MATRiX-ASSISTED LASER DESORPTION/IONIZATION
MAsS SPECTROMETRY OF CARBOHYDrATES

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This review describes the application of matrix-assisted laser desorption/ionization (MALDI) mass spectrometry to carbohydrate analysis and covers the period 1991–1998. The technique is particularly valuable for carbohydrates because it enables un derivatised, as well as derivatised compounds to be examined. The various MALDI matrices that have been used for carbohydrate analysis are described, and the use of derivatization for improving mass spectral detection limits is also discussed. Methods for sample preparation and for extracting carbohydrates from biological media prior to mass spectrometric analysis are compared with emphasis on highly sensitive mass spectrometric methods. Quantitative aspects of MALDI are covered with respect to the relationship between signal strength and both mass and compound structure. The value of mass measurements by MALDI to provide a carbohydrate composition is stressed, together with the ability of the technique to provide fragmentation spectra. The use of in-source and post-source decay and collision-induced fragmentation in this context is described with emphasis on ions that provide information on the linkage and branching patterns of carbohydrates. The use of MALDI mass spectrometry, linked with exoglycosidase sequencing, is described for N-linked glycans derived from glycoproteins, and methods for the analysis of O-linked glycans are also covered. The review ends with a description of various applications of the technique to carbohydrates found as constituents of glycoproteins, bacterial glycolipids, sphingolipids, and glycolipid anchors. © 1999 John Wiley & Sons, Inc., Mass Spec Rev 18: 349–450, 1999

I. INTRODUCTION

Carbohydrates are the most abundant and structurally diverse compounds found in nature. Unlike the linear polymers, such as proteins and nucleic acids, oligo- and polymeric carbohydrates can form branched structures because linkage of the constituent monosaccharides can occur at a number of positions. It has been calculated (Laine, 1994) that for a simple hexasaccharide, there are in excess of $1.05 \times 10^{12}$ possible isomeric structures. This “isomer barrier” means that a single-method analytical technique is probably an unreasonable goal for carbohydrate analysis. Fortunately, however, the biosynthetic pathways creating most complex carbohydrates are specific and limited by the available glycosyltransferases. Consequently, only a very few of the theoretically possible isomers are ever encountered, a property that can be utilized structurally if the source of the glycan is known.

II. STRUCTURES AND NOMENCLATURE OF THE MAJOR COMMONLY OCCURRING CARBOHYDRATE TYPES

A. Monosaccharides

Most monosaccharide constituents of oligo- and polysaccharides consist of compounds with the general formula $C_nH_{2n}O_n$ where $n = 3$ (trioses) to 9 (nonoses), with $n = 6$ (hexoses) being by far the most abundant. These compounds contain $n−1$ hydroxy groups and one aldehyde (reducing sugars) or keto group. Common modifications include the absence of one or more oxygen atoms (deoxysugars), oxidation of a primary hydroxyl group to a carboxylic acid (uronic acid), reduction to an alcohol, replacement of a hydroxy group with a primary amine or acylamino-group, or methylation of one or more hydroxy groups. Each of these monosaccharides exists in a number of isomeric forms because of the constituent asymmetrically substituted carbon atoms; aldoses contain $n−2$ and ketoses $n−3$ chiral carbon atoms. The number of isomers, thus, increases rapidly with $n$; for example, aldohexoses contain $n−2 = 4$ chiral carbons, giving rise to 16 stereoisomers. Cyclization of the linear forms of these compounds creates a further anomeric center (termed anomericity) and can result in rings of different sizes, depending on the hydroxy groups involved. Hexoses commonly form six- (pyranose) or five- (furanose) membered rings, indicated by italicized $p$ and $f$, respectively, after the abbreviated name of the monosaccharide. Rings can also exist in different conformations, e.g. chair or boat. Each isomer is named individually, and those encountered in this review are listed as structures 1–16 (only one isomer is shown) with their common abbreviations. Symbols are often used to specify monosaccharide types in complex structures, but there is no consensus as to which symbol is used for any particular sugar. The symbols used in this review are shown in the legends to the appropriate figures.
B. Oligo- and Polysaccharides

Oligo- (2–10 units) and poly-saccharides (>10 units) are formed by a condensation reaction between the anomeric hydroxyl group of one monosaccharide and any of the other hydroxy groups of a second residue. Formation of a bond to the anomeric carbon prevents ring opening and fixes the ring size and conformation of that monosaccharide. Branching arises when two or more sugars are linked to a monosaccharide. Naturally occurring sugars can contain a small number of monosaccharide residues (di-, tri-, tetra-, etc.), giving rise to specific structures, or they can form very large, less specific, polymers. Examples of such polymers are cellulose (d-Glcβ(1→4)– linear repeat), dextran (d-Glcα(1→6)–, d-Glcα(1→3)– branched), glycogen (d-Glcα(1→6)–, d-Glcα(1→4)– branched), chitin d-GlcNAcβ(1→4)– linear), chondroitins (2-amino-2-deoxy-d-Gal and d-GlcA, linear), inulin (d-Fruβ(2→1)– linear), or hyaluronic acid (2-amino-2-deoxy-d-Glc and d-GlcA, linear). All of these large polymers constitute major structural or storage molecules of most living organisms.

C. Glycoconjugates

1. Glycoproteins

Most proteins are glycosylated to a greater or lesser extent. Mammalian glycoproteins contain three types of glycan, the so-called “N-linked glycans”, which are linked via an amide bond to asparagine in a Asp-X-(Ser or Thr) motif, where X can be any amino acid other than proline; “O-linked glycans”, where there is a glycosidic bond to serine or threonine; and glycosylphosphatidylinositol (GPI) lipid anchors. The N-linked glycans contain a common trimannosyl chitobiose core with two or more antennae (Scheme 1) and are biosynthesized as described by Kornfeld & Kornfeld (1985). Glycans that contain only mannose (5) substituents are known as ‘high mannose’ glycans, those whose six antennae contain only mannose and three antennae contain N-acetyl lactosamine (d-Galβ(1→4)d-GlcNAcβ-) are called ‘hybrid’ glycans, and those with N-acetyl lactosamine on both antennae are referred to as ‘complex’ sugars. A GlcNAc (8) residue attached to the 4-position of the core mannose is known as a “bisecting” GlcNAc. Each potential glycosylation site in the glycoprotein may or may not be occupied, or it may only be partially occupied. Furthermore, each glycosylation site on the

SCHEME 1. Structure features of a typical (biantennary) N-linked glycan.
glycoprotein can contain a range of glycans, resulting in a large number of different glycosylated forms of each protein. It has been estimated, for example, that Desmodus rotundus salivary plasminogen activator, with its two N- and four O-linked sites, contains in excess of 330,000 individual molecular species if the glycans were randomly distributed (Chakel et al., 1997, Apffel et al., 1996). These different species are known as ‘glycoforms’.

O-linked glycans of glycoproteins are generally smaller and more varied in structure (Hounsell, Davies, & Renouf, 1996). There is no common ‘core’ structure or a structural motif for locating the glycans on the protein chain other than the glycans that are attached to serine or threonine. It is common to find several O-linked glycosylation sites in close proximity. The glycan chains of the proteoglycans (conjugates that contain mainly carbohydrate), on the other hand, tend to be much larger. The molecules consist of a protein backbone to which is attached a large number of short to medium-length sugar chains, in many cases giving the appearance of a “bottle brush” type structure. Representative types include the mucins that contain short, often highly branched sugars, and the glycosaminoglycans that contain linear chains of hexuronic acids, hexoses, and hexosamines, many of which contain sulfate.

GPI anchors (17) are glycolipids, attached to the carboxy terminus of certain proteins and whose function is to anchor those proteins to membranes. They consist of a diacylphosphatidylinositol moiety attached to a substituted tetrasaccharide which, in turn, is attached to the protein via ethanolamine phosphate. Additional sugars, acyl groups, or ethanolamine phosphate groups can be attached to this basic structure, depending on the source of the anchor (Ferguson, 1991).

In Gram-positive bacteria and archaea, the carbohydrates are attached to proteins, but with N- and O-linkages are different from those discussed above. Such linkages are Glc → Asn, Gal → Thr, GalNAc → Asn (Halobacterium halobium), Rha → Asn (Mathanothrix soehngenii), and Glc → Tyr, Gal → Tyr (Thermoanaerobacter thermosulfuricus).

2. Glycolipids

Two types of glycolipids (other than the GPI anchors) will be covered in this review, the sphingolipids and bacterial glycolipids such as lipopolysaccharides. Sphingolipids contain acyl-sphingosine (ceramide, Cer) linked to a (usually) short carbohydrate chain. Seven series are recognized, depending on the structure of the sugar chain. Sphingolipids of the ganglio-series are the most common, and they contain a D-Galβ(1 → 3)-D-GalNAcβ(1 → 4)-D-Galβ(1 → 4)-D-Glcβ(1 → ) chain that bears one or more NeuNAc (sialic acid) residues (11) (see Section X.A for structure).

Bacterial lipooligosaccharides from Gram-negative bacteria usually consist of multiple repeats of a short oligosaccharide chain attached to a core oligosaccharide which, in turn, is linked to an anchoring moiety, known as lipid A (18), via the acidic sugar, 2-keto-3-deoxyoctulosonic acid (Kdo) (13). These polysaccharides frequently contain monosaccharide residues such as rhamnose (10) and quinovosamine (amino-14), which are not found in higher organisms (Messner et al., 1997). Lipid A is a glycolipid that consists of two 1,6-linked glucosamines substituted with two phosphate groups and up to six N- and O-linked fatty acids and hydroxy fatty acids, whose function is to anchor the molecule into the cell membrane. For more information of carbohydrate structure, see, for example, Kennedy (1988).
III. MALDI MASS SPECTROMETRY

With such a wide range of structural types, carbohydrate analysis by mass spectrometry can involve a large number of techniques with no single method being ideal for all compounds. Electron impact ionization, for example, is only applicable to the smaller molecules, most of which require derivatization. Matrix-assisted laser desorption/ionization (MALDI), on the other hand, is more versatile because most compounds give signals in their native state. As with other types of mass spectrometry, MALDI can provide valuable information on several aspects of structural analysis, such as the determination of sequence, branching, and linkage. However, for much isomer differentiation, MALDI is probably best combined with one or more chromatographic techniques such as those described by Guile et al. (1996) and Tomiya et al. (1988).

This review describes the areas of carbohydrate analysis most amenable to MALDI analysis and shows how the technique has been used to solve structural problems for a number of structural types. Earlier reviews and summaries of methods have been published (Bahr, Karas, & Hillenkamp, 1994; Harvey, 1996a; 1997; Harvey, Naven, & Küster, 1996; Harvey, Küster, & Naven, 1998; Harvey et al., 1999a; Garozzo, 1997; Burlingame, 1996).

MALDI mass spectrometry was developed in the middle to late 1980s (see Karas & Bahr, 1997; Karas & Hillenkamp, 1988; Karas, Bachmann, & Hillenkamp, 1985; Karas et al., 1987), for the analysis of molecules with large molecular weights. To obtain a signal, the analyte is mixed with a matrix compound, usually a small organic molecule, allowed to crystallize by evaporation of the solvent (the “dried droplet” technique), and subjected to a pulse from a laser. Most of the laser energy, which is usually in the ultraviolet (UV) range, is absorbed by the matrix and “transferred” to the analyte, which is ionized by processes such as hydrogen or alkali metal attachment. The UV absorption characteristics of common matrices deposited onto targets have recently been investigated (Allwood et al., 1996), and found to be similar to those recorded from solution.

Although developed primarily as a technique for the analysis of proteins, even the early reports demonstrated that the method was applicable to carbohydrates. Stachyose (19), a phytochemical tetrasaccharide, appears to have been the first compound examined (Karas et al., 1987). Although this carbohydrate was capable of giving a spectrum without a matrix, the signal strength was reported to be considerably enhanced when the solid matrices, nicotinic acid (20) or tryptophan, were used with a frequency-quadrupled Nd–YAG laser (266 nm). The major carbohydrate ion produced by use of these matrices was $\text{MNa}^+$, in contrast to those from two liquid matrices, 3-nitrobenzyl alcohol (21) and 3-nitrophenyl octyl ether (22), which produced a radical molecular ion or an $[\text{M-17}]^+$ ion, respectively. Most matrices discovered since this early work ionize carbohydrates as $\text{MNa}^+$ ions.
MALDI has been reported to be 10–100 times more sensitive than fast atom bombardment (FAB) mass spectrometry for the analysis of glycoproteins (Huberty et al., 1993), derivatives of N-linked glycans (Shen & Perreault, 1998), and cyclic glucans (Garozzo et al., 1994). It is also more sensitive than $^{252}$Cf plasma desorption (PD) mass spectrometry (Tsarbopoulos et al., 1994) for proteins and glycoproteins. The latter study also found that MALDI was able to produce spectra from tryptic glycopeptides in situations where PD failed. Garozzo (1997) has reported a study in which MALDI was able to produce oligomers to higher mass than FAB for a series of cyclic glucans. However, ionization of carbohydrates as M$\text{Na}^+$ species, is not as sensitive as ionization of peptides, where the most prominent species is MH$^+$. Consequently, higher laser powers are normally used, with the result that sample spots are evaporated more rapidly than with peptide analysis. As a result, the laser spot usually has to be moved across the target surface in order to maintain the signal. Although relatively insignificant for acquiring the signal from a molecular ion of a time-of-flight (TOF) instrument, this effect acquires increased significance for instrument-focusing and for acquiring fragmentation spectra.

The ionization mechanisms involved in MALDI are still largely unknown. Ions may be pre-formed in the solid state or may be formed in the gas phase by ion–molecule reactions immediately following desorption by the laser. In fact, both processes probably occur to different extents, depending on the sample and matrix. Lehmann, Knochenmuss, & Zenobi (1997) believe that much of the ionization is present in the solid state for mixtures that contain transition metal complexes, whereas other samples, such as those containing polystyrene, are ionized predominantly in the gas phase. Knochenmuss et al. (1996) also believe in rapid solid rather than gas phase ionization, and they have proposed a mechanism that accounts for protonation, cationization, and matrix suppression effects that involves photochemically excited, but not ionized, matrix molecules as the induced reactive species. Vertes, Irinyi, & Gijbels (1993) have used a hydrodynamic model to describe the plume emitted from the surface upon firing the laser. The high gas densities of the initial plume are consistent with the occurrence of fast collision-induced reactions and adduct formation whereas the subsequent rapid cooling of the plume accounts for the stability of the ions and the lack of additional thermal reactions. The necessity for the matrix to absorb light at the laser wavelength is emphasized by a study by Metzger et al. (1993), in which it was shown that the use of popular MALDI matrices provided no signal enhancement for molecules when they were ionized by PDMS.

A. Lasers
Nitrogen lasers that emit at 337 nm (UV range) are now almost universally employed for MALDI analysis, and are supplied as standard on all commercial instruments. Thus, all of the work that is reported in this review used these lasers unless otherwise stated. However, infrared (IR) lasers have also found a limited role in glycoprotein and glycan analysis and, before the advent of MALDI, were used extensively for ionization of lipid-A and related compounds (see below). Berkenkamp et al. (1997) have recently examined the utility of two lasers that emit in the mid-IR range, an Er-YAG (2.94 $\mu$m, 80–90 nsec pulse width) and an Er-YSGG laser (2.79 $\mu$m, 80 nsec) for the analysis of biological macromolecules such as the glycoprotein, immunoglobulin G (IgG). Glycerol and succinic acid were used as the matrices. Sample consumption per laser shot exceeded that for UV lasers by at least two orders of magnitude. Glycerol, used as the matrix, gave good sample reproducibility and mass accuracy, but the solid matrix, succinic acid, gave poorer results. Metastable fragmentation from compounds with masses below ca. 20 kDa was comparable to that seen with a UV laser, but was considerably less for compounds with higher masses, thus giving improved mass resolution and an extended mass range.

B. Instrumentation
The pulsed nature of a laser ion source and its ability to ionize very large molecules is ideal for coupling to a time-of-flight (TOF) analyzer. This arrangement also produces very high sensitivity because essentially most ions generated by the source can be recorded by the detector. The first commercial MALDI–TOF instrument was produced in 1990 (Cottrell, 1992), but like all linear instruments of its type, the resolution was poor (in the range of 2–300). Higher resolutions of around 2000 FWHM (full-height at half-maximum) were soon achieved with magnetic sector instruments and were used for work on carbohydrates (Bordoli et al., 1994, 1996). These instruments had to be fitted with an array detector in order to accommodate the pulsed nature of the MALDI ion source. Soon, however, commercial instruments fitted with reflectrons were able to provide even higher resolutions, combined with the advantage of high sensitivity. Still further improvement was achieved with
the introduction of “delayed extraction” or “time-lag focusing” ion sources (Vestal, Juhasz, & Martin, 1995), which is a concept that was inherent in Wiley and McLaren’s original design for a high resolution TOF ion source (Wiley & McLaren, 1955). Today, resolutions of 10,000 or more, providing mass accuracies of a few parts per million (ppm), are becoming routine with TOF instruments, thus rendering the use of sector instruments with array detectors obsolete in this context.

However, two alternative approaches to the recording of MALDI mass spectra with a magnetic sector instrument, employing a conventional point detector, have been reported. In the first of these approaches, developed by Kolli & Orlando (1996), the instrument was set to scan slowly (0–2400 mass units in 1 min), and many spectra were recorded and averaged. With a sufficient number of spectra, enough small spectral segments were acquired to enable the full spectrum to be obtained. In order for the technique to work, it was necessary to develop a matrix that enabled a signal to be maintained for several minutes. The ‘liquid’ matrix, consisting of α-cyano-4-hydroxycinnamic acid (4-HCCA, 23) and 3-aminoquinoline (24), described below, proved to be satisfactory for carbohydrate research. Use of the magnetic sector instrument enabled resolutions of up to 8000 (FWHM) to be achieved with a mass accuracy of ±2 ppm. MS/MS spectra of maltodecaose were obtained with helium as the collision gas (Kolli & Orlando, 1997).

