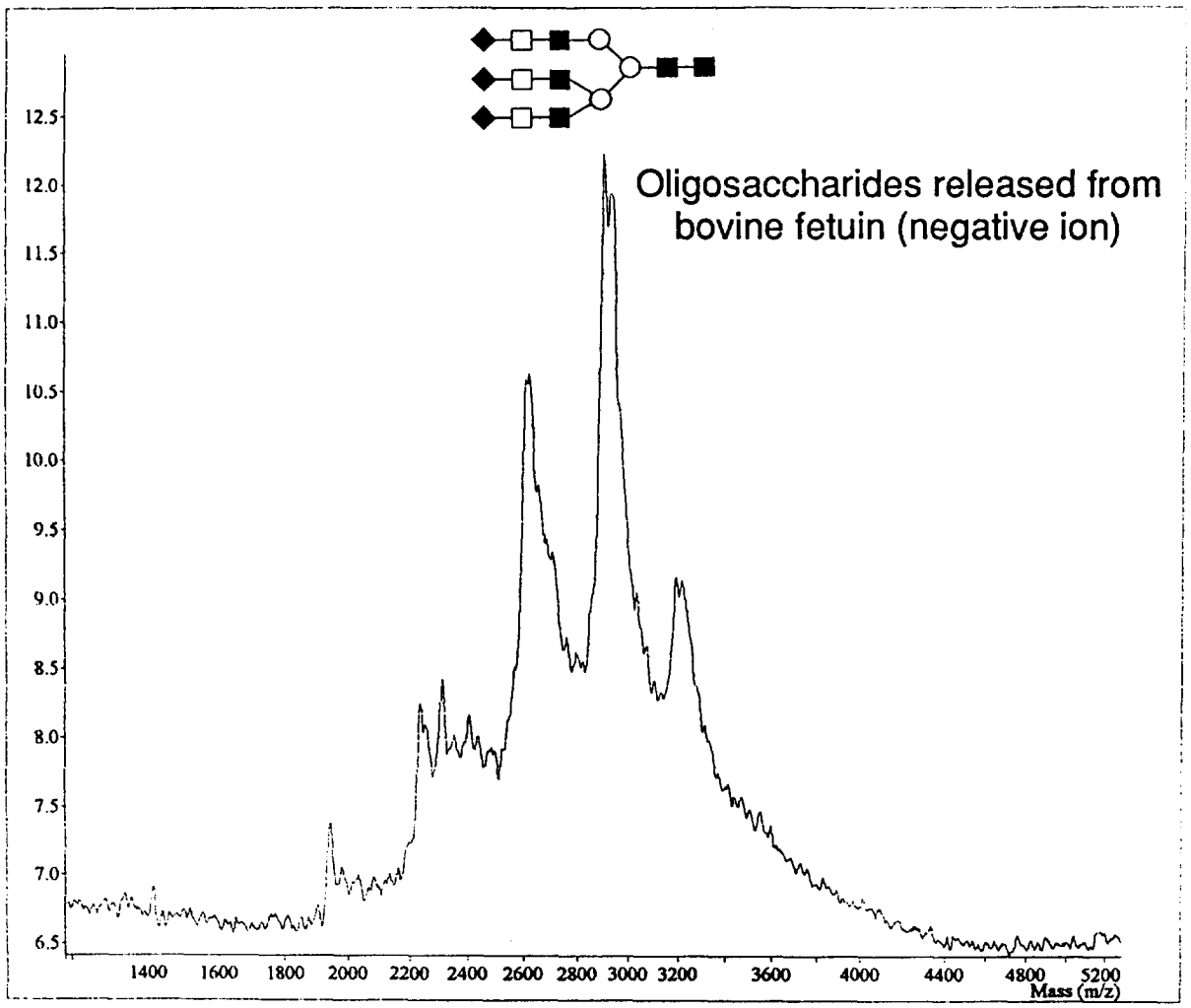

Fig. 9. Negative ion MALDI-TOF spectrum of the oligosaccharides released by hydrazinolysis from bovine fetuin and ionised with 2,5-DHB.

When examined with a magnetic sector instrument, sialylated oligosaccharides are observed to undergo extensive fragmentation, mainly by loss of sialic acid but also by decarboxylation. Thus, fetuin oligosaccharides, of which the triantennary compound is the major species, looses most of the sialic acid to give major ions corresponding to the unsialylated sugars. Fig. 8 shows this clearly. Much stronger spectra are obtained in the negative ion mode although resolution by linear TOF instruments is not good. Fig. 9, for example shows the negative ion spectrum from bovine fetuin and clearly shows the presence of the mono-, di-, tri- and tetrasialylated triantennary oligosaccharides. The latter compound has been difficult to detect by electrospray mass spectrometry.