In the second approach, the scan speed of the instrument was reduced such that six to ten laser pulses were fired across each mass peak (Harvey & Hunter, 1998a, b) (Fig. 1). The resulting spectra were smoothened to reveal the peak profile. In order to maintain the ion beam, the above liquid matrix was used. Although slow,

![Figure 1](image_url)
this scanning method has the advantage that spectra can be recorded in a single scan and can utilize the full resolving power of the mass spectrometer. Using maltoheptaose and a biantennary N-linked glycan, resolutions of ca. 20,000 were obtained.

MALDI ion sources have also been interfaced to ion-trap mass spectrometers (Qin, Steenvoorden, & Chait, 1996). In these instruments, the time between ion production and detection is sufficiently long for considerable fragmentation to occur, as demonstrated by Qin & Chait (1997) for the 5 kDa glycoprotein, amylin. As described below, fragmentation under these conditions involves mainly rupture of glycosidic bonds, and the major ion in the amylin spectrum was due to the loss of a sialic acid residue (291 mass units). Fourier–transform ion cyclotron resonance (FT–ICR) instruments have also been used (Carroll et al., 1996), and have given exceptional resolution. For example, the molecular ion of permethylated β-cyclodextrin was shown with a resolution of 210,000 in the original paper. The other advantage that this type of instrument offers is very efficient collisionally induced dissociation to produce abundant structurally informative fragment ions.

C. Matrices

The nature of the matrix and the method of sample preparation are of critical importance for obtaining strong signals from carbohydrates because some compounds, which act as very effective matrices for some compound types, are ineffective for others. Discussed below are those matrices found most useful for carbohydrate analysis.

1. Matrices for Free Neutral Carbohydrates

a. Substituted benzoic acids

The first matrix specifically designed for MALDI analysis of carbohydrates was 3-amino-4-hydroxybenzoic acid (25) (Mock, Davy, & Cottrell, 1991). It produced a MNa\(^+\) ion and gave signals from as little as 1 pmol of dextran hydrolysate and N-linked glycans released from glycoproteins. This matrix was soon superseded by 2,5-dihydroxybenzoic acid (2,5-DHB, 26) (Stahl et al., 1991), a matrix originally introduced for proteins by Strupat, Karas, and Hillenkamp in 1991 (Strupat, Karas, & Hillenkamp, 1991). It is still in use today as the most popular matrix for carbohydrates. Again, it produces MNa\(^+\) species as the major ion. This ion is often accompanied by a weaker MK\(^+\) ion, and other species, such as MLi\(^+\) can be generated by the addition of the appropriate inorganic salt to the matrix (Stahl et al., 1991). Mohr, Börnsen, & Widmer (1995) have studied the relative affinities of different alkali metal ions for carbohydrates, and have found that the affinity order is Cs > K > Na > Li > H (Fig. 2). Cesium, however, although the most efficient at producing ions, is unable to ionize small carbohydrates (Cancilla et al., 1996). Di- and trivalent metals are also capable of ionizing carbohydrates, but only singly charged molecular ions are formed (Wong & Chan, 1997).

2,5-DHB typically crystallizes from mixtures of acetonitrile, or ethanol and water as long, needle-shaped crystals that originate at the periphery of the target spot and project towards the center. The central region usually contains an amorphous mixture of sugar, contaminants, and salts. In order to produce a more even film of crystals, the spot may be re-dissolved in dry ethanol and allowed to recrystallize (Harvey, 1993; 1996b). This technique not only produced a thin, even film of small crystals, but also increased the sensitivity by about an order of magnitude, probably due to more efficient mixing of sample and analyte from the single solvent. Evidence for fractionation of sample constituents in 2,5-DHB has been provided by Stahl et al. (1994a). They observed that, in a mixture of sugars and glycoproteins, spectra of the sugars could be obtained from the amorphous central region of the target, whereas the glycoproteins gave spectra from the peripheral crystals.

Other isomers of 2,5-DHB produce relatively poor signals from carbohydrates unless they contain an ortho-hydroxy group (Krause, Stoeckli, & Schlunegger, 1996). The authors of that paper (Krause, Stoeckli, & Schl-
negger, 1996) have proposed that, to be a matrix, the compound must be able to undergo an intramolecular hydrogen transfer between the phenolic and carboxyl groups. Only the ortho-substituted isomers are able to achieve this transfer. Of the twelve hydroxybenzoic acids tested, all of the ortho-hydroxylated compounds were effective matrices, whereas other compounds were not. The 2,5-dihydroxybenzoic acid was the most effective of the ortho-substituted compounds tested. The reason for the 2,5-isomer being the most effective is not clear, but may be related to its further photochemical decomposition (Harvey, 1993). In response to UV light, benzoic acids tend to eliminate CO₂. Further oxidation of the aromatic moiety of 2,5-DHB would produce the relatively stable ₂⁺-benzoquinone. None of the other isomers studied can react in this way to produce a stable product. Based on this proposal, other potential matrices capable of forming stable conjugated species have been investigated, and compounds such as 1,4-dihydroxy-2-naphthoic acid (27) have been found to be suitable (Harvey, 1993).

Mixtures of several other compounds with 2,5-DHB have given improved sensitivity or resolution. Thus, Karas et al. (1993) have added small amounts of other substituted benzoic acids or related compounds with the aim of causing disorder of the crystal lattice, allowing ‘softer’ desorption. The most effective additive was 10% 2-hydroxy-5-methoxybenzoic acid (28), giving a mixture commonly referred to as ‘super 2,5-DHB’. An increase in sensitivity of 2–3 fold was reported for a standard dextran 1000 mixture with a concomitant resolution increase being attributed to a reduction in metastable ion formation. This matrix has also been reported to give enhanced signals from tryptic glycoproteins (Tsaropoulos et al., 1994) and has been used by a number of investigators as will be evident later in this review.

Carbohydrates themselves have been used as matrix additives. Thus, Gusev et al. (1995) have obtained improved reproducibility and resolution with the addition of α-L(−)-fucose to 2,5-DHB, and even better results when 2,5-DHB was doped with 2-hydroxy-5-methoxybenzoic acid and fucose. Accelerated drying of the target spot also improved performance. The fucose was thought to decompose to yield gaseous products such as carbon dioxide and water during the desorption process, creating a dense environment that could cool the analyte molecules (Köster, Castoro, & Wilkins, 1992). The addition of the co-matrices appeared to improve crystal homogeneity rather than change the morphological structure of the crystal.

Mohr,Bornsen, & Widmer (1995) have found that a mixture of 2,5-DHB and 1-hydroxyisoquinoline (HIQ, 29) in the molar ratio of ca. 3:1 was very effective at ionizing carbohydrates. Fast vacuum drying was used to produce fine crystals, and this drying led to a strong signal. 1-Hydroxyisoquinoline itself was a poor matrix for carbohydrates, but its presence in the 2,5-DHB caused much finer crystals to form than when 2,5-DHB was used alone. Being less soluble in the water:acetonitrile solvent, the HIQ crystallized first and was thought by Mohr, Bornsen, & Widmer (1995) to seed the crystallization of the 2,5-DHB. The resulting spectra, however, were devoid of matrix peaks from 2,5-DHB, although peaks from the HIQ were present. The matrix was found to be tolerant to the presence of a number of salts and additives and even to compounds such as sodium dodecylsulfate (SDS). So tolerant, in fact, did the matrix prove to be, that a strong signal from maltose oligomers up to the 35-mer was obtained from the confectionery “Gummy Bears” by simply dissolving it in water, filtering through a 22 μm Millipore filter, and mixing with the matrix.

Mechref & Novotny (1998a) have used the base spermine (30) as a co-matrix with 2,5-DHB for the examination of sialylated glycans in the negative ion mode. One of the problems with the spectra of sialylated glycans is the tendency of the carboxylic group of the sialic acids to form salts with alkali metals, giving multiple peaks in the spectra. Addition of spermine to 2,5-DHB was found to reduce sodium salt formation and to provide a good crystalline surface. Detection limits of ca. 50 fmol were reported. Calibration of the instrument in the negative ion mode was achieved with a dextran ladder labeled by reductive amination (see later) with 8-aminopyrene-2,3,4-trisulfonic acid (31) in order to introduce an anionic group.
2-(p-hydroxyphenylazo)benzoic acid (HABA, \textbf{32}) gives a fine crystalline surface but weaker signals than those from 2,5-DHB. Fragmentation, however, is much more pronounced. Unfortunately, this matrix produces relatively abundant matrix ions up to about \textit{m/z} 1000 (Mohr, Börnsen, & Widmer, 1995), and is, thus, of little use for small glycans.

\textbf{b. 3-Aminoquinoline}

Metzger et al. (1994) have successfully used 3-aminoquinoline (\textbf{24}) for ionization of plant inulins with masses of up to 6 KDa. A lower background and an increased resolution over those obtained with 2,5-DHB was reported. On the other hand, Papac, Wong, & Jones (1996) have found that this matrix sublimes too rapidly to be of great practical use, although Stahl et al. (1994a) have reported that it is superior to 2,5-DHB for ionization of sialylated sugars. A 5–10 fold increase in sensitivity was achieved. Less fragmentation was produced by this matrix than from 2,5-DHB, but the matrix appeared to be more sensitive to the presence of contaminants (Stahl et al., 1997). Kolli & Orlando (1996) have used 3-aminoquinoline, mixed with 4-HCCA (\textbf{23}), to produce a “liquid” matrix that was capable of giving long-lasting signals from a single laser spot. Results of our experiments show that, the matrix has the appearance of a viscous gum rather than a liquid but it enables a signal to be recorded from a single spot for several minutes. Its general applicability for glycan analysis, however, appears to be limited because we have observed that reducing sugars are able to form Schiff bases with 3-aminoquinoline, particularly in the acidic environment created by the presence of 4-HCCA (Harvey & Hunter, 1998a). The success of the matrix for ionization of inulins may stem from the fact that they have a blocked reducing terminus, and, thus, are not susceptible to reaction with the basic amino group of 3-aminoquinoline.

\textbf{c. Other liquid matrices}

A mixture of potassium hexacyanoferrate and glycerol was developed as a liquid matrix by Zöllner, Schmid, & Allmaier (1996), and was found to produce signals at the femtomole level from hydrophobic compounds. Although signals from underivatized glycans were weak, strong signals were obtained from glycolipids. Premixed sample:matrix mixtures deposited onto the target were found not to give a signal. The recommended sample preparation technique was to deposit the matrix, dissolved in methanol, onto the target, allow it to dry, and finally to cover the surface with the sample dissolved in water or chloroform. The best signals were obtained directly after inserting the target into the mass spectrometer. Although signals could be obtained for several hours, the sensitivity progressively fell as the result of evaporation of the glycerol. Spectra consisted of single potassium adduct peaks with no evidence of fragmentation. Peracetylated and partially benzylated carbohydrates gave stronger signals than underivatized sugars, due to solubility of the analyte in chloroform (Zöllner et al., 1997). Only 5–50 pmol was needed to produce a strong signal.

A “pseudo-liquid” matrix, consisting of a concentrated solution of hygroscopic tetrabutylammonium bromide, chloride, or acetate, has been described by Breuer, Knochenmuss, & Zenobi (1998). Because the salt solution did not absorb UV light, it was mixed with dark silicon particles. Carbon was found to be as effective but it produced intense carbon-cluster ions. The matrix was prepared by adding ca. 70% by volume of water to the salt crystals, followed by an equal volume of silicon particles. After being shaken for at least 15 min, 0.5 \textmu L of the slurry was applied to the MALDI target and was allowed to dry. Analyte solutions were then applied to the target. Ionization was thought to occur by a chemical ionization-type process. The negative ion MALDI spectrum of \textit{g}-cyclodextrin recorded from the bromide salt showed a prominent \([\text{M+Br}]^-\) ion with no fragmentation. However, the ion from the acetate, which had a higher proton affinity, gave \([\text{M-H}]^-\) as the molecular ion with a considerable amount of fragmentation in the form of B and C glycosidic cleavages (see below). The chloride salt, which had a proton affinity between that of the bromide and acetate, produced a \([\text{M+Cl}]^-\) and a \([\text{M-H}]^-\) ion with, again, glycosidic fragmentation.

\textbf{d. Mercaptobenzothiazoles}

Of five mercaptobenzothiazoles examined by Xu et al. (1997a), only 5-chloro-2-mercaptobenzothiazole (CMBT, \textbf{33}) proved effective for carbohydrates. However, it was
found to be superior to 2,5-DHB for analysis of high-mannose N-linked glycans because it produced a much improved signal:noise ratio and somewhat better resolution. A strong signal was observed from 100 fmol of (Man)$_{5}$(GlcNAc)$_{2}$, whereas no signal could be detected at this level from 2,5-DHB. However, although signals from compounds with masses of up to 5500 Da were observed from dextran 5000, some discrimination against the higher mass ions was reported.

![33. 5-Chloro-2-mercaptobenzothiazole (CMBT)](image)

e. **β-Carbolines**

The β-carbolines harmane (34), nor-harmane (35), harmine (36), harmol (37), harmaline (38), and harmalol (39) have been shown to be useful matrices for cyclic (cycloextrinsics in the 1000 Da region) and acyclic (MW 342–828) carbohydrates (Nonami et al., 1998). They produced signals of comparable intensity to those obtained from 2,5-DHB, but, unusually, they produced MH$^+$ rather than MNa$^+$ ions in the positive ion mode from cycloextrinsics. In our hands, however, they give MNa$^+$ ions from maltooligosaccharides and N-linked glycans, accompanied by a prominent fragment ion due to loss of water. Furthermore, these matrices have been reported to produce [M–H]$^-$ ions from small, neutral glycans. No negative ion signal is normally observed from neutral sugars with matrices such as 2,5-DHB. Hao et al. (1998) have also observed negative ion spectra from neutral sugars, but in their case the compounds (dextran) were much larger (7500 Da). Interestingly, the ions were produced from 2,5-DHB; that observation led the authors to believe that the MALDI process for polysaccharides must differ somewhat from that for smaller sugars.

![Image](image)

![Image](image)

f. **Osazones**

Osazones have been reported to give superior spectra than those produced by 2,5-DHB (Chen, Baker, & Novotny, 1997). In particular, resolution was improved and the signal:noise ratio was higher, leading to better detection limits (50 fmol) for neutral sugars. The research that resulted in the development of these matrices was initiated by the observation that osazones produce some of the best crystallization characteristics known, and also absorb in the UV range. Several osazones were synthesized and tested, and those synthesized from D- or L-arabinose (40) with phenylhydrazine were found to be the best. Those osazones from hexoses were not soluble enough in water to be used with aqueous sugar solutions. The spectrum of laminarin, a linear D-glucan from brown algae that contains 1,3- and 1,6-linkages, showed larger peaks from the higher mass constituents when recorded from arabinosazone than from 2,5-DHB. This effect was attributed to less fragmentation as the result of the lower laser energies needed to produce signals with arabinosazone. Fragment ions were, in fact, seen at [M–98]$^+$ from maltooligosaccharides that contain 1,4-linked glucose. A loss of 98 mass units is difficult to explain from the MNa$^+$ ion produced from these compounds, but could be accounted for by an $0.4X$ cross-ring fragmentation (see below) from a MH$^+$ ion. Although these MH$^+$ ions were not observed, a similar type of behavior has been observed in the electrospray spectra of these compounds, where prominent MNa$^+$ ions are observed together with glycosidic fragments of the absent MH$^+$ ion. Ngoka, Gal, & Lebrilla (1994) have reported that protonated sugars decompose at much greater rates than sodiated glycans; that finding supported this proposal. Possibly of diagnostic significance, the [M–98]$^+$ ions observed by Chen, Baker, & Novotny (1997), were present only in the spectra of 1,4-linked oligomers. Wheeler and Harvey (unpublished) have found that arabinosazone produces strong negative ion spectra ([M–H]$^-$ molecular ions) from small glycans that contain sialic acid, and that the linkage of the sialic acid can be deduced from the production of diagnostic fragment ions.

![Image](image)
g. Ferulic acid
Ferulic acid (41) is an uncommon matrix for sugars, but has been used to study the (p-methylbenzylidene) complex of sorbitol (42) (Kim, Shin, & Loo, 1998). Because of the insoluble nature of the analyte, a vapor deposition method of sample preparation was developed. Sample and matrix were heated at 100°C whereupon they sublimed and condensed on the sample plate.

h. Hydroxyacetophenones
Several substituted acetophenones have been shown to be efficient matrices for peptides (Krause, Stoeckli, & Schlunegger, 1996), and would also appear to be suitable for carbohydrates. As with the substituted benzoic acids, only those compounds that possess an ortho-hydroxy group were effective for ionization of peptides, but unlike the benzoic acids, acetophenones were much more effective at producing negative ion signals. The most efficient compound, in the positive and negative ion modes, was 2,5-dihydroxyacetophenone (DHAP) (43). Although, in our hands, this compound does act as a matrix for neutral carbohydrates, it does not appear to offer any significant advantages over 2,5-DHB.

j. Ineffective compounds
The following matrices, which are effective for compounds other than sugars, have been reported to give weak or no signals from neutral carbohydrates 2-amino-5-nitropyridine, anthranilic acid, 3-hydroxypicolinic acid, 3-hydroxypicolinic amide, ferulic acid, salicylic acid (Mohr, Börnsen, & Widmer, 1995), 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, and sinapinic (sina-pic) acid (Harvey, 1993; Mohr, Börnsen, & Widmer, 1995).

2. Matrices for Free Acidic Carbohydrates
Acidic glycans, such as the sialylated N-linked sugars, generally give poor MALDI spectra when ionized with matrices such as 2,5-DHB. Positive (MNa⁺) and negative ions ([M–H]⁻) are both produced, and multiple peaks are present as the result of sodium and potassium salt formation. Fragmentation of sialylated glycans and some sulfated carbohydrates by loss of either sialic acid or CO₂ (from sialic acids) or sulfate, can also be a problem (see below). Carbohydrates that contain uronic acids, however, appear to be stable under MALDI conditions (Simpson et al., 1998). Oligomers that contain as many as 12 galacturonic residues give strong signals in the positive ion mode with 2,5-DHB as the matrix (Daas et al., 1998) (Fig. 3). However, many of the problems associated with loss of sialic acids by fragmentation can be overcome by a suitable choice of matrix. Alternatively, it has recently been reported (Cramer et al., 1998) that glycoproteins that contain sialylated glycans can be ionized intact with an IR laser.
a. 6-Aza-2-thiothymine

Papac, Wong, & Jones (1996) have found that 6-aza-2-thiothymine (45), a matrix earlier found effective with gangliosides (Juhasz & Costello, 1992), gives a significant increase in sensitivity for acidic glycans over that produced by the more common matrices. Sialylated biantennary N-linked glycans were reported to give a detection limit of 50 fmol (signal:noise ratio = 6:1), which is ca. 10-fold greater than that obtained from 2,5-DHB. However, fragmentation by loss of HCOOH and, sometimes, a small amount of sialic acid was seen from spectra recorded in the linear mode from the [M−H]− ion. With the reflectron operating, a major loss of sialic acid and a weaker loss of CO2 was seen (Fig. 4).

b. Hydroxyacetophenones

Another matrix found to be effective for the analysis of sialylated glycans is a mixture of 2,4,6-trihydroxyacetophenone (THAP, 44) and ammonium citrate (1 mM THAP in 1 mL of a 1:1 mixture of acetonitrile:20 mM ammonium citrate) (Papac, Wong, & Jones, 1996). The ammonium citrate was thought to prevent salt formation from the carbohydrate (Zhu et al., 1996). This mixture gave a single negative ion peak from sialylated N-linked glycans at the 10 fmol level in linear mode with no evidence of fragmentation. However, in the reflector mode, loss of sialic acid was again seen. The conditions used for sample preparation were found to be crucial for obtaining maximum sensitivity. Vacuum drying of the sample spot was used to prevent large crystals from forming, and then the sample was allowed to absorb water to promote the formation of small crystals. The percentage of water in matrix crystals has been reported by others (Figueroa, Torres, & Russell, 1998) to affect signal strength as well as crystal formation. The utility of the matrix was demonstrated with glycans from tissue plasminogen activator (tPA) which gave a profile typical of that reported by others. THAP also gave good signals from glycopeptides separated by high performance liquid chromatography (HPLC) under conditions where 4-HCCA failed.

It was noted during the research with free glycans that acid-catalyzed lactonization of the sialic acids could give an ion that appears at 18 mass units lower than the molecular ion. However, lactone formation could be prevented if ammonium hydroxide was added to the sample. Another possible disadvantage of this matrix is that the formation of citrate adducts ([M+191]−) from high-mannose glycans has sometimes been noted (Papac et al., 1998). Another problem is that THAP has been reported to be ineffective for the ionization of sulfated carbohydrates (Nonami, Fukui, & Erra-Balsells, 1997).
3. Matrices for Sulfated Carbohydrates

Many sulfated glycans are particularly difficult to study by MALDI, partly because of the labile nature of the sulfate groups in certain linkages. Although signals are produced by most of the normal carbohydrate matrices, results can be erratic and a number of laboratories have attempted to develop alternative methods. Thus, Nonami, Fukui, & Erra-Balsells (1997) showed that β-carbolines such as harmane (34) and nor-harmane (35) produced negative ion signals from β-carrageenans. However, out of a series of carbazoles, only 1-nitrocarbazole (46) was effective. Dai et al. (1997) have found that the basic compound 7-amino-4-methylcoumarin (47) is a good matrix for monosulfated disaccharides. Molecular ions could also be obtained from HABA and sinapinic acid (48), but the presence of abundant matrix ions in the region of the molecular ion interfered with detection. The nature of the sugar was found to affect the efficiency of ionization. For example, sulfated tri- and tetra-saccharides and those containing sialic acid ionized better from a mixture of 7-amino-4-methylcoumarin (47) and 6-aza-2-thiothymine (45) than from 7-amino-4-methylcoumarin alone. 7-Amino-4-methylcoumarin is hydrophobic and was found to produce strong signals from other hydrophobic compounds such as sugars that contain a methoxycarbonyloctyl group at the reducing terminus. This strategy for increasing hydrophobicity could probably be used to advantage in combination with other hydrophobic matrices. Another approach for the ioniza-
tion of sulfated glycans involving ion-pairing with bases is discussed later in the section on glycosaminoglycans (VIII.C.3).

a. Ineffective compounds

The following matrices have proved to be ineffective for sulfated carbohydrates: anthranilic acid, several hydroxyacetophenones, hydroxybenzophenones, harmalol, substituted carbazoles, and indoleacrylic acid (Nonami, Fukui, & Erra-Balsells, 1997).

4. Matrices for Glycoproteins

Although some of the above matrices are capable of ionizing glycoproteins and glycopeptides, most large glycoproteins require a different range of compounds. 2,5-DHB and 4-HCCA effectively produce molecular ions from glycoproteins up to a molecular weight of about 5000 Da, but above this mass, signal strength tends to fall. The presence of basic groups in the peptide encourages the formation of $\text{MH}^+$ rather than $\text{MNa}^+$ ions, and this trend increases with the percent of peptide in the molecule. 2,5-DHB, however, tends to produce more $\text{MNa}^+$ ions than does 4-HCCA for a given glycopeptide. Kussmann et al. (1997a) have described several methods for sample preparation with 4-HCCA. In their “thin layer” method, a thin film of matrix solution is allowed to dry on the target before the analyte solution is placed on top. In the “thick layer” method, 4-HCCA is mixed with nitrocellulose in acetone, applied to the target, and allowed to dry before addition of the sample. A “sandwich” method is also described in which an extra layer of matrix is added to the thin layer preparation.

These three sample preparation methods can be used with 4-HCCA but not with 2,5-DHB, which only appears to work well as a dried droplet. For small glycopeptides, Kussmann et al. (1997a) preferred 2,5-DHB, followed by 4-HCCA. Larger glycoproteins usually give enhanced signal strengths if examined with matrices such as sinapinic acid, as found by Beavis & Chait (1989). Unfortunately, there appeared to be no particular sample preparation technique that worked well with all samples. Each sample had to be treated individually.

Pitt & Gorman (1996) have confirmed the utility of 2,6-dihydroxyacetophenone (49) as a useful matrix for glycoproteins. The compound was mixed with diammonium hydrogen citrate (10%, 1 M), and was shown to give much sharper peaks from fetuin glycopeptide (5–6 kDa) than from either 2,5-DHB or 4-HCCA. The reason was thought to be the much smaller amount of metastable fragmentation produced by the acetophenone. In the reflector mode, 2,5-DHB and 4-HCCA both failed to produce molecular ions from this glycopeptide, whereas strong peaks were produced from the 2,6-dihydroxyacetophenone/ammonium citrate mixture. This mixture was also shown to produce stronger signals from bovine serum albumin (BSA) than those obtained from sinapinic acid, which is usually considered the most effective matrix for proteins.

The resolution of existing TOF mass spectrometers does not allow any isotopic resolution for glycoproteins with masses above about 10 kDa. With larger molecules, resolution of salts, adducts, fragment ions, and other components such as glycoforms becomes increasingly difficult and can result in mass measurement inaccuracies. Fragmentation will give peak broadening on the low mass side of the peak, and adduct formation (from matrix or alkali metals) will cause the peak to broaden towards high mass. Thus, matrices should be chosen to reduce the production of these unresolved components. In many cases, this choice can only be made on a “trial and error” basis because the reason for peak broadening for any particular glycoprotein is usually not obvious. Results, however, can sometimes be dramatic. For example, ribonuclease B, a glycoprotein of ca. 15 kDa, that carries five neutral glycans, has been reported to give a spectrum with resolved glycoform components on a linear TOF instrument when recorded from 2,4,6-THAP (44), whereas from sinapinic acid the presence of glycoforms was not apparent (Sottani, Fiorentino, & Minoia, 1997). Similar results were obtained with ovalbumin (Fig. 5). With the acidic glycoprotein, erythropoietin (28 kDa), ferulic acid gave a more resolved spectrum than sinapinic acid. The addition of a small amount of formic acid also improved the resolution in this case, presumably by
reducing alkali metal adduction. MALDI was stated to be the only technique capable of producing a useable spectrum from this glycopeptide; the glycoform population was too complex to be resolved by electrospray.

As an illustration of the effect of fragmentation on mass measurement accuracy, Chinese hamster ovary (CHO)-derived human interleukin receptor protein (23,671 Da), a glycoprotein that contains a high proportion of sialic acid, gave a mass that was low by the equivalent of 11.3 sialic acids when measured on a reflectron instrument from super-DHB (Tsarbopoulos et al., 1995) (Fig. 6). The mass deficit was caused by the metastable loss of one or more molecules of sialic acid. When measured on a linear instrument, the correct mass was obtained (Fig. 6c). The colder matrix, 3-hydroxypicolinic acid (314), which does not catalyze fragmentation, does not produce the mass deficit on reflectron instruments, but unfortunately forms adducts with the glycoprotein to produce a mass excess.

Juhasz, Costello, & Biemann (1993) have reported that HABA is a superior matrix for large glycoproteins than sinapinic acid, 2,5-DHB, or 4-HCCA. HABA does not show the same discrimination against high-mass compounds as the other matrices, and it also provides higher resolution. Its very even crystalline surface gives little spatial variation in ion signal over the target surface. Strong signals have also been reported from glycolipids with this matrix (Juhasz, Costello, & Biemann, 1993).

![FIGURE 5. Linear MALDI mass spectrum of ovalbumin recorded from (a) sinapinic acid and (b) 2,4,6-THAP. (From Sottani, Fiorentino, & Minoia, 1997).](image)

![FIGURE 6. MALDI mass spectrum of recombinant human interleukin-4 receptor recorded with (a) a reflectron TOF instrument with super-DHB as the matrix, (b) a reflectron TOF instrument with 3-hydroxypicolinic acid as the matrix, and (c) a linear TOF with super DHB as the matrix. The correct mass is shown by the vertical line. (From Tsarbopoulos et al., 1995).](image)

\[\text{HABA} \]

50, 3-Hydroxypicolinic acid
IV. QUANTITATIVE ASPECTS OF MALDI MASS SPECTROMETRY

In common with that of proteins (Jespersen et al., 1995), the signal strength of glycans ionized by MALDI appears to reflect accurately the amount of material on the target, providing that the correct matrix is chosen (Harvey, 1993). 3-Amino-4-hydroxybenzoic acid (25) is a poor quantitative matrix because it appears to show a saturation effect at relatively low analyte concentrations with N-linked glycans. 2,5-DHB, on the other hand, does not show such an effect, and produces a linear response from samples over several decades of concentration (Fig. 7). However, in the presence of an internal standard of similar structure, even matrices such as 3-amino-4-hydroxybenzoic acid can be used for quantitative work if peak ratios are used. Thus, the use of a fucosylated analog as an internal standard for quantification of a biantennary glycan has given a linear concentration–response relationship with a correlation coefficient of 0.999. The amount of the biantennary glycan could, thus, be determined reasonably accurately. Similar results have been obtained with cyclodextrins, using maltohexaose as the internal standard and 4-HCCA as the matrix (Bartsch et al., 1996). In this latter work, comparisons were made between peak height and peak area measurements, but no significant differences between them were seen. A linear relationship has also been found for N-linked glycans derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) (see Section V.B.3) when measured against PMP-(Glc)₆ with 2,6-DHAP as the matrix (Pitt & Gorman, 1997).

Quantitative measurements can also be made on sugars extracted from biological media as illustrated by a method for measuring a trisaccharide product (Fucα(1→2)Galβ(1→3)Gal) of a reaction to study galactosyl transferase. The tetrasaccharide (Fucα(1→2)-Galβ(1→3)Fucα(1→4)GlcNAc) was used as the internal standard (Whittal et al., 1995). The compounds were derivatized as their tetramethylrhodamine (TMR) analogs to increase sensitivity (see Section V.B.3), and were measured directly in serum following an on-probe clean-up method discussed below. A linear calibration was obtained over the range 0.2–2 pmol/μL.

It is essential in quantification studies to compensate for the inhomogeneity of the target surface by averaging signals from several laser shots fired at multiple spots. Harvey (1993) used five shots fired at each of 16 spots for each measurement, and Bartsch et al. (1996) used ten shots fired at ten spots. Such protocols usually generate standard deviations in the region of 6–10% from sugars and other compounds such as proteins (Jespersen et al., 1995). Also of importance is the need to use a matrix that provides a homogeneous surface. 2,5-DHB is not good in this respect, although it may be improved as discussed above, by recrystallization of the dried spot from ethanol (Harvey, 1993). 4-HCCA gives a better surface, but it has been noted that there are solubility problems above 10 nmol/μL for cyclodextrins, and that below 100 pmol/μL, signal strengths are poor (Bartsch et al., 1996).

For mixture analysis, it is essential that there should be an equal response from each constituent and that the response is independent of the molecular weight and structure. With MALDI, this independence appears to be the case for the ionization of carbohydrates. No mass-dependent variation in ion abundance has been observed up to ca. 10,000 Da (Stahl et al., 1991; 1997) from 2,5-DHB or 3-AQ. For inulins in this mass range, MALDI and high pH anion exchange chromatography (HPAEC) have been found to give similar profiles (Stahl et al., 1997). However, for larger polymer mixtures, there is a fall in signal intensity with increasing mass. Garrozzo et al. (1995) have investigated dextran mixtures with masses of up to 100 kDa, ionized from 2,5-DHB, and found that, for mixtures spanning a wide molecular weight range, there was a pronounced loss of signal from the larger molecules. This effect was attributed to signal suppression by the smaller components and verified by a prior fractionation of the mixture. After this procedure, the fractions that contained only a small range of the larger compounds gave strong signals. Similar results have been obtained by Hao et al. (1998). This suppression effect was also noted by Mohr, Börnsen, & Widmer (1995) with dextran mixtures of up to 40 kDa, but was attributed to the aggregation of the larger molecules as seen with synthetic polymer samples. Garrozzo et al. (1994) have compared the MALDI responses given by β(1→2) cyclic glucans with molecular weights of 2–4 kDa on three commercial

![FIGURE 7. Relationship between the signal strength of a biantennary N-linked glycan and the amount deposited onto the MALDI target with 2,5-DHB as the matrix. (From Harvey, 1993).](image-url)
instruments and found no significant difference. Furthermore, the profiles did not show any significant difference from those recorded from the same compounds by HPLC. Similarly, the profile of high mannose glycans released from ribonuclease B and examined by MALDI have shown no significant differences from those obtained by capillary electrophoresis, gel filtration (P-4), and HPLC (Rudd et al., 1992).

In addition to the absence of an effect of molecular weight on signal strength, there also appears to be no significant effect of structure on the efficiency of ionization (Stahl et al., 1991) of sugars. This lack of these two effects is unlike the situation with peptides, where the signal intensity is proportional to the proton affinity of the peptide; this difference suggests that formation of the MNa\(^+\) ion from sugars proceeds to a similar extent with most compounds. A comparative study of the signal strength produced by equimolar amounts of twenty-five N-linked glycans (Naven & Harvey, 1996b) has shown no significant difference for compounds with masses over about 1 kDa. For glycans with lower masses, the signal strength fell progressively with decreasing molecular weight when recorded on a linear TOF instrument. This effect was attributed to the transient saturation of the detector by the matrix ions, and with malto-oligosaccharides, was so marked that the monomer, glucose, could hardly be detected in a mixture of mono- to penta-oligomers. The signal strength from glucose was ca. 100-fold less than that of the pentamer. Kazmaier et al. (1998) have also reported that MALDI gives a lower response for smaller glycans in a comparative study with capillary electrophoresis and HPAEC. On the other hand, oligomers of higher molecular weight could be detected, with a higher resolution, by MALDI than by the other techniques. Furthermore, the compounds did not require derivatization. For larger glycopeptides (2.5–6 kDa) ionized from 2,5-DHB, excellent correlations have been allowed to dry as normal, and spectra were obtained (Mock, Sutton, & Cottrell, 1992). Although salts and most buffers can be removed from glycoproteins by washing the dried target in cold water (Beavis & Chait, 1990) or dilute trifluoroacetic acid (TFA) solution (e.g., Strub et al., 1996), carbohydrates are often too soluble for this strategy to succeed. Nevertheless, carbohydrates may be cleaned satisfactorily by drop dialysis on a membrane with a reasonably low molecular weight cut-off (Marusyk & Sergeant, 1980; Görisch, 1988) prior to deposition onto the target; 500 Da is a good cut-off size for the membrane for use with N-linked glycans. A small piece of the membrane, ca. 1 cm\(^2\), is floated on the surface of water, hydrophilic side down, and ca. 1 \(\mu\)L of the carbohydrate solution is placed on top. The apparatus is covered to prevent evaporation, and is left for 10–60 min. The solution can then be removed and treated as normal.

Börnsen, Mohr, & Widmer (1995) have used a Nafion-117 membrane to purify sugars. This membrane

\[ \text{MALDI MASS SPECTROMETRY OF CARBOHYDRATES} \]

### V. SAMPLE PREPARATION

Many naturally occurring carbohydrates occur as mixtures, and it is important to ensure that isolation and purification techniques do not cause any fractionation of the sample with loss of quantitative information. Sialic acids, for example are often lost from glycoproteins if the pH becomes too low or the sample temperature is too high. Care should be taken to ensure that residual enzymatic activity is not present in the sample because enzymolysis can result in altered glycan profiles. In a recent paper, for example, Field, Papac, & Jones (1996) report the co-purification of a \(\beta\)-galactosidase with recombinant TNK-tPA prepared in CHO cells. MALDI analysis of the released glycans over 65 days showed a substantial loss of galactose from the biantennary N-linked glycan as the result of the action of this enzyme.

### A. Contaminant Removal

The condition of the sample is of critical importance for obtaining high quality MALDI spectra. Compounds other than the analyte, such as salts and buffers, generally have an adverse effect on ion yield and crystal formation, and they should be removed. However, MALDI analysis of proteins and glycoproteins appears to be less affected by the presence of these contaminants than do most other forms of ionization. The maximum tolerated amounts of common buffers etc. have been investigated by Mock, Sutton, & Cottrell (1992). For peptides measured from 2,5-DHB, tolerance to buffers has been found to be buffer-dependent and dramatically improved at high matrix-analyte ratios (Yao et al., 1998). Carbohydrates appear to be somewhat more susceptible than proteins to the effects of salts and other compounds although the presence of small amounts of sodium or other alkali metals is essential for ionization. Metals have recently been found to cause clustering between the matrix and sample, with adverse effects on resolution. They also cause the matrix multimers, frequently seen with 2,5-DHB in particular, in the region of mass 200–1000 (Dubois et al., 1996).