4. Use of MALDI for the analysis of glycolipids

4.1. Neutral glycolipids

Ceramides and glycolipids with complex oligosaccharides give abundant $[M + Na]^+$ ions when examined by MALDI MS [64]. On magnetic sector instruments, the spectra also contain several matrix-dependent and structurally informative fragment ions. Thus, with α-cyano-4-hydroxycinnamic acid, both the sugar moiety and the acyl group are lost to leave a sphingosine-derived ion, the mass of which defines the sphingosine carbon number. A second, and usually more abundant ion, is derived from this ion by loss of water. When 2,5-DHB is used as the matrix, these two ions are weak. On the

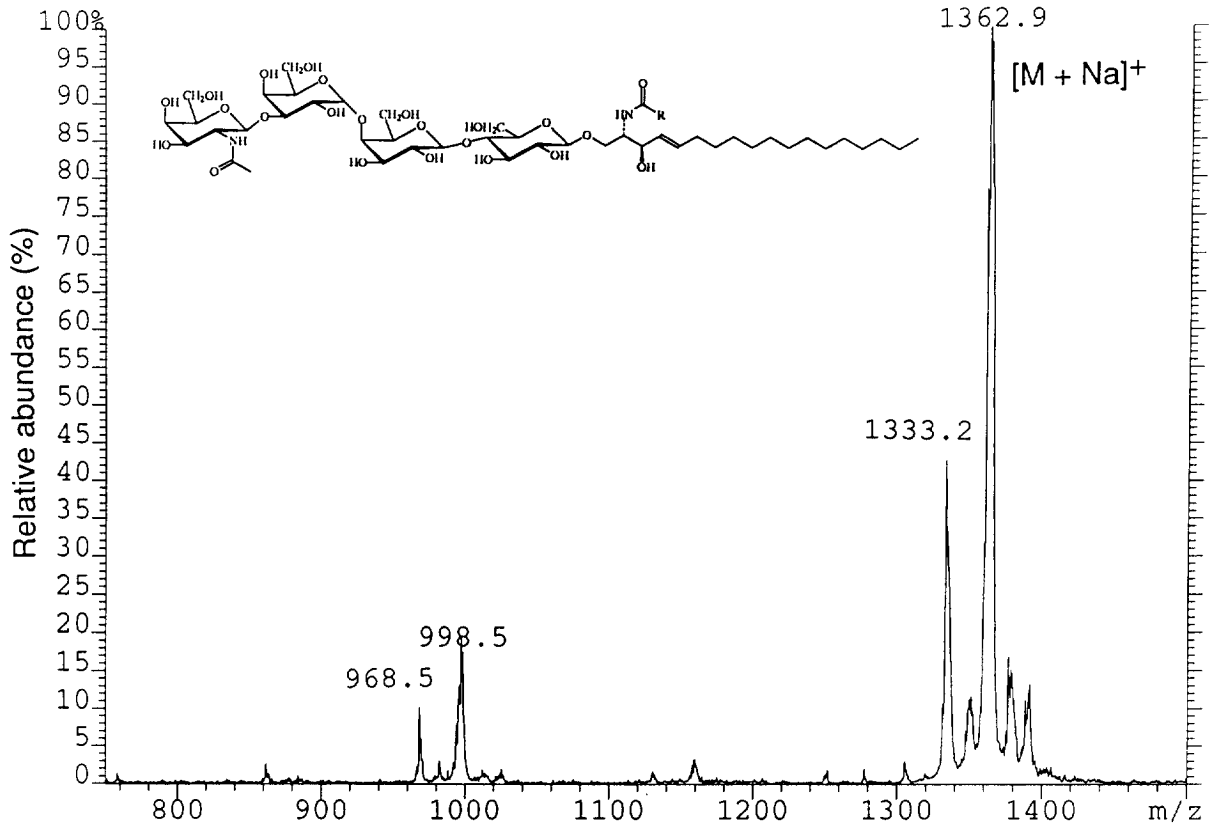

Fig. 10. Positive ion MALDI spectrum of globosides from human erythrocytes recorded on a magnetic sector instrument with α-cyano-4-hydroxy-cinnamic acid as the matrix.

other hand, with glycosphingolipids containing no acetylamino sugars in the oligosaccharide portion, the ion produced by cleavage of the oligosaccharide is very prominent. When acetylamino sugars are present, this cleavage ion is comparatively weak, but more prominent ions are produced by cleavage at other glycosidic linkages, leading to considerable sequence information (Fig. 10).