Many methods for removing salts and buffers have been reported. For example, salts and buffers have been removed from glycoproteins by first adsorbing the glycoprotein onto a gold or better, onto an electrosprayed nitrocellulose target, washing with water, and adding the matrix solution. After about two minutes, the target was allowed to dry as normal, and spectra were obtained (Mock, Sutton, & Cottrell, 1992). Although salts and most buffers can be removed from glycoproteins by washing the dried target in cold water (Beavis & Chait, 1990) or dilute trifluoroacetic acid (TFA) solution (e.g., Strub et al., 1996), carbohydrates are often too soluble for this strategy to succeed. Nevertheless, carbohydrates may be cleaned satisfactorily by drop dialysis on a membrane with a reasonably low molecular weight cut-off (Marusyk & Sergeant, 1980; Görisch, 1988) prior to deposition onto the target; 500 Da is a good cut-off size for the membrane for use with N-linked glycans. A small piece of the membrane, ca. 1 cm\(^2\), is floated on the surface of water, hydrophilic side down, and ca. 1 \(\mu\)L of the carbohydrate solution is placed on top. The apparatus is covered to prevent evaporation, and is left for 10–60 min. The solution can then be removed and treated as normal.
was first pre-treated by heating at 80°C in nitric or hydrochloric acid for two hours to saturate all of the sulfate groups with protons before the membrane was washed with water. The sample was spotted onto the membrane surface, which was floated on water as above. This membrane had the additional advantage of adsorbing proteins and peptides (Fig. 8), and was extremely useful for removing these compounds from glycan mixtures. It has also been used, together with mixed ion-exchange chromatography, for cleaning the released glycan mixtures produced by automated hydrazine release (Harvey et al., 1999a) (Fig. 9). Another use has been to exchange sodium for other alkali metals in order to produce ions other than MNa⁺. Instead of hydrogen, the membrane was saturated with an alkali metal salt and floated on a 100 mM salt solution. After about one minute, the sample droplet was recovered with a micropipette and was mixed

![Graph](image)

**FIGURE 8.** (a) Positive ion MALDI spectrum of a mixture of maltoheptaose and angiotensin I recorded from 2,5-DHB/HIC with no pre-treatment. (b) The same sample with diammonium hydrogen citrate added. The glycan peak is suppressed. (c) The sample after purification on a Nafion membrane, showing a complete removal of the peptide. (From Börnson, Mohr, & Widmer, 1995).

with matrix as normal. The resulting MALDI ions consisted almost entirely of adducts of the salt used.

Instead of membranes, some investigators prefer short columns of ion-exchange or hydrophobic resins, packed into disposable pipette tips for contaminant removal. For example, Kussmann et al. (1997a) use micro-columns (~1 µL) of Poros 50 R1 packed into Eppendorf GELoader tips. This method is convenient because several resins can be employed in one tip to remove a variety of charged or hydrophobic compounds in one stage.

The use of graphite for desalting carbohydrates has been extensively investigated by Packer et al. (1998). It appears to be a robust method that can be used to clean carbohydrates released from glycoproteins by hydrazinolysis (see below), enzymatic glycan release, or β-elimination. The glycans were applied to a short carbon column in aqueous solution, and all but the neutral
monosaccharides were retained. Neutral oligosaccharides were eluted with a 25% solution of acetonitrile in water, and acidic glycans were recovered by the further addition of 0.05% TFA to this solution. The recovery was ca. 100%. The technique was claimed to remove salts, detergents (SDS and Triton X-100), proteins, hydrazine, and sodium borohydride, and could be used to purify glycans directly from biological fluids such as urine.

Desalting of glycans following separation by HPAEC is a particularly difficult problem. In the method devised by Thayer et al. (1998), an anion-exchange micromembrane system was used to exchange sodium ions in the eluant for hydrogen ions flowing on the opposite side of the membrane. An electric field was placed perpendicular to the liquid flow. For MALDI applications, the volatile TFA replaced the more usual sulfuric acid because sulfate ions have been observed to cross such membranes. The system was reported to remove 99.5% of the sodium.

In order to avoid sample losses by adsorption onto surfaces, the amount of equipment used during clean-up stages should be minimized, and to achieve this reduction, several investigators have removed contaminants directly from the sample probe tip. Thus, Whittal et al. (1995) first deposited a thick layer of 4-HCCA followed by a serum sample that contained the target carbohydrates derivatized with tetramethylrhodamine and diluted into 50% ethanol/water. Just before the sample dried, the probe was immersed in water for 45 sec. The excess of water was removed by touching the probe against a wiper. The method enabled the sugar to be detected in serum directly, without the need for an extraction step and with a detection sensitivity of hundreds of fmol/μL. In the method described by Rouse & Vath (1996), the carbohydrate solution was first deposited on the tip and dried with a stream of air. The matrix, super-DHB, was added, followed by various adsorbing media, depending on the contaminants present. The sample was again dried and the adsorbent was loosened with a microspatula before being removed with a forced air stream. Detergents were removed with Extracti-gel-D resin, cations with AG-50W-X8, and anions with Mono-Q resin. Acetate ions were more effectively removed, however, by drop dialysis on a Millipore 25 mm V-series membrane. The authors recommended this technique prior to their on-probe sample cleanup for any sample, such as those from HPAEC, which contains acetate ions.

B. Derivatization

Carbohydrates are not ionized as efficiently as compounds such as proteins that can be protonated, neither do they appear to be transferred to the vapor phase as effectively. Consequently, derivatization has frequently been used in attempts to redress these problems. Permethylation with methyl iodide, catalyzed either by the methylsulfenyl carbanion (Hakomori, 1964) or by sodium hydroxide (Ciucanu & Kerek, 1984), can improve the sensitivity by ca. an order of magnitude, but at the expense of a considerable increase in the molecular weight. Peracetylation is also effective and can be conveniently accomplished cleanly in the gas phase (Perreault et al., 1994).

Reducing sugars contain a single reactive carbonyl group that can be derivatized separately from the many hydroxy groups. This property has been used by several investigators to improve ionization, to assist desorption, or to add fluorescent tags for detection by HPLC. Several types of reaction are employed, of which reductive amination is the most common. Other reactions include formation of carbonyl-type derivatives, such as oximes and hydrazones. In order to increase sensitivity, the carbohydrate may be derivatized with a reagent that already contains a charged functional group, or with one that can easily be protonated. This former strategy avoids the inefficient sodium adduct formation that is necessary for ionization of neutral glycans.

1. Derivatives Formed by Reductive Amination

Many of these derivatives have been discussed in a recent review (Hase, 1996). The sugar, usually in mild acid solution to promote ring opening, is reacted with a large excess of amine (to form a Schiff base) in the presence of a reducing agent (Scheme 2). The product is a secondary

![Scheme 2](image)
amine, and although this amine can accept a proton during MALDI ionization, higher sensitivity can be achieved if groups that are more basic are incorporated into the derivative.

Takao et al. (1996) have used 4-aminobenzoic acid 2-(diethylamino)ethyl ester (ABDEAE 51) to prepare derivatives of maltoseptaose, dextran, and the N-linked glycan, (Man)_3(GlcNAc)_2, by reductive amination, and they report sensitivity increases of 1000-fold over that of the free glycan. An MH^+ ion rather than the more normal MNa^+ ion was formed, as expected, and probably accounted for the gain in ion yield. This large increase in sensitivity, however, does not appear to have been matched by other derivatives. Okamoto et al. (1997), for example, have compared the signals from 2-aminopyridine (2-AP, 52), 4-aminobenzoic acid ethyl ester (ABEE, 53), and trimethyl-(4-aminophenyl)amino (TMAPA, 54) derivatives of maltopentaose recorded from 2,5-DHB or 4-HCCA. 2,5-DHB proved to be the best matrix, and a 100-fold increase in sensitivity was found with the 2-AP derivative. The ABEE and TMAPA derivatives showed sensitivity increases of 30- and 10-fold, respectively. The poor result from the TMAPA derivative was somewhat surprising because it contains a constitutive positive charge. Derivatives of this type are observed to increase sensitivity dramatically when carbohydrates are examined by electrospray (Okamoto, Takahashi, & Doi, 1995; Suzuki, Kakehi, & Honda, 1996). However, this 10-fold gain was similar to that reported by Naven & Harvey (1996b) for sugars derivatized with Girard’s T reagent (55). Because this derivative also carries a constitutive cationic charge (Fig. 10), the modest increase in sensitivity suggests that other factors such as hydrophobicity also contribute significantly to gas-phase ion yields.

FIGURE 10. (a) Positive ion MALDI mass spectrum of N-linked glycans (45 pmol) from ribonuclease B recorded with a magnetic sector instrument with 2,5-DHB as the matrix. (b) The same glycans derivatized with Girard’s T reagent. (From Naven & Harvey, 1996b).
Although the derivatives formed from 2-aminopyridine (52) (Hase, Ibuki, & Ikenaka, 1984) have been in use for many years for chromatographic studies, they have not found extensive use for MALDI mass spectrometry. Tokugawa, Oguri, & Takeuchi (1996) have found that they produce mainly a MH\(^+\) ion rather than the normal MNa\(^+\) species with biantennary N-linked glycans ionized from 2,5-DHB, but sensitivity studies were not performed. The derivative was synthesized by reductive amination, but a borane-dimethylamine complex rather than the more usual cyanoborohydride was used for reduction of the Schiff base because this complex was more appropriate for the large-scale synthesis required. However, this reducing agent also appears to work well for analytical-scale reactions.

The 2-aminobenzamide (2-AB, 56) derivatives are a later development, and are popular for conferring fluorescence to the glycans for HPLC detection (Bigge et al., 1995). Unlike the 2-AP derivatives, they do not produce any appreciable MH\(^+\) ions, and they do not appear to offer any advantage for MALDI detection such as increasing sensitivity. Indeed, as discussed below, they are disadvantageous for many fragmentation studies because the formation of several important cross-ring fragment ions from the reducing-terminal sugar is suppressed.

Another derivative that has been developed for HPLC separations and that has given good MALDI spectra is that formed with 2-aminoacridone (AMAC, 58) (Okafo et al., 1996; 1997). The derivatives gave strong spectra that contained MH\(^+\) in addition to MNa\(^+\) and MK\(^+\) ions. Addition of lithium chloride to the 2,5-DHB:1-HIQ matrix suppressed these ions and converted all of the ionization into the MLi\(^+\) form without any apparent loss in sensitivity (North et al., 1997). In the negative ion mode, these derivatives gave [M−H]\(^-\) ions from N-linked sugars. Mono- and di-sialylated species could be differentiated by the ability of the di-sialylated glycans to form a mono-lithium salt; the hydrogen from the other acid group was preferentially lost during ion formation. AMAC derivatives have been used to investigate the structure of glycans from chicken ovalbumin (North, Birrell, & Camilleri, 1998) and, in combination with microbore reversed-phase HPLC, to examine glycans from ribonuclease B and human IgG (Grimm et al., 1998).

Aromatic sulfonate derivatives have recently been introduced as charged fluorophores for electrophoresis. 1-Aminopyrene-3,6,8-trisulfonate (APTS, 59) has been used as a derivative for capillary electrophoresis (CE), and Suzuki et al. (1997) have investigated its use for the detection, by MALDI, of peaks from the CE-separated compounds. The derivatives were prepared by reductive amination but were found to give multiple peaks with the conventional matrices due to formation of salts. However, the use of a new matrix, consisting of a 1:1 mixture of 3- (50) and 6-hydroxypticolinic acid (60), combined with on-probe sample clean-up by a NH\(^+\)\(\text{4}^-\)-loaded cation exchange resin (Rouse & Vath, 1996) gave a single peak for maltoheptaose in the negative ion mode with a detection limit of 30 fmol.
2. Carbonyl Derivatives

Zhao, Kent, & Chait (1997) used substituted-oxime formation for adding a basic peptide residue in its aminooxyacetyl form to the reducing terminus of several neutral N-linked glycans, and they reported sensitivity increases of between 50- and 1000-fold. 4-HCCA was used as the matrix, compared with 2,5-DHB/HIQ for the underivatized sugars. Although this technique appeared to work well with pure sugars, samples derived from biological sources invariably contained other carbonyl-containing compounds that appeared to react preferentially with the reagent, resulting in weak or absent signals from the analyte.

Substituted hydrazones were investigated by Naven & Harvey (1996b) in an attempt to avoid the reduction step of reductive amination with its subsequent problems of reagent removal. The reaction with Girard’s T reagent (55) was found to be particularly beneficial in producing reasonable increases in sensitivity (10-fold) because of its constitutive cationic charge (see Fig. 10).

3. Other Derivatives

The reaction of reducing sugars with 1-phenyl-3-methyl-5-pyrazolone under basic conditions leads to the formation of a di-substituted PMP derivative (61) (Honda et al., 1989), which gives good chromatographic and detection properties. Pitt & Gormon (1997) have found that this derivative is also useful for the MALDI analysis of maltoligosaccharides and N-linked glycans. The matrices 2,5-DHB and 4-HCCA yielded MNa⁺ ions, but 2,6-DHAP, in the presence of diammonium hydrogen citrate, produced predominantly MH⁺ ions. The derivatives were claimed to be useful for obtaining signals in the presence of contaminating material. Shen & Perreault (1998) found that they gave ionization efficiencies that were ten-fold better than those provided by the 2-AP derivatives, and that they were also easier to prepare. Although the derivatives were also useful for FAB and ESI studies, the signal under FAB conditions was only one hundredth as strong as that produced by MALDI from 4-HCCA. Tetramethylrhodamine derivatives (TMR, 62), originally synthesized for fluorescence studies, have been found to give an increase in sensitivity of 100-fold over that of the underivatized glycans (Whittal et al., 1995).

An alternative approach to improving the MALDI spectra of sialic-acid-containing carbohydrates is to methylate the carboxylic acid group of the sialic acid to produce a neutral sugar (Powell & Harvey, 1996). This methylation can be accomplished by first converting the acid into its sodium salt with an AG-50 ion-exchange resin in its sodium form, and reacting the resulting sialic acid salt with methyl iodide in dry dimethylsulfoxide (DMSO) for two hours. The free acid can also be methylated, but the reaction takes 48 h to complete. Formation of the methyl ester improves the signal in four ways. Firstly, it converts the entire ion current into the positive mode, thus avoiding the splitting of the signal between positive and negative ionization. Secondly, it enables the sialic acid-substituted glycans to be measured in the same spectrum as the neutral glycans with equivalent ionization efficiency and, thus, quantitative relationship. Thirdly, it prevents salt formation, with the result that only one peak is produced from each compound, and finally, it stabilizes the sialic acid and prevents sialic acid loss by fragmenta-
tion (Fig. 11). Permethylation, although conferring the same properties and somewhat increasing the sensitivity, is less useful for glycan profiling on account of the presence of abundant in-source fragment ions, some of which are isobaric with neutral sugars.

An alternative method for studying sialic acid composition but not their linkage to larger glycans, is to remove them and observe the released monosaccharides rather than the residual neutral oligosaccharide. Stehling et al. (1998) have used mild acid hydrolysis to remove sialic acids from bovine submaxillary gland mucins, and examined the released acids as their diaminomethylene-benzene (DMB) derivatives (prepared as derivatives for HPLC). The mild conditions employed allowed profiles of N-acetyl, N-glycoyl, and O-acetyl sialic acids to be observed. Corresponding profiles obtained by HPLC and MALDI suggested that the techniques were quantitatively accurate.

**VI. FRAGMENTATION**

Fragmentation of carbohydrates has been extensively studied following ionization by methods such as FAB, electrospray, and MALDI. In general terms, the types of fragmentation observed under different ionization conditions are similar and depend on factors such as the type of ion formed (MH\(^+\), MNa\(^+\), etc.), the charge state, the energy deposited into the ion, and the time available for fragmentation. Several reviews have appeared (e.g. Reinhold, Reinhold, & Costello, 1995). In general, the fragmentation pathways can be classified into two groups; glycosidic cleavages that result from the breaking of a bond linking two sugar rings, and cross-ring cleavages that involve the breaking of two bonds. The glycosidic cleavages provide information mainly on sequence and branching whereas the cross-ring cleavages reveal more details on linkage.
The accepted nomenclature for describing the fragmentation of carbohydrates is that introduced by Domon & Costello (1988). Ions that retain the charge at the reducing terminus are designated X (cross-ring), Y, and Z (glycosidic) whereas the complementary ions are A (cross-ring), B, and C (Scheme 3). Sugar rings are numbered from the non-reducing end for A, B, and C ions and from the reducing end for the others. Ions are designated by a subscript number that follows the letter to show the fragment type. Fragments from branched chain glycans are distinguished by Greek letters, with \( \alpha \) representing the largest chain. Cross-ring fragments are given superscript numbers showing the bonds cleaved.

The type of parent ion produced from the carbohydrate has a significant effect on fragmentation, but the resulting spectrum appears to be relatively independent of the way in which the parent ion is formed. Ngoka, Gal, & Lebrilla (1994) and Cancilla et al. (1996) have found that protonated species decompose much more readily than metal-cationized species, and that the order in which the cationized species decompose is \( \text{Li}^+ > \text{Na}^+ > \text{K}^+ > \text{Cs}^+ \). The exothermicity of cation binding also follows this order, suggesting that the protonated and lithiated species are formed with excess energy, thus promoting fragmentation. Furthermore, Cancilla et al. (1996) note that linear carbohydrates produce more fragmentation than branched ones. They attribute these observations to the coordination of the metal ion to the sugar and note, also, that the larger cations such as cesium are unable to ionize the smaller sugars. Molecular models that employ molecular dynamics calculations on the complex formed between cesium and the highly branched octasaccharide, difuco-syllacto-\( \text{N} \)-hexaose, show that the cesium atom sits near a branch point and coordinates as many as five pyranose rings; this gives the oligomer-cesium complex stability. Lithium, on the other hand, is relatively mobile and coordinates with only two rings. It is usually found near a relatively basic glycosidic oxygen, where it can presumably initiate a charge-site directed cleavage. Most fragmentation from the relatively rigid cesium complexes is, thus, probably due to charge-remote cleavage. The type of fragmentation seen with these species also varies. Many more cross-ring cleavages are produced from the metal-containing ions, particularly those containing lithium, than from the protonated species (Orlando, Bush, & Fenselau, 1990); these observations are common with those made for ions produced by other ionization techniques.

A. Post-source Decay (PSD) Fragmentation

A number of options are available for obtaining fragmentation from MALDI spectra. Huberty et al. (1993) first noted that, for linear TOF instruments, much of the signal produced by glycopeptides that contained sialylated glycans consisted of fragment ions formed by loss of sialic acid, as could be seen by switching on the reflectron. These ions, formed after ion extraction from the ion source, are termed post-source decay (PSD) ions in contrast to ions formed rapidly within the ion source, which are known as in-source decay (ISD) or prompt ions. ISD ions can be observed as focused ions in the linear spectrum. By stepping the reflectron voltage, so that fragment ions were focused over the entire mass range, Huberty et al. also observed strong glycosidic PSD fragment ions from a neutral biantennary glycan. These ions resulted from cleavage between the constituent monosaccharides, leading the way for general fragmentation studies following MALDI ion production. Talbo & Mann (1996) observed similar PSD ions from the disialylated analog of this biantennary glycan.

The extent of sialic acid loss and of many other fragmentations in PSD spectra is dependent on the laser power (Szilágyi et al., 1998) and on the matrix. With high laser powers, more ions are seen at lower mass, presumably as the result of successive cleavages. As an example of the effect of the matrix on the type of ion formed, sialic acid loss has been shown to be prominent...

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**SCHEME 3.** Scheme showing the nomenclature for describing the major fragment ions from carbohydrates. (From Domon & Costello, 1988, With permission from Elsevier Science BV).
with 2,5-DHB, but less significant with 6-aza-2-thiothymine (45) when spectra of biantennary glycans are recorded in the linear mode (Papac, Wong, & Jones, 1996). However, in the reflectron mode, significant sialic acid loss is observed from both matrices (see Fig. 4). PMP-derivatized N-linked glycans behave in a similar manner (Pitt & Gorman, 1997). Because of the problem of sialic acid loss by post-source decay, most studies of sialylated glycans are conducted in the linear mode (see Tsarbopoulos et al., 1997) so that the PSD ions are not separated.

Matrix-dependent PSD fragmentation can also degrade the resolution and mass measurement of glycoproteins by increasing the velocity distribution of the ions. The effect can be much greater in reflectron instruments when PSD fragmentation occurs during the path of the ions through the reflectron (Karas et al., 1995). With glycoproteins ionized with 4-HCCA or 2,5-DHB, considerable metastable defocusing occurs in contrast to ionization with 3-hydroxypicolinic acid (50) that induces little. These observations have prompted Karas et al. (1995) to propose that the fragmentation is produced by bimolecular collisions within the matrix plume, resulting in proton transfer from the matrix to the analyte. The theory is supported by the fact that 4-HCCA, the matrix that produces the most intense fragmentation, is also the strongest gas-phase acid of the common matrices. In general, matrices with low proton affinities, such as 4-HCCA (Table 1), are very effective at ionization and in producing extensive fragmentation (Burton et al., 1997; Jorgensen, Bojesen, & Rahbek-Nielsen, 1998).

The type of fragmentation observed also depends on the type of molecular ion formed, which to some extent depends on the matrix. This dependence can be illustrated by the PSD spectra of 2-AP (52) derivatives of maltoheptaose recorded by Okamoto et al. (1997). 4-HCCA efficiently protonated the nitrogen atom of the derivative, and the resulting MH$^+$ ion subsequently fragmented to give only a Y-series of fragments as the result of the charge localization on the reducing terminus. 2,5-DHB, on the other hand, produced only a MNa$^+$ ion with a non-localized charge. This ion fragmented to give a complex mixture of B and Y ions together with some cross-ring fragments (Fig. 12). Charged derivatives behaved in a similar manner (Okamoto et al., 1997) with the production of only the reducing-terminal-containing Y and Z-ions. The relative stabilities of MNa$^+$ and MH$^+$ ions in 4-HCCA is dramatically illustrated for the ABDEAE (51) derivatives of (Man)$_6$(GlcNAc)$_2$ (Mo et al. 1998). This derivative produces a strong NH$^+$ ion, but when recorded on a linear instrument appeared out of focus compared to that of the MNa$^+$ ion as the result of extensive PSD (Fig. 13). A strong PSD spectrum recorded in the reflectron mode showed mainly Y-type cleavage ions.

PSD spectra of the MNa$^+$ ions from neutral carbohydrates tend to be dominated by glycosidic and internal cleavage reactions with only weak contributions from cross-ring products (Spengler et al., 1995; Harvey et al., 1995). The abundant internal cleavage ions make spectral interpretation particularly difficult because many deductions tend to be ambiguous. Major ions are usually

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**TABLE 1.** Proton affinities of common MALDI matrices.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Proton affinity (kcal mol$^{-1}$)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Nicotinic acid</td>
<td>215 ± 4</td>
<td>Burton et al., 1997</td>
</tr>
<tr>
<td>Picolinic acid</td>
<td>216</td>
<td>Jorgensen, Bojesen &amp; Rahbek-Nielsen, 1998$^1$</td>
</tr>
<tr>
<td><em>trans</em>-3-Indoleacrylic acid</td>
<td>217</td>
<td>Jorgensen, Bojesen &amp; Rahbek-Nielsen, 1998</td>
</tr>
<tr>
<td>1,8,9-Anthracenetriol</td>
<td>215 ± 4</td>
<td>Burton et al., 1997</td>
</tr>
<tr>
<td>3-Hydroxypicolinic acid</td>
<td>209 ± 2</td>
<td>Burton et al., 1997</td>
</tr>
<tr>
<td>2,5-DHB</td>
<td>214</td>
<td>Burton et al., 1997</td>
</tr>
<tr>
<td>Sinapinic acid</td>
<td>204 ± 4</td>
<td>Burton et al., 1997</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>212</td>
<td>Burton et al., 1997</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>183 ± 2</td>
<td>Burton et al., 1997</td>
</tr>
<tr>
<td>4-HCCA</td>
<td>207</td>
<td>Jorgensen, Bojesen, &amp; Rahbek-Nielsen, 1998</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>183 ± 2</td>
<td>Burton et al., 1997</td>
</tr>
<tr>
<td>HABA</td>
<td>225</td>
<td>Burton et al., 1997</td>
</tr>
</tbody>
</table>

$^1$Values in this paper in kJ mol$^{-1}$ have been converted into kcal mol$^{-1}$ for comparison.
the result of B- and Y-cleavages and carry information on sequence and branching. Linkage information is more difficult to extract because it is carried mainly by the rather weak cross-ring cleavage ions. Nevertheless, linkage can affect the relative abundance of glycosidic cleavage ions, but the information is often difficult or impossible to extract without the spectra of both isomers for comparison. Spengler et al. (1995) have reported the PSD spectra of the MNa\(^+\) ions from two isomeric pentasaccharides that differ only in the \(\alpha1\rightarrow3\)- or

FIGURE 12. MALDI–PSD mass spectrum of the 2-AP derivative of maltopentaose recorded from (a) 4-HCCA and (b) 2,5-DHB. (Reprinted with permission from Okamoto et al., Anal Chem 69:2919–2926, Copyright 1997, American Chemical Society).
The $\alpha 1 \rightarrow 6$ linkage between two mannose units. It was found that the most significant difference was in the relative abundances of the C- and Z-ions formed by cleavage of the mannose–mannose bond. In the $\alpha 1 \rightarrow 6$-linked isomer, the C-ion was the more abundant, whereas in the $\alpha 1 \rightarrow 3$-isomer the position was reversed.

We have found that one of the most reliable ions for distinguishing differences between the 3- and 6-antennae of N-linked glycans is that ion formed by loss of the 3-antenna together with the chitobiose core (Scheme 4). It will be referred to here as ion D. Preferential losses of groups attached to the 3-position of hexoses have frequently been observed in the fragmentation of sugars (Egge & Peter-Katalinic, 1987; Dell & Thomas-Oates, 1989; Domon, Müller, & Richter, 1990; Laine et al., 1991; Garozzo et al., 1992). Because ion D contains the

![Figure 13](image_url)
complete 6-antenna, the composition of this antenna can easily be determined, as can that of the 3-antenna, by difference. It would appear from some reports, however, that not all asymmetrically substituted N-linked glycans can be differentiated by the relative abundance of ions of type D. For example, although the spectra of the two isomeric triantennary glycans with either a branched 3- or 6- antenna (Fig. 14) were found to contain this ion \((m/z\) 713 and 1079, respectively), there were no significant differences in its relative abundances (Rouse et al., 1998) in the two spectra. This lack of a difference is probably because of the strong tendency of glycans of this type to fragment preferentially by cleavage of the bond adjacent

**SCHEME 4.** Proposed structure for ion D. Symbols are defined in the legend to Fig. 11.

**FIGURE 14.** MALDI–PSD spectra of a complex 3-branched triantennary N-linked glycan (a) and a complex 6-linked glycan (b). Symbols for the constituent monosaccharides are ■ = GlcNAc, □ = fucose, ○ = mannose, ◦ = core mannose, ● = galactose. (From Rouse et al., 1998, with permission from Academic Press).
to a GlcNAc residue, and that this fragmentation takes precedence. Ions produced by such cleavages are prominent in the two spectra (Fig. 14), and their relative abundance reflects the isomeric difference. Most prominent among these differences are the ions produced by loss of successive Gal–GlcNAc fragments from the B4 ion (loss the chitobiose core); these ions are more abundant in the spectrum of the isomer with the branched 3-antenna. Although it would appear that these triantennary glycans cannot be differentiated on the relative abundance of ions of type D, several other complex N-linked glycans that contain GlcNAc residues in their antennae do produce diagnostically abundant type D ions. Such compounds are the highly branched glycans from ovalbumin and related egg-white glycoproteins (Harvey et al., 1999b).

High-mannose glycans with differing numbers of mannose residues, on the other hand, do not have GlcNAc residues in their antennae, and are easily differentiated by the mass of ion D, as illustrated by isomers of Man7 (Fig. 15). The isomer that contains four mannose residues in the 6-antenna and two in the other (Fig. 15b), contains a prominent ion at \( m/z \) 834 that contains five mannose residues. The isomer that contains three mannose residues in each arm does not possess a prominent ion at this mass.

**FIGURE 15.** MALDI–PSD spectra of (Man)7(GlcNAc)2 (D1) (a) and a mixture of this compound (51%) and the two isomers (D2, 18.5% and D3, 25.5%) (b) Symbols for the constituent monosaccharides are the same as in Fig. 14. (From Rouse et al., 1998, with permission from Academic Press).
Unfortunately, comparison of ion abundance in most PSD spectra is not an absolute measurement because these spectra are usually acquired in segments by stepping the reflectron voltage. Consequently, the ions in question might appear in different segments where ionization conditions and, consequently, the signal strength, might differ. The logical solutions to this problem would be to take the mean value of the ion abundances measured in several spectra or to use an instrument equipped with a curved-field reflectron, which records all fragment ions in a single spectrum.

In some cases, isomeric glycans fragment to give unique glycosidic cleavage ions that define linkage. For example, although the position of fucose substitution in a series of four isomeric pentasaccharides that contain the sequence Gal(1→3(4))GlcNAc(1→3)Gal(1→4)Glc is relatively easy to deduce from the mass of the B- and Y-ions, the linkage is not. Garozzo et al. (1997) have found that for the pair of sugars Gal(1→3)(Fuc(1→4))GlcNAc(1→3)Gal(1→4)Glc and Fuc(1→3)(Gal(1→4))GlcNAc(1→3)Gal(1→4)Glc, containing fucose in either a 1→3 or 1→4 linkage to the GlcNAc residue, further fragmentation of the B2-ion (loss of the Gal(1→4)Glc moiety) enabled a differentiation to be made. Ions at m/z 353.9 in the spectrum of the former, Fuc(1→4), isomer and at m/z 370 in the latter, although weak, were unique (Fig. 16). Their formation could be rationalized by loss of the intact sugar attached to C-3 of the GlcNAc residue of the B-ion. It was noted that the spectra contained the same fragment ions as those from 2,5-DHB, but that their intensity was higher. It was also noted that no rearrangement ions were present in these PSD spectra. Such ions appear to be rare, although they have been proposed to occur in the fragmentation of MH+ (but not MNa+) ions in FAB and CID spectra. Their formation was thought to involve the loss of an internal monosaccharide residue (Kováčik et al., 1995, Brühl et al., 1997; Warrack et al., 1998). A recent study by Brühl et al. (1998) has confirmed their absence from the fragmentation spectra of MNa+ ions.

Cross-ring fragments are of particular importance in PSD spectra for determining linkage positions, but their relative abundance tends to be low because their formation appears to require somewhat higher energy than that necessary for the formation of glycosidic fragments. The exception is the 0,2A ion produced from the reducing terminal residue of many carbohydrates, where it is usually among the most abundant ions in the spectrum. Ions of this type are characteristic of 1→4-linked sugars, and are also seen as fragments of C-type glycosidic cleavage ions. Their mechanism of formation appears to be a 2-stage process that involves ring-opening followed by a retro-aldol reaction (Spengler, Doice, & Cotter, 1990). The most useful cross-ring cleavage ions in the spectra of N-linked glycans are the 3,5A and 0,4A ions produced by cleavage of the core-branching mannose residue. These ions contain only the antenna attached to the 6-position and, thus, provide valuable information on the composition of each antenna. Spengler et al. (1995) have found 0,4A ions in the PSD spectra of bi- and tri-antennary N-linked glycans. Zal et al. (1998) have used both of them to determine the antenna configuration in high-mannose N-linked glycans derived from haemoglobin extracted from tube worms (Riftia pachyptila) that live around deep-sea hydrothermal vents from the East Pacific Rise (Fig. 17). These spectra also contained prominent internal fragments of type D, which, as described above, allowed the composition of the 6-antenna to be determined.

The effect of derivatization on PSD fragmentation has been studied by Lemoine, Chirat, & Domon (1996) from 2,5-DHB for a series of linear and branched glycans. Permethylated sugars gave poor fragmentation with cleavage of glycosidic bonds to sialic acid and GlcNAc.
producing the only significant fragments. This reduced fragmentation is probably due to the lack of free hydroxy groups. Fragmentation of underivatized glycans is facilitated by a hydrogen transfer that involves one of the labile OH groups (Hofmeister, Zhou, & Leary, 1991) and the presence of the methyl groups in the permethylated glycans prevents this reaction. Peracetylated glycans fragmented slightly better than permethylated ones to give B- and Y-ions, but the fragments were usually accompanied by additional ions produced by loss of acetic acid. Permethylation gave a product in which there was only one mass unit difference between a derivatized hexose and an N-acetylamino sugar. It was pointed out that the calibration of most PSD spectra is not usually accurate enough to give an unambiguous distinction between these two species. In order to overcome this problem, perdeuteroacetylation was recommended. It was also noted that there was little difference in the type of fragmentation produced by the MNa$^+$ and MLi$^+$ species.

An attempt to produce MH$^+$ species following derivative formation by reductive amination with either aniline or 2-aminobenzamide failed because only the MNa$^+$ ions were seen, even though a proton acceptor had been introduced into the molecule. Glycans were, thus, converted into the more basic benzylamino derivative that were observed to produce MNa$^+$ and MH$^+$ species. The MH$^+$ ions were formed specifically from the outer, crystalline region of the 2,5-DHB target, whereas MNa$^+$ ions were produced from the amorphous central region. Kussmann et al. (1997a) have also reported a similar fractionation with glycopeptides. The peaks from the MH$^+$ ions of the benzylamino derivatives were narrower than those from the MNa$^+$ ions, probably reflecting the fact that they were produced with lower laser power, and PSD fragmentation was simpler. Both spectra contained the same B and Y-ions but the MNa$^+$ ions gave an additional series of C-ions (Fig. 18). Fragmentation of the MH$^+$ ions of branched glycans gave clear indications of the points of attachment of fucose in the spectra of the pentasaccharides shown in Fig. 19 (Lemoine, Chirat, & Domon, 1996).

### B. In-Source Decay Fragmentation

The rate of a fragmentation reaction is of critical importance in determining whether or not that reaction will be observed under specific instrumental focusing conditions. Very fast reactions, such as the loss of carbon dioxide from sialic acids (Pitt & Gorman, 1997), can be observed under prompt (continuous) extraction conditions with linear instruments because they occur within the ion source before ion extraction. On the other hand, the glycosidic cleavages, such as those responsible for the loss of sialic acid, require more time because a hydrogen transfer between suitably positioned functional groups is required. These glycosidic cleavage reactions, therefore, mainly produce post-source fragments. In instruments fitted with delayed extraction, it is possible to monitor the relative rates of formation of these types of ion. Those ions formed by glycosidic cleavages can be observed as
in-source fragments if the delay is long enough and their relative abundance has been found to increase with the delay time (Naven et al., 1997). Long delay times, thus, have an adverse effect on the relative abundance of PSD ions. Kaufmann et al. (1996) have proposed that an additional loss of PSD fragments can be due to a reduced collisional activation in delayed-extraction sources. Cross-ring fragmentation appears to occur in a shorter time frame than glycosidic cleavages, and has been observed to produce fragments in the in-source decay (ISD) spectra (Naven & Harvey, 1997). Such ions are very prominent in laser desorption spectra (no matrix) that use a CO₂ laser (Martin et al., 1989).