4.2. Acidic glycolipids

Gangliosides appear to be even less stable than sialylated *N*-linked sugars under MALDI-TOF conditions [36,64]. Although reasonably abundant molecular ions are observed from 2,5-DHB in both positive and negative ion modes, most other matrices cause extensive loss of sialic acids which appears in the spectra as broad, unresolved peaks (Fig. 11). Presumably, fragmentation is occurring during acceleration resulting in the fragment ions possessing a considerable energy spread. When examined with a magnetic sector instrument, on the other hand, the ions are clearly resolved (Fig. 12) as a result of the energy filtering properties of the instrument's electrostatic analysers. In addition, the molecular ions are weaker than in the TOF spectra, presumably as the result of their longer

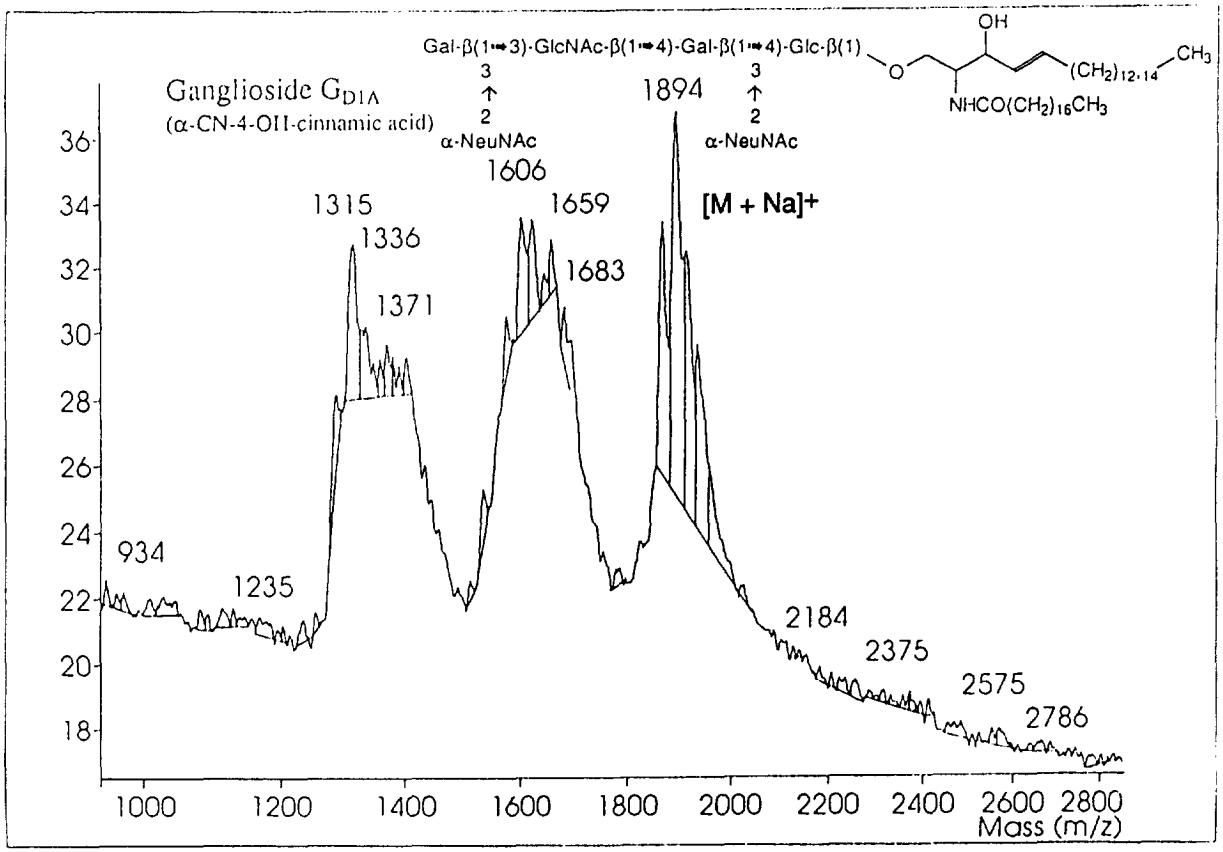

Fig. 11. Positive ion MALDI spectrum of the ganglioside G_{D1a} recorded on a TOF instrument with α-cyano-4-hydroxycinnamic acid as the matrix. Reprinted from Ref. [46].