ISD and PSD fragments can often be observed in the same spectrum when recorded with reflectron-delayed extraction TOF instruments operated in the reflectron mode. The fragment ions produced by ISD appear as sharply focused peaks with, at higher apparent mass, broader and diffuse metastable peaks produced by the corresponding unfocused PSD ions (Fig. 20). The intensity ratio of the two peaks changes as a function of the delay, with the PSD peak dropping in intensity as the delay increases (Fig. 21a). The apparent mass of the metastable ion also changes with the ion source focusing conditions and the instrument used to record the spectra (Fig. 21b). Harvey et al. (1999c) have found a second order relationship (Equation 1) among the masses of the parent, fragment, and metastable ions. The equation contains terms, reduced to the term \( r \) in Equation 1, that reflect the dimensions of the instrument, and is, thus, much more complex than that relating metastable, parent and fragment ions in magnetic sector instruments \([M = M_{\text{f(rag)}}^2/M_{\text{parent}}]\). However, for a given set of focusing conditions, \( r \) can be determined experimentally. By rearranging the equations (Equations 2 and 3), the mass of any one of the three ions can be calculated given the mass of the other two.

\[
M_i = M_a \left[1 + \frac{M_b}{M_a} \cdot r\right]^2
\]  

(1)
Where $M_a =$ mass of parent ion, $M_b =$ mass of focused fragment ion, and $r =$ instrumental constant (function of field-free region length, reflectron dimensions, voltages etc.).

The appearance of these pairs of fragment ions in a spectrum can have considerable diagnostic potential in glycan spectra because the mass difference between them and their relative abundance reflects the type of fragmentation involved. Loss of the acidic groups, sialic acid and sulfate, produces metastable ions in particularly high abundance. In the MALDI spectra of the disialylated biantennary glycan (24), shown in Fig. 22, metastable (unfocused PSD) ions at $m/z$ 2008.2 and 1986.4 link the focused ISD ions formed by loss of one sialic acid ($m/z$ 1976.8 and 1954.7) to the $\text{MNa}^+$ molecular ion of the free acid ($m/z$ 2245.8) and its monosodium salt ($m/z$ 2266.8). No link is present to the disodium salt, showing that the hydrogen transferred from the eliminated sialic acid is probably that from the carboxylic acid group. The ion formed by loss of two sialic acid groups ($m/z$ 1663.9) is formed by two routes, as shown by the appropriate metastable ions. These routes are from the $\text{MNa}^+$ molecular ion of the free acid ($m/z$ 2245.8) and from the fragment that has lost one sialic acid by an in-source cleavage ($m/z$ 1954.7). For very unstable compounds, such as disulfated glycans, the presence of metastable ions has proved to be the only means of identifying the presence of the second sulfate group because the molecular ions are too unstable to be observed directly (Harvey et al., 1999c).

ISD fragmentation of N-linked glycans has also been observed with a magnetic sector instrument (Harvey et al., 1995). Again, the most abundant fragment ions resulted from glycosidic cleavage, but prominent $0,2,2A$ and $2,4A$ ions were observed from the reducing terminal GlcNAc of N-linked glycans (see Fig. 23). Loss of the antenna from C-3 of the core mannose was generally more favorable than loss from C-6, as illustrated in Fig. 23 ($Y_{3^a}$-ion at $m/z$ 1136.5). Unfortunately, with this technique, the fragment ions in the region of the spectrum below about $m/z$ 500 were weak and generally became lost among the matrix background ions.

C. Fragmentation Produced by Collision-induced Decomposition

A magnetic sector mass spectrometer fitted with a MALDI source, a collision cell, and an orthogonal-TOF analyzer (Clayton & Bateman, 1992) has been used to produce high-energy (800 eV) collision-induced decomposition (CID) fragmentation spectra. In addition to the normal series of B- and Y-ions, these spectra contained many more abundant cross-ring fragments than are normally produced under ISD and PSD conditions (Harvey, et al., 1995; Harvey, Bateman, & Green, 1997). Of particular significance were the very abundant $1,5X$-
ions (Fig. 24). Their masses could be used to determine the branching pattern of the glycans such as the high-mannose sugars. $^{3,5}A$-ions were frequently present and were of particularly significant at the core-branching mannose residue, allowing the composition of the antennae to be determined. These cross-ring ions, however, were usually rather weak, and of more general use was an internal cleavage ion of type D discussed above. Its relative abundance in the spectra of monogalactosylated biantennary glycans from human IgG has been used to determine the site of attachment of the galactose (Wormald et al., 1997). Another ion formed by loss of water from ion D was also present in the spectra of N-linked glycans that lacked a bisecting GlcNAc residue. However, when a bisecting GlcNAc residue was present, it was lost as a fragment of 221 mass units (Fig. 24). B-type cleavage ions were particularly abundant adjacent to GlcNAc residues, notably between the GlcNAc residues of the chitobiose core and in the antennae of complex N-linked glycans.

Reducing-terminal derivatives have been found to modify the pattern of fragmentation ions formed under these conditions (Küster, Naven, & Harvey, 1996a). Fewer B- and Y-ions were seen with peptide substituents, but there was a greater tendency for formation of A-type cleavage ions, particularly from the reducing terminus (Fig. 25). In fact, the $^{0.2}A$-ion was the base peak in some spectra because of formation of the conjugated neutral species (64, Scheme 5). Of the series of derivatives studied by Küster, Naven, & Harvey (1996a), the popular 2-AB derivatives gave the least informative spectra.

MALDI CID spectra of milk sugars and N-linked carbohydrates have been reported by Penn, Cancilla, and Lebrilla (1996), using a Fourier transform mass spectrometer fitted with an external ion source. As with the high energy CID spectra recorded on a magnetic sector-TOF hybrid instrument, fucose loss was observed as the most favorable glycosidic cleavage, and was found to have the lowest relative dissociation threshold. This threshold increased when cesium was used as the cationization species, and was attributed to complexation between the ions (Fig. 24). Their masses could be used to determine the branching pattern of the glycans such as the high-mannose sugars. $^{3,5}A$-ions were frequently present and were of particularly significant at the core-branching mannose residue, allowing the composition of the antennae to be determined. These cross-ring ions, however, were usually rather weak, and of more general use was an internal cleavage ion of type D discussed above. Its relative abundance in the spectra of monogalactosylated biantennary glycans from human IgG has been used to determine the site of attachment of the galactose (Wormald et al., 1997). Another ion formed by loss of water from ion D was also present in the spectra of N-linked glycans that lacked a bisecting GlcNAc residue. However, when a bisecting GlcNAc residue was present, it was lost as a fragment of 221 mass units (Fig. 24). B-type cleavage ions were particularly abundant adjacent to GlcNAc residues, notably between the GlcNAc residues of the chitobiose core and in the antennae of complex N-linked glycans.

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![Image](image-url)
cesium and fucose. Dissociation thresholds for the appearance of cross-ring cleavage ions were found to be higher than those of glycosidic cleavages, correlating well with the appearance of these ions in the CID spectra discussed above from the sector instrument. The ability to measure dissociation thresholds with the Fourier transform instrument suggested that such measurements might be used to determine the composition of a carbohydrate by distinguishing, for example, between galactose and mannose, at present achievable only by chromatographic techniques. Another useful feature of this instrument was the ability to store ions for a considerable period of time, making possible the selection and further fragmentation of primary fragments. This feature was demonstrated for the B$_3$ fragment ion from the trimannosyl-chitobiose core oligosaccharide of N-linked glycans. This ion was selected and fragmented in an MS$^3$ experiment to produce a further diagnostic loss of mannose. Although the instrument had the capability to perform MS$^n$ experiments, where $n > 3$, it was not exploited to the same extent as with recent experiments using an ion trap (electrospray ion introduction), where $n$ values of 8 have been achieved (Weiskopf, Vouros, & Harvey, 1997).

D. General

As noted above, several of the common fragmentation routes displayed by carbohydrates, particularly the formation of Y-type ions, produce species that are isobaric with native sugars. Thus, if these fragments are the result of in-source (prompt) fragmentation, then they will be focused with the molecular ions of native glycans and will be indistinguishable from them. This problem is particularly significant for the analysis of mixtures, where it is important to ensure that the spectrum accurately reflects the profile of the original glycans. Fragment ions formed by PSD reactions will not present problems in linear spectra, but could potentially give erroneous results in spectra recorded in the reflectron mode. Loss of sialic acid, as noted above, is particularly relevant in this context. Mortz et al. (1996a) have addressed this problem with a mixture of N-linked glycans from human.

![FIGURE 22. MALDI mass spectrum of the sialylated biantennary glycan (NeuNAc)$_2$(Gal)$_2$(Man)$_3$(GlcNAc)$_4$ recorded from 2,5-DHB with a delayed extraction reflectron-TOF mass spectrometer. (From Harvey et al., 1999c, with permission from Elsevier Publishing Co.).]
interferon-γ, and have concluded that in-source fragmentation does not make a significant contribution to the profile, even when several sialic acids are present. PSD fragmentation was also not a problem in the reflectron spectra with “cool” matrices such as 2,5-DHB or THAP, but 4-HCCA produced significant sialic acid loss. Two types of ion can be used to test for the occurrence of fragmentation: unfocused PSD ions and the cross-ring 0,2-A-ion from the reducing terminus. If these ions are absent, then little, if any, fragmentation is usually present.

VII. ANALYSIS OF FREE CARBOHYDRATES WITH MALDI MASS SPECTROMETRY

A. Carbohydrates from Plants

Plants contain many large carbohydrate polymers that act as structural and storage molecules. Most are too large for direct analysis by mass spectrometry, and are usually degraded to smaller molecules by chemical or enzymatic techniques prior to mass spectrometry. Many compounds also contain modifications of the basic monosaccharide units, such as methylation, which present additional analytical problems. The following examples illustrate how some of these problems have been overcome.

1. Fructans

Fructans, of which the inulins mentioned above are a specific type, are linear, branched or occasionally cyclic polysaccharides that contain predominantly fructose. Together with starch, inulins constitute the main storage carbohydrate of higher plants. Fructans from *Dahlia variabilis* consist mainly of inulin, \([\text{Glcβ}(1\rightarrow2)(\text{Frcβ}(1\rightarrow2(\text{or } 6)))_n\rightarrow2\text{Frc}]\), and have been studied with MALDI by Stahl et al. (1997), using 3-aminoquinoline as the matrix on a reflectron-TOF instrument. Although peaks from ca. 50 compounds were seen with masses up to 10,000 Da, they were only resolved to about 7000 Da. Fructans from *Allium cepa* L. (onion) are more varied in structure, but of lower molecular weight. When examined by MALDI from 2,5-DHB, the major ions were due to MK⁺ rather than the more normal MNa⁺, reflecting the

![FIGURE 23. MALDI mass spectrum of the neutral triantennary glycan (Gal)₃(Man)₃(GlcNAc)₅ recorded from 2,5-DHB with a magnetic sector mass spectrometer. Symbols for the monosaccharides are as in Fig. 11. (From Harvey et al., 1995).](image-url)
high content of potassium in the onion. Stahl et al. (1997) also observed fructans directly from onion skin and parenchyma by fixing small pieces of tissue to the target and covering them with a solution of 2,5-DHB.

2. Pectins

Pectins are large polysaccharides found in the cell walls of higher plants. These compounds consist of chains of partially methylated galacturonic acids (smooth region) interrupted by L-rhamnose-containing branched neutral sugar side-chains (hairy region). Partial enzymatic hydrolysis has been used to release oligogalacturonic acids of random length for examination by MALDI (Daas et al., 1998) using 2,5-DHB as the matrix. Pectin from lemons was examined, and some of the uronic acid groups were shown to be methyl esterified. Körner et al. (1998) have used partial digestion with pectin lyase to reduce molecular weights to those that could be examined by mass spectrometry, and the products were examined by MALDI in the positive and negative ion modes with several matrices. The best matrix was found to be a mixture of 2,4,6-THAP (44) and nitrocellulose in a thin-layer preparation. Spectra were recorded in the negative ion mode. Methylation patterns could be discerned in some cases, and oligomers with degrees of polymerization of up to 45 (8000 Da) were detected after enrichment of the larger oligomers by propan-2-ol precipitation (Fig. 26).

3. Xylans

Xyloglucans are hemicellulosic polysaccharides present in the cell walls of most higher plants and in algae. They are related to cellulose, but contain additional side-chains at the 6-position of the glucose residues. The location of these side-chains in solanaceous plants has been investigated by York et al. (1996), using a combination of chromatography, MALDI, FAB, and NMR. Polymeric chains were cleaved with a fungal endo-β(1→4)-d-
glucanase into oligosaccharides with masses of about 1 kDa. Xyloglucans from tobacco and tomato were found to contain α-D-Xylp and α-L-Araf-(1→2)-α-D-Xylp side chains, but tomato was also found to contain the more complex β-D-Galp(1→2)-α-D-Xylp and β-Araf-(1→3)-α-L-Araf-(1→2)-α-D-Xylp chains. In hardwood species such as birch, the side chain is an O-acetyl-2-O-(4-O-methyl-α-D-glucurono)-β-D-xylan. This chain becomes extensively modified during kraft pulping, and the resulting compounds have recently been studied by capillary zone electrophoresis (CZE) and MALDI. Cleavage of the polymeric chains was achieved with a *Trichoderma reesei* endoxylanase to give fragments of 400–1000 Da (Rydlund & Dahlman, 1997). This enzyme was also used by York et al. (1995) to cleave xyloglucans from *Acer pseudoplatanus* cells into fragments that contain from seventeen to twenty glycosyl residues. These fragments were reduced with borohydride before being examined by MALDI, FAB, and NMR. The side-chains were found to contain xylose, galactose, and fucose. PSD has been used by Yamagaki, Mitsuishi, & Nakanishi (1997) to study the heptasaccharide repeat unit from xyloglycans of tamarind seeds prepared by treatment with galactosidase and *Trichoderma* glucanase. This glycan has four β1–4-linked glucose residues, three of which carry 1–6-linked xylose residues. Ions were almost entirely produced from Y-type cleavages and internal fragments. 1→6-Linked glycans were observed to be lost in preference to 1→4-linked sugars (Yamagaki, Mitsuishi, & Nakanishi, 1998a, 1998b).

### 4. Borate Complexes

A borate complex with sugars has been ionized from 2,5-DHB from radish root cells, and was found to have a mass of 9894 Da (Kobayashi, Matoh, & Azuma, 1996). This mass was reduced to 4927 Da by acid hydrolysis, indicating that the complex was formed from two sugar molecules linked by boron. Penn et al. (1997a) have produced borate complexes of mannitol and sorbitol by...
treating celery (*Apium graveoleus*) with boric acid, and obtained MALDI spectra in negative ion mode. They note that 2,5-DHB also forms a complex with boron to produce a peak at \( m/z \) 315, and thus 3-aminoquinoline (24), which contains no oxygen, was used. Again, complexes of two sugars with one boron atom were observed. Larger sugars tended to form several complexes of two sugars with a varying number of boron atoms. Maltoheptaose, on the other hand, formed a single complex with only one sugar coordinated with a single boron atom.

**B. Carbohydrates from Algae**

1. Xylans

Xylans from the marine alga *Caulerpa brachypus* have been shown by Yamagaki et al. (1996a) to consist of a linear polymer of at least twenty-five xylose residues in \( \beta1 \rightarrow 3 \)-linkage. The compounds were extracted with hot water, and were subjected to partial acid hydrolysis before being examined by MALDI from super-DHB. Linkage analysis was performed by combined gas chromatography/mass spectrometry (GC/MS).

2. \( \beta \)-glucans

Laminarans are \( \beta \)-glucans found in brown algae, and generally consist of polymeric chains of two types: G-chains that contain only GlcP residues, and M-chains that are terminated with a d-mannitol residue. Limited branching at the 6-position can sometimes occur, and chain lengths are typically in the range of 20–50 units. Chizhov et al. (1998) have compared MALDI (Fig. 27) and FAB mass spectrometry for the analysis of these compounds, and have concluded that MALDI gives superior results because it is not subject to the same degree of signal loss at higher mass values that is seen with FAB. Laminarans from eight species were studied, and shown to have different profiles, particularly in the mass of the most abundant oligomer. The spectra of native laminarans generally gave ions up to about \( m/z \) 6000, corresponding to polymer chains in the region of 40 residues. Permethylation was used to increase the mass.
FIGURE 25. High-energy (800 eV) MALDI–CID spectra of (a) underivatized (Man)₅(GlcNAc)₂ and the same compound derivatized with (b) 2-AB, (c) asparagine, and (d) the peptide Asn–Leu–Thr–Lys. (From Küster, Naven, & Harvey, 1996a).
Evidence was found for HexNAc-capped chains in laminarans from *Cystoseira barbata* and *C. crinita* and for cyclization (ions 46 mass units below those of the main peaks) in some laminaran samples. Chen, Baker, & Novotny (1997) have investigated osazones for the ionization of these compounds, and claim that better spectra are obtained than with 2,5-DHB on account of reduced fragmentation.

**C. Carbohydrates from Milk**

Neutral and acidic fucosylated lactose polymers with molecular weights of up to 8000 have been identified in human milk. 2,5-DHB and super-DHB gave the best signals from the neutral sugars, whereas 3-aminoquinoline was found to be the most appropriate matrix for the acidic glycans (Stahl et al., 1994a).

**D. Carbohydrates from Beer**

Oligosaccharides from several varieties of beer have been examined by Vinogradov & Bock (1998). Isomaltoligosaccharides were labeled with 7-amino-4-methylcoumarin by reductive amination, and were examined by HPLC and MALDI. Chains with up to ten \( \alpha-(1 \rightarrow 4) \)-linked glucose residues were found with a single maltose or maltotriose residue substituted at any 6-position except on the reducing terminal residue. In order to reduce the size of the molecules, cleavage of the 6-linked side-chains was achieved with the enzyme pullulanase.

**E. Cyclodextrins**

These molecules cannot be sequenced by exoglycosidase digestion because of their cyclic structure (see 65). However, fragmentation does yield structural information, even though two bonds have to be broken in order to yield fragment ions. MALDI can also be used to profile commercial mixtures. Linnemayr, Rizzi, & Allmaier (1997) have used 4-HCCA and 2,5-DHB to define the profiles of alkylated (methyl, hydroxypropyl, and carboxymethyl) \( \beta \)-cyclodextrins, and to show that most commercial samples are mixtures. No suppression of the signal from any of the components was observed, unlike the situation in the spectra produced by FAB or PD mass spectrometry. MALDI thus provides a simple and rapid method for checking the purity of commercial samples. The \( \beta \)- and \( \gamma \)-cyclodextrins substituted at the 6-position of the sugar rings with anionic sulfalkyl ethers (ethyl, \( n \)-propyl, \( n \)-butyl) and with 2-hydroxy-3-trimethylammoniumpropyl ether (Chankvetadze et al., 1996) behave likewise. Positive ion spectra (MNa\(^+\) ions) of the sodium salts of cyclodextrins with up to eight propylsulfate groups were obtained from 2,5-DHB/HIQ doped with 30 mM sodium chloride. The same matrix was used by Jung and Francotte (1996) to characterize synthetic mixtures of the sulfobutyl ether of \( \gamma \)-cyclodextrin, desalted by drop dialysis on a cellulose membrane filter (pore size 0.025 \( \mu \)m). In addition to substitution at C-6, small amounts of compounds that contain sulfobutyl groups at C-2 and C-3 of the glucose residues, were also
FIGURE 27. Positive-ion MALDI–TOF mass spectrum of laminarans from *Laminaria cichorioides* recorded from 2,5-DHB. (From Chizhov et al., 1998) with permission from Elsevier Science Ltd.).
FIGURE 28. (a) Positive ion MALDI–PSD mass spectrum of γ-cyclodextrin recorded on an instrument with a curved-field reflectron. (b) The spectrum of the branched cyclodextrin G2-a-CD recorded under the same conditions. (From Yamagaki et al., 1996b).
detected. Although, as discussed above, some sulfates are unstable under MALDI conditions, no evidence of fragmentation was seen with these samples. However, because the spectra were recorded on a linear TOF instrument, only in-source fragments would have been visible.

\(\gamma\)-Cyclodextrin (cyclic-1,4-glucopyranose)\(_n\), 65) has been found to give a series of five glycosidic PSD ions from 2,5-DHB (Yamagaki et al., 1996b). Two isomeric, branched cyclodextrins that contain hexacyclic rings and either two 1,6-linked glucose or one diglucose chain also gave five ions of the same mass, but with very different relative abundance (Fig. 28). Loss of one glucose from both branched isomers gave the most abundant fragment rather than loss of three or four glucose residues that gave the most abundant ion from the unbranched compound. The difference was thought to be related to the fact that two bonds had to be broken in the cyclic isomer in order to produce a fragment. Only a single bond cleavage would be needed to release a glucose residue from the branched compounds. No cross-ring fragments were seen. Studies with cyclodextrin, substituted with trisaccharides containing 1→4- or 1→6-linkages, have suggested that the relative abundance of ions produced by cleavage of 1→4-glycosidic linkages is greater than that produced by cleavage of 1→6-linked sugars (Yamagaki et al., 1997). The opposite has been reported by these authors from xyloglucans (Yamagaki & Nakanishi, 1998). In the spectra of cyclodextrins substituted with \(\alpha\)1→6-linked glucose, galactose, or mannose, fragment ions produced by loss of mannose were much weaker than those produced by loss of either of the other two sugars (Yamagaki & Nakanishi, 1998). The reason for that difference was thought to reflect the stereochemistry of the carbon atom at position 2 in D-mannopyranose, which is different from that in the other two sugars. Thus, the hydrogen attached to this carbon, which is the one thought to migrate in the glycosidic cleavage reaction, was less favorably placed than in glucose or galactose, accounting for the less abundant reaction.

Quantitatively, cyclodextrins appear to behave analogously to other carbohydrates (Bartsch et al. 1996). Excellent linear correlations \((r^2 > 0.995)\) were found between concentration and response with linear dextrans as internal standards. Signals from partially methylated cyclodextrins, recorded on a Bruker Biflex mass spectrometer, showed some peak overlap between the MK\(^+\) ion and the MNa\(^+\) ion of the compound that contained one extra methyl group (two mass units difference). This problem was overcome by adding lithium chloride to the target, whereupon only MLi\(^+\) ions were formed.

Although most of the above research has been performed on synthetic cyclodextrins, MALDI has also been used for the analysis of naturally occurring compounds. For example, cyclic-\(\beta\)-glucans that contain 13 and 16 glucose residues have been identified in trichloroacetic acid extracts of Burkholderia solanacearum and Xanthomonas campestris, respectively (Talaga et al., 1996). All linkages were \(\beta1 \rightarrow 2\) except one, which was \(\beta1 \rightarrow 6\). 2,5-DHB was found to be the most effective matrix.

**VIII. ANALYSIS OF GLYCOPROTEINS AND PROTEOGLYCANS WITH MALDI MASS SPECTROMETRY**

The analysis of the glycans, particularly the N-linked glycans of glycoproteins, is the area in which MALDI mass spectrometry has had the most impact on carbohydrate analysis. Intact glycoproteins and released glycans have been investigated, and techniques are now available to examine these compounds at the low femtomole level.

### A. Intact Glycoproteins

The resolution of TOF instruments is generally insufficient to deconvolute the glycoforms of any but the smallest glycoproteins, and then only if they contain a limited number of glycans. In other cases, only broad, unresolved peaks are produced. Ribonuclease B (15 kDa) with its five glycans at a single glycosylation site, can be resolved well enough to determine the glycosylation pattern (Mock, Davy, & Cottrell, 1991; Bonfichi et al., 1995) even with linear instruments. However, molecules such as \(\alpha\)1-acid glycoprotein (35 kDa) (Bonfichi et al., 1995) are reaching the limits of resolution. Tsarbopoulos et al. (1997) have resolved glycoforms of the 30 kDa SF9-derived IL-4 receptor, but only when delayed extraction was used.

MALDI mass spectrometry can, however, be used with larger molecules to determine the presence or absence of glycosylation, or other post-translational modifications, as illustrated by the study of the carbohydrate-deficient glycoprotein syndrome reported by Wada et al. (1994). Human serum transferrin from normal controls was shown to produce an ion of 79.6 kDa with sinapinic acid as the matrix. Transferrin from patients with the syndrome produced additional peaks, appearing as shoulders on the main peak, at 77.4 and 75.2 kDa, corresponding to species that lack one and two sialylated biantennary oligosaccharides, respectively (Fig. 29). Resolution, however, was inferior to that obtained in an electrospray study, which resulted in a complete separation of the glycoforms (Yamashita et al., 1993). Glycoforms have been resolved by Nakaniishi et al. (1994) from immunoprecipitated transferrin, using sinapinic acid. The signal of the tri-positive ion (26.4 kDa), recorded from 4-HCCA, showed a slightly higher resolution of the
carbohydrate-deficient species. Carbohydrate-deficient transferrin has also been found in the sera of patients following severe alcohol abuse. In this study, the transferrins were first isolated by Mono-Q chromatography before mass measurement from sinapinic acid. Species that lack one or both biantennary glycans were found together with analogs that lack sialic acid from the antennae (Peter et al., 1998). In another example, Ownby et al. (1993) found that the α-chain (17.5 kDa) from the extracellular haemoglobin of the earthworm, *Lumbricus terrestris*, is heterogeneous, with three forms differing in the number of hexose residues in its constituent glycan.

With molecules that are too large for a direct examination of glycoforms, two options are open: removal of the glycan, or cleavage of the peptide into smaller units. The measurement of protein molecular weight before and after removal of the attached glycans by either chemical or exo- and endo-glycosidase treatment provides information on the state of glycosylation, even though the individual glycoforms may not be resolved. The utility of this approach obviously depends on the molecular weight—more detailed information is obtained for smaller molecules. Using this technique, information on the extent of sialylation and O-glycosylation of the low molecular weight glycoprotein, insulin-like growth factor II (~10 kDa), has been obtained following incubations with neuraminidase and O-glycanase, respectively (Jespersen et al., 1996). The first accurate mass measurement to be made of a hydroxyproline-rich glycoprotein, that from the Douglas fir (*Pseudotsuga menziesii*) before and after deglycosylation with HF, gave a mass of 73,186 ± 146 and 53,953 ± 108, respectively. When combined with an earlier carbohydrate compositional analysis, these measurements enabled the carbohydrate composition to be determined as (arabinose)$_{127}$(galactose)$_{13}$ (Kieliszewski et al., 1995).

If the glycoprotein contains a single glycan, then the mass difference before and after deglycosylation gives the mass, and in most cases the composition of the glycan. Since N-linked glycans contain a relatively small number of isobaric monosaccharides [hexose (galactose, glucose, mannose,), N-acetylaminohexose (GlcNAc), deoxyhexose (fucose), and sialic acid], this mass measurement leads directly to a composition in terms of these isobaric sugars. Table 2 lists the residue masses of monosaccharides that are commonly found in carbohydrates together with masses of their permethyl and peracetyl derivatives. The glycan mass may be calculated by adding the masses of the relevant monosaccharides to the mass of the terminal groups, given in Table 3, and to that of the MALDI adduct given in Table 4. The masses of common modifications found on carbohydrates are given in Table 5.

An example of this technique is provided by the MALDI (4-HCCA) analysis of the latent transforming growth factor-β binding protein-1, which revealed two peaks (13,266 and 13,345); the difference of 79 Da is attributable to another post-translational modification. Digestion with peptide N-glycosidase-F (PNGase-F)
reduced the masses to 12,230 and 12,309, respectively, and provided a mass of 1036 Da for the glycan. This mass is consistent with the presence of an N-linked glycan of composition (Hex)\(\text{3}(\text{GlcNAc})\text{2}(\text{Fuc})\text{1}\) (Gleizes et al., 1996). Apffel et al. (1996) have measured the mass of recombinant reduced and alkylated Desmodus rotundus salivary plasminogen activator by MALDI from sinapinic acid (53,407 Da), and found a mass difference of 3899 Da from the predicted protein mass. The difference was assumed to be due to carbohydrate, and was confirmed by incubations with sialidase, PNGase-F, or an O-glycanase. However, because several sites are glycosylated in this molecule, masses of individual glycans could only be obtained after further cleavage of the protein.

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Residue formula</th>
<th>Residue mass</th>
<th>Methyl Residue Mass</th>
<th>Methyl No.</th>
<th>Methyl Me</th>
<th>Acetyl Residue mass</th>
<th>Acetyl No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxy-pentose</td>
<td>C(_5)H(_8)O(_3)</td>
<td>116.047</td>
<td>130.063</td>
<td>1</td>
<td>158.058</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pentose</td>
<td>C(_6)H(_6)O(_4)</td>
<td>132.042</td>
<td>160.074</td>
<td>2</td>
<td>216.063</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Deoxy-hexose</td>
<td>C(_6)H(_10)O(_4)</td>
<td>146.078</td>
<td>174.089</td>
<td>2</td>
<td>230.079</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Hexose</td>
<td>C(_6)H(_10)O(_5)</td>
<td>162.053</td>
<td>204.100</td>
<td>3</td>
<td>288.084</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>C(_6)H(_11)NO(_4)</td>
<td>161.069</td>
<td>217.131</td>
<td>4</td>
<td>287.100</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>HexNAc</td>
<td>C(_6)H(_13)NO(_5)</td>
<td>203.079</td>
<td>245.126</td>
<td>3</td>
<td>287.100</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Hexuronic-Acid</td>
<td>C(_6)H(_8)O(_6)</td>
<td>176.032</td>
<td>218.079</td>
<td>3</td>
<td>260.053</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Kdo</td>
<td>C(_8)H(_12)O(_7)</td>
<td>220.058</td>
<td>276.121</td>
<td>4</td>
<td>346.090</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>N-acetyl neuraminic acid</td>
<td>C(_11)H(_17)NO(_8)</td>
<td>291.095</td>
<td>361.174</td>
<td>5</td>
<td>417.127</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>N-glycoyl neuraminic acid</td>
<td>C(_11)H(_17)NO(_9)</td>
<td>307.090</td>
<td>391.184</td>
<td>6</td>
<td>475.133</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^1\)Top figure = monoisotopic mass; lower figure = average mass.

<table>
<thead>
<tr>
<th>Table 3. Masses of terminal groups to be used with masses listed in Table 2 to obtain the molecular weights of carbohydrates.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing end</td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>Free</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Reduced</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Top figure = monoisotopic mass; lower figure = average mass.

<table>
<thead>
<tr>
<th>Table 2. Residue masses of common monosaccharides and their methyl and acetyl derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligosaccharide</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Deoxy-pentose</td>
</tr>
<tr>
<td>Pentose</td>
</tr>
<tr>
<td>Deoxy-hexose</td>
</tr>
<tr>
<td>Hexose</td>
</tr>
<tr>
<td>Hexosamine</td>
</tr>
<tr>
<td>HexNAc</td>
</tr>
<tr>
<td>Hexuronic-Acid</td>
</tr>
<tr>
<td>Kdo</td>
</tr>
<tr>
<td>N-acetyl neuraminic acid</td>
</tr>
<tr>
<td>N-glycoyl neuraminic acid</td>
</tr>
</tbody>
</table>

\(^1\)Top figure = monoisotopic mass; lower figure = average mass.

<table>
<thead>
<tr>
<th>Table 5. Masses of additional groups found on carbohydrates.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Methyl</td>
</tr>
<tr>
<td>Acetyl</td>
</tr>
<tr>
<td>Sulfate</td>
</tr>
<tr>
<td>Phosphate</td>
</tr>
<tr>
<td>Inositol</td>
</tr>
</tbody>
</table>

\(^1\)Most abundant isotope.

<table>
<thead>
<tr>
<th>Table 4. Masses of common adducts for carbohydrates ionized by MALDI.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adduct</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>Hydrogen</td>
</tr>
<tr>
<td>Sodium</td>
</tr>
<tr>
<td>Lithium</td>
</tr>
<tr>
<td>Potassium</td>
</tr>
<tr>
<td>Cesium</td>
</tr>
</tbody>
</table>

\(^1\)Top figure = monoisotopic mass; lower figure = average mass.

\(^2\)Assuming that no derivatization occurs.
B. N-Linked Glycans

1. Analysis of Glycopeptides Derived from Glycoproteins

Cleavage of large glycoproteins, either chemically or enzymatically, can overcome the problem of mass-limited resolution by reducing the molecular weight of glycosylated fragments. It also serves as a means of isolating glycosylation sites into different glycopeptides that can be separated by HPLC and analyzed individually. Removal of the glycan with a suitable glycosidase (see below) and repeating the mass measurement reveals the mass of the peptide and enables the presence of N- or O-linked glycans to be determined (Yang & Orlando, 1996a; Zaia et al., 1997) as described above for intact glycoproteins. Such reactions can be performed in solution or, as Kussmann et al. (1997a) have demonstrated for the membrane-bound glycoprotein neurolin, digestion can be performed with trypsin within a SDS gel. These authors note that the SDS was able to replace detergents that rendered the protein immune to attack by peptidases. However, an attempt to identify the N-glycosylation sites by digestion with PNGase-F within the gel before trypsin treatment was unsuccessful because the amount of sample available did not enable the relevant peptides to be identified.

An alternative approach to reducing the protein molecular weight is to digest it with pronase and leave only a single amino acid or a short peptide attached to the glycan. This method identifies the linking amino acid, but does not localize it in the protein chain. Juhasz & Martin (1997) have used this approach for the glycoproteins ribonuclease B and ovalbumin with a single glycosylation site and α1-acid glycoprotein with several sites. The glycoproteins were digested with pronase for 36–48 h. Neutral glycopeptides were examined directly from super-DHB and acidic ones from 2,4,6-THAP/ammonium citrate. Glycopeptides were distinguished from peptides by (a) their relatively high mass, (b) a mass that was not predicted from the protein sequence, and (c) a fractional mass that was deficient with respect to peptides of a similar size on account of the relatively large amount of oxygen. Ribonuclease glycans were found attached to a single dipeptide (Arg–Asn), but the ovalbumin glycans were found with three peptides. In general, peptides were examined preferentially in the negative ion mode because it gave stronger signals than the positive ion spectra and cleaner peaks due to the reduction of sodium salt formation. Ribonuclease was an exception to this generalization because the basic arginine in the peptide portion gave a strong positive ion signal (Fig. 30). Analysis of the acidic α1-acid glycoprotein was not as satisfactory. The linear, rather than the reflectron, mode had to be employed to avoid an excessive metastable loss of sialic acid. It was observed that not all glycans at any one site could be seen conjugated with the various peptides produced by the digest, and it was speculated that different glycans affected the extent to which the protein was digested by the pronase.

2. Detailed Glycan Analysis by Use of Exoglycosidase Sequencing

Although the determination of the mass of a glycan or glycopeptide can often lead to a composition in terms of...
its isobaric monosaccharide composition, additional structural information must be obtained by other methods. Fragmentation has already been discussed, but often the amounts of material available are not sufficient for a full characterization, and as will be apparent from the discussion, conclusions can often be ambiguous. Techniques such as NMR, although often able to provide a complete structure, are rarely appropriate because of low sample amounts. Fortunately, however, mass spectrometry can be combined with the use of structurally specific glycosidases to provide the necessary information, and this technique is currently the most powerful for providing sequence, branching, and linkage data. It also provides data on the nature of the constituent monosaccharide residues—information that is virtually impossible to acquire from fragmentation studies. However, as emphasized in the Introduction, no single technique can yet provide unambiguous proof of structure.

Most methods that use enzymes for structural characterization involve an incubation of the glycan with the enzyme—usually a specific exoglycosidase, and observation of the number of monosaccharide residues removed. The specificity of the enzyme often uniquely determines the monosaccharide linkage. Successive digestions are performed until the glycan is reduced to an identifiable oligosaccharide or to a single monosaccharide. Various sequences of enzymes can be devised to examine the fine structure of, for example, the individual antennae in N-linked glycans. Endoglycosidases, such as endo-β-galactosidase, can be used to identify antennae from these compounds that contain N-acetylaminolactosamine (Galβ1→4GlcNAc) extensions. As an alternative to successive exoglycosidase digestion, arrays of enzymes can be used (Edge et al., 1992); the so-called reagent array analysis (RAAM) method. In this technique, the glycan is divided into several fractions, and each fraction is incubated with a mixture of exoglycosidases in which one of the total range of enzymes is missing. Digestion, therefore, occurs until the point that needs the missing enzyme occurs. The mixtures of partially digested glycans can be examined individually or in combination. A single mass measurement of the constituents of the resulting glycan profile gives a glycan signature from which the original glycan can be identified by matching to a computer-generated library. Common enzymes for use in this technique are listed in Table 6.