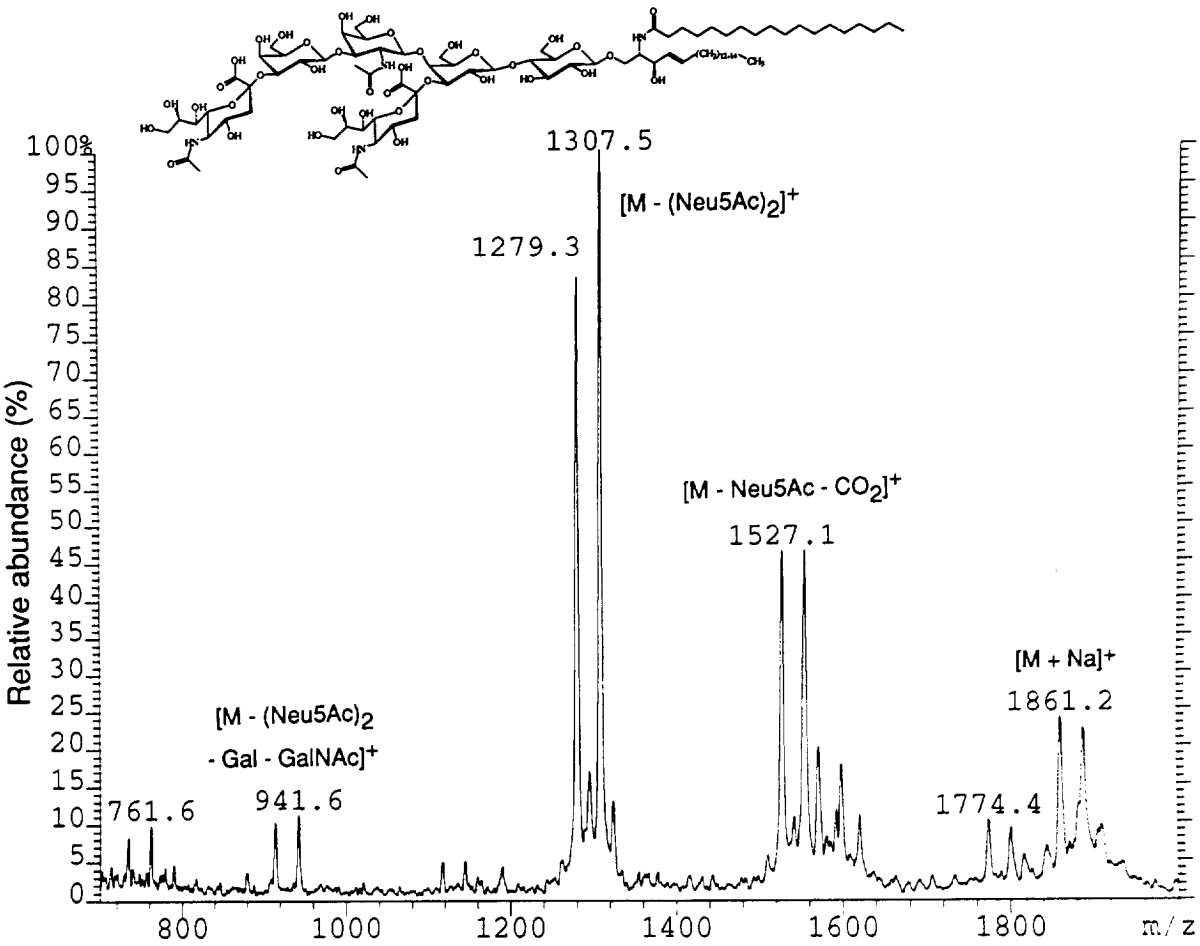

Fig. 12. Positive ion MALDI spectrum of the ganglioside G_{D1a} recorded on a magnetic sector instrument with 2,5-DHB as the matrix.

pre-acceleration lifetime caused by the lower acceleration voltage. Even though the fragment ions are broad and ill defined on TOF instruments, their relative abundance has been reported to yield some structural information, Thus, Juhasz and Costello [36] have found that the gangliosides G_{D1a} and G_{D1b} can be differentiated on the bases of the relative abundance of the ions formed by elimination of one and two sialic acid residues. Ganglioside G_{D1b} has both of its sialic acids linked together whereas in G_{D1a} the sialic acids are on different sugars. Not surprisingly, therefore, the loss of two sialic acids is more prominent from G_{D1b}.

Several matrices are suitable for ionization of gangliosides. In addition to 2,5-DHB, *a*-cyano-4-hydroxycinnamic acid and esculetin discussed above, Juhasz et al. [36,37] have examined 6-aza-2-thiathymine (XII), 1,5-diaminonaphthalene (XIII), 4-hydrazinobenzoic acid (XIV) and 2-thiohydantoin (XV). Although 2,5-DHB and 6-aza-2-thiathymine gave positive ion spectra, better defined spectra were recorded in the negative mode. Sinapinic acid was ineffective.

Juhasz and Costello. [36] also report that derivatization of the gangliosides by permethylation leads to considerable increases in sensitivity.

5. Conclusions

MALDI has proved to be an invaluable technique for the examination of oligosaccharides, glycopeptides and glycolipids. Although resolutions on TOF instruments can be limiting, improvements can be made by using other types of mass spectrometers such as TOF instruments fitted with reflectrons, ICR and magnetic sector instruments. Fragmentation gives considerable structural information under suitable conditions but there is scope for improvement in this area. Alternatively, structural information can be obtained by using the technique as a detector in the more traditional method of exoglycosidase digestion.

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