The validity of exoglycosidase digestions to provide detailed information on the glycan structure relies on the availability of highly purified enzymes. Some commercial preparations have been found to contain contaminating enzymes, whose presence has been revealed by MALDI analysis of the glycan products. For example, certain preparations of S. pneumoniae β-N-acetylhexosaminidase have been found to be contaminated with an enzyme with endoglycosidase-H-like activity (Sutton, O’Neill, & Cottrell, 1994; Roberts et al., 1995). Also, chicken α-fucosidase, said to be specific for fucose α1→2-, 4- or 6-linked to GlcNAc, was also found to remove a consider-

### Table 6. Exoglycosidases used for carbohydrate sequencing.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>EC number</th>
<th>Source</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-d-Sialidase</td>
<td>3.2.1.18</td>
<td>Arthrobacter ureafaciens</td>
<td>NeuNAc/Galβ3→6,3,8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Newcastle disease virus</td>
<td>NeuNAc/Galβ3→6,8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clostridium perfringes</td>
<td>NeuNAc/Galβ3→3,6,8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vibrio cholerae</td>
<td>NeuNAc/Galβ3→3,6,8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovine testis</td>
<td>Galβ1→3,4,6</td>
</tr>
<tr>
<td>β-d-Galactosidase</td>
<td>3.2.1.23</td>
<td>Streptococcus pneumonia</td>
<td>Galβ1→4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jack bean</td>
<td>Galβ1→6,4 ≥ 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Canavalia ensiformis)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Escherichia coli</td>
<td></td>
</tr>
<tr>
<td>α-d-galactosidase</td>
<td>3.2.1.22</td>
<td>Green coffee bean</td>
<td>Galβ1→4Glc</td>
</tr>
<tr>
<td>β-N-Acetyl-d-hexosaminidase</td>
<td>3.2.1.30</td>
<td>Jack bean</td>
<td>Galβ1→3,4,6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.01 U/mL)</td>
<td>Glc(Gal)NAcβ1→2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10 U/mL)</td>
<td></td>
</tr>
<tr>
<td>α-N-Acetyl-d-hexosaminidase</td>
<td>3.2.1.49</td>
<td>Chicken liver</td>
<td>GallNacβ1→</td>
</tr>
<tr>
<td>α-d-Mannosidase</td>
<td>3.2.1.24</td>
<td>Jack bean</td>
<td>Manβ1→2</td>
</tr>
<tr>
<td>β-d-Mannosidase</td>
<td>3.2.1.25</td>
<td>Aspergillus phoenicis</td>
<td>Manβ1→4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Helix pomatia</td>
<td>Manβ1→4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Achatina fulica</td>
<td>Manβ1→2</td>
</tr>
<tr>
<td>α-l-Fucosidase</td>
<td>3.2.1.51</td>
<td>Bovine epididymis</td>
<td>Fucβ1→6,2,3,4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Almond emulsin II</td>
<td>Fucβ1→2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Almond emulsin III</td>
<td>Fucβ1→3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Charonia lampas</td>
<td>Xylβ1→2</td>
</tr>
<tr>
<td>β-l-Xylosidase</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Endo-β-d-galactosidase</td>
<td>3.2.1.103</td>
<td></td>
<td>RGalβ1→4GlcNAc</td>
</tr>
</tbody>
</table>
able amount of galactose (Roberts et al., 1995). Another problem with commercial enzyme preparations is that they frequently contain buffers and salts such as sodium citrate/phosphate or NP-40 (nonylphenoxoy polyethoxy ethanol) that are incompatible with MALDI and are often difficult to remove. Furthermore, analysis relies on a continuing commercial supply of the enzymes.

The use of MALDI mass spectrometry combined with exoglycosidase sequencing to analyze N-linked glycans was first demonstrated by Sutton, O’Neill, and Cottrell (1994), using glycans from the recombinant human tissue inhibitor of metalloproteinasases (TIMP). The two N-linked sites were isolated by trypsin digestion, followed by reversed-phase HPLC. MALDI analysis was performed on the glycopeptides, using a reflectron-TOF instrument and 4-HCCA as the matrix. The complex glycans in the mixture were analyzed by using mixtures of enzymes as follows: A. ureafaciens sialidase to remove sialic acids from the ends of the antennae, sialidase plus S. pneumoniae β-galactosidase to remove galactose, and sialidase plus β-galactosidase plus chicken liver N-acetylhexosaminidase to remove GlcNAc residues. Manose substitution was investigated with Jack bean α-mannosidase (0.1 U/mL), which cleaves only mannose in α1→6 linkage and at 5 U/mL for cleavage of α1→3 and α1→6 linked mannose. It should be noted that, in addition to Jack bean α-mannosidase, other enzymes can be used at different concentrations to increase their specificity (see Table 6). The resulting spectra of the TIMP glycans showed them to consist of complex mixture of sialylated fucosylated bi-, tri-, and tetraantennary compounds. The peptide molecular weight was confirmed by incubation with PNGase-F to remove the intact glycans, and the method was verified by using the well-characterized glycoproteins fetuin, α1-acid glycoprotein, and TPA. TIMP glycans have also been analyzed directly following removal with PNGase-F (Harvey et al., 1999a).

The exoglycosidases used above are fairly typical of those normally employed for sequencing N-linked glycans, but occasionally glycans are found in which the antennae are capped with α-linked galactose, which requires additional enzymes to be employed. Glycans that contain α-linked galactose usually occur in situations where sialic acid would be unstable, such as the stomach, and are found on the β-subunit of rabbit H,K-ATPase (Tyagarajan, Townsend, & Forte, 1996). All seven N-linked sites of this glycoprotein were found to be occupied, some with very large tetra-antennary glycans (e.g., Hexα16HexNAcα3Fuc) with masses as high as 5 kDa (Tyagarajan et al., 1997). Another such glycoprotein is haemagglutinin from influenza A virus (Mir-Shekari et al., 1997), where the absence of sialic acid was attributed to the presence of viral neuraminidase. Tryptic peptides (4.0→4.6 kDa) from site 129 of this glycoprotein were profiled from 4-HCCA matrix on a magnetic sector mass spectrometer, and were sequenced by exoglycosidase digestion as free glycans (2,5-DHB matrix) following release by hydrazinolysis.

Exoglycosidase digestions are normally performed in sodium-containing buffers that generally have to be removed prior to MALDI analysis. Yang & Orlando (1996b) have, however, shown that 25 mM ammonium acetate, adjusted to the required pH, can be used instead to avoid the intermediate clean-up stage. Samples were analyzed as glycopeptides with sinapinic acid as the matrix.

The advantage of using the glycopeptide rather than the released glycans for MALDI analysis is that the peptide is more readily ionized than the sugar, thus giving higher sensitivity. On the negative side, the higher molecular weight results in poorer resolution. In addition, unambiguous compositional data on the glycans require knowledge of the sequence and, thus, molecular weight of the peptide. Other problems result from difficulty in separating some complex mixtures and that, in the presence of peptides, glycopeptides frequently produce relatively weak signals by MALDI analysis. One solution to the first of these problems is to use a two-stage separation procedure, such as the one described by Wu (1997) in which the second stage involved capillary electrophoresis. Separation at the second stage reflected the number of sialic acid residues. MALDI spectra in this study, recorded from 4-HCCA and 2,5-DHB, showed loss of sialic acid whereas those recorded from 1,3,5-trihydroxyacetophenone or by electrospray, did not. A solution to the second problem is to use the electrospray method developed by Carr, Huddleston, & Bean (1993), which employs selected ion monitoring of diagnostic fragments of the glycan, produced in the ion source region of the mass spectrometer, to localize the glycoprotein. This method has, for example, been used in a study of glycans from endopolygalacturonase II (EPG II)—an enzyme produced by plant pathogens that degrades pectins in the cell wall (Yang et al., 1997). The single glycopeptide peak was identified by plotting the chromatogram of the ion at m/z 204, diagnostic for GlcNAc. In order to avoid these problems associated with analysis of glycans as glycopeptides, sequencing can be performed on the released glycans.

3. Analysis of Released Glycans
   a. Chemical release
   N- (Takasaki, Misuochi, & Kobata, 1982; Patel et al., 1993) and O-linked (Patel et al., 1993) glycans can both be removed from glycoproteins with hydrazine. This reagent cleaves peptide bonds, including that between the N-linked glycan and asparagine. O-linked glycans are
specifically released at 60°C, whereas 95°C is needed to release the N-linked sugars. Although all types of glycan can be released in this way, unlike some enzymatic release methods, the technique has several major disadvantages. Because all peptide bonds are destroyed, all information relating to the protein, such as the site of glycan attachment, is lost. Secondly, the acyl groups are cleaved from the N-acetylamino sugars and sialic acids. Normally, these acyl groups are replaced chemically in a reacetylation step on the assumption that they were originally acetyl. Although this assumption is true for the N-acetylamino-hexoses, it is not always true for the sialic acids that frequently contain N-glycoyl groups and, thus, this information is also lost. The reacetylation step also often adds a small amount of acetyl substitution to hydroxyl groups. Thirdly, the reducing terminus of some of the glycans contains residual hydradize or amino groups. Acetylation of the amino group produces a compound with a mass increment that is equivalent to that between a hexose and an N-acetylaminoxhexose and, thus, can lead to the apparent appearance of additional glycans in a profile. Bendiaik & Cumming (1985) have examined the hydrazinolysis/reacetylation reaction in detail, and have calculated that as much as 25% of the total glycans are converted into products that bear nitrogen-containing groups at the reducing terminus. They further conclude that these compounds can never be converted into the parent sugar. Finally, if the hydrazinolysis conditions are too vigorous, then the N-acetylamino group can be removed from the reducing terminus. There have also been reports of the reducing-terminal GlcNAc residue being removed from, for example, plant glycans that contain an α1-3-linked fucose residue (Costa et al., 1997a).

b. Enzymatic release
Several enzymes are available for releasing N-glycans. The most popular is peptide N-glycosidase F (PNGase-F) (Tarentino, Goméz, & Plummer, 1985), which cleaves the intact glycan as the glycosylamine and leaves aspartic acid in place of the asparagine at the N-linked site of the protein. The mass difference of one unit in the protein/peptide mass is readily detectable by mass spectrometry, and provides information on the extent of occupancy at that site. If the peptide sequence is unknown, then the aspartic acid can be identified by partial 18O incorporation if the digestion is performed in 40% 18O-enriched water (Gonzalez et al., 1992). The released glycosylamine readily hydrolyzes to the glycan except if the reaction is performed in ammonium-containing buffers (Küster & Harvey, 1997) when a considerable amount of residual glycosylamine has been detected by MALDI owing to its mass difference of one unit.

PNGase-F releases most glycans except those that contain fucose α1-3 linked to the reducing-terminal GlcNAc (Tretter, Altmann, & März, 1991) as commonly found in plants. In these situations, PNGase-A is usually effective. This enzyme is also capable of releasing glycans as small as GlcNAc, whereas PNGase-F appears to require at least two GlcNAc residues for effective release. In other respects, the glycan specificity of these enzymes appears to be similar although the rates of release of related glycans vary greatly as a result of differing protein structure (Altmann, Schweizer, & Weber, 1995). PNGase-A has been used (Yang, Gray, & Montgomery, 1996a) to study glycans from horseradish peroxidase. Like many plant-derived N-linked glycans, they were mainly truncated N-linked structures that contain xylose (3) attached to the core mannose. Twelve glycans were found, and profiles were examined as potassium adducts in order to avoid any coincidences of mass between potassium adducts and sodium adducts of glycans that contain one extra oxygen atom. Costa et al. (1997a) have found an almost complete resistance towards PNGase-F of glycans from the 31 and 15 kDa subunits of the aspartic proteinase, cardosin A from Cynara cardunculus L. due to the presence of an α1→3-linked fucose residue. However, PNGase-A also failed to release the glycans from the intact glycoproteins, which had to be partially digested with pronase before incubation with PNGase-A was successful. Glycan profiles measured from the 2-AB derivatives were similar to those obtained by automated hydrazinolysis, although the latter technique was observed to cause substantial decomposition of the glycans at 95°C. N-Linked glycans from the Fe(III)–Zn(II) purple acid phosphatase of the red kidney bean (Phaseolus vulgaris) have been identified, following cyanogen bromide and trypsin digestion to isolate the glycosylation site (Stahl et al., 1994b) prior to incubation with PNGase-A. Glycosylation of soybean peroxidases have also been studied and found to contain mannose-type glycans with xylose attached to the core (Gray & Montgomery, 1997).

Endoglycosidase-H (endo-H) is another popular enzyme for releasing N-linked glycans. It hydrolyses the bond between the two GlcNAc residues of the chitobiose core, leaving the core GlcNAc with any attached fucose attached to the protein. Information on the presence of core fucosylation is, thus, not available from the spectra of the resulting glycans. Another potential disadvantage of this enzyme is that it releases only high-mannose and hybrid glycans although this property does yield some structural information.

c. Detailed N-linked glycan analysis by use of exoglycosidase sequencing
The detection of the glycans following digestion presents problems, because they contain no chromophore. Conse-
sequently, they must be labeled with a UV-absorbing, fluorescent, or radioactive tag at the reducing terminus. Profiling can be undertaken by either chromatographic techniques or mass spectrometry. Chromatographic techniques such as Bio-Gel P4 gel filtration chromatography was once popular, but it is a procedure applicable only to neutral glycans, one that takes many hours and that provides limited resolution. Although HPLC is better, greatly increased resolution (in the sense of the numbers of compounds detected) in a small fraction of the time taken by chromatographic separation can now be provided by MALDI mass spectrometry. In addition, there is no need for labeling. The resolution provided by HPLC is not sufficient to separate very complex glycan mixtures such as those found in the membrane-bound form of the glycoprotein CD59 obtained from human erythrocytes. These glycans, however, present no problem when examined by MALDI, as demonstrated by Rudd et al. (1997). Hydrazine-released glycans from the single glycosylation site of this glycopeptide were examined on a magnetic sector instrument from 2,5-DHB. Over one hundred bi- and poly-antennary glycans, some with long polylactosamine extensions and multiple fucosylation, were found (Fig. 31). The relative abundance of the extended glycans dropped with increasing complexity until the signals fell below the noise level at around m/z 5000.

On the other hand, HPLC, or another chromatographic technique, is essential for the analysis of compounds that contain isomeric glycans because mass spectrometry cannot differentiate between these isomers unless their spectra are very different. Such isomeric glycans are found at the single glycosylation site of the glycoprotein ribonuclease B (15 kDa). These glycans have compositions of (Man)₅₋₉(GlcNAc)₂ of which three, (Man)₅(GlcNAc)₂, (Man)₆(GlcNAc)₂ and (Man)₉-(GlcNAc)₂, are single compounds. The other two are each mixtures of 3 isomers, as revealed in a detailed study by MALDI and NMR (Fu, Chen, & O’Neill, 1994). In order to examine these mixtures, they must first be separated by chromatography as demonstrated by Townsend et al. (1996) as 2-AB derivatives, using multimode chromatography with analysis by MALDI, and with porous graphite (Lipiunius et al., 1996).

The first report of the application of MALDI to exoglycosidase sequencing of free glycans was that reported by Harvey et al. (1994). Biantennary N-linked glycans were released from human IgG with hydrazide and successively incubated with bovine testis β-galactosidase to remove the one or two galactose residues, jack bean N-acetyl-β-hexosaminidase to remove two or three HexNAc moieties, and Charonia lampas fucosidase to hydrolyze the fucose. MALDI analysis with 2,5-DHB was performed at each stage (Fig. 32). Although the products of each incubation were isolated after each digestion, with consequent losses the signal:noise ratio of the MALDI spectra actually rose as the result of the progressive collapse of the several peaks in the original mixture to the single peak of the final incubation product.

Küster, Naven, & Harvey (1996b) have simplified this approach by reducing the volume of enzyme buffer and performing the incubations on the MALDI targets. Their rapid method relies on the fact that, as the buffer volume is reduced, the substrate concentration approaches the Michaelis constant (Km) of the enzyme and reactions proceed more rapidly. Exoglycosidase reactions were found to be complete in one (S. pneumoniae β-galactosidase and Jack bean α-mannosidase) to eight hours (Jack bean β-N-acetylhexosaminidase) rather than the standard “overnight” period. In addition, because no reaction products were formed, which could alter the pH of the incubation mixture, there was no need for strongly buffered systems and, thus, 20 mM sodium acetate was used. Following incubation, the sample solution (1–3 μL) was transferred to a drop dialysis membrane (500 Da cut-off) for 10 min to remove buffer salts, and mixed with 2,5-DHB for MALDI analysis. The matrix was removed by further drop-dialysis, and the sample was re-incubated.
with the next exoglycosidase. The procedure was repeated until sequencing was complete. Starting with 100 pmol of glycan, it was possible to conduct three successive enzyme digestions before the sample became too dilute to give a MALDI signal. It was estimated that about 25 pmol of material was lost in each round of exoglycosidase digestion and measurement. The method was used to investigate glycans from human IgG and ovalbumin.

Mechref & Novotny (1998b) have extended this method by eliminating the extraction steps and performing all reactions on the MALDI target with enzyme arrays in 10 mM phosphate buffer. Three sample spots were prepared. All contained PNGase-F (30 milliunits) for releasing the glycans, together with A. ureafaciens neuraminidase or with mixtures of neuraminidase and D. pneumoniae β-galactosidase or neuraminidase, β-
galactosidase, and *D. pneumoniae* β-D-glucosaminidase. Incubations were conducted for three hours, and the glycans were examined by MALDI from arabinosazone (40). The glycoproteins, ribonuclease, ovalbumin, bovine fetuin, human α1-acid glycoprotein (AGP), and porcine kidney diamine oxidase, were examined and a pH of 6.5 was found to be optimal for maximum reaction of the neuraminidase. MALDI profiles from the ribonuclease and ovalbumin corresponded well with those from published studies. In the case of human AGP, however, glycan abundances appeared to be biased towards the biantennary glycan, with none of the reported tetra-antennary glycan apparent in the profile. The result with AGP probably reflects a non-optimized reaction time for PNGase-F with this glycoprotein. Hirani, Bernasconi, & Rasmussen (1987) have also reported problems with incomplete deglycosylation of this glycoprotein with PNGase-F at 5 U/mL.

In order to obtain strong MALDI signals from small biological samples, isolation and clean-up methods should be as simple as possible, and conducted in apparatus appropriate to the size of the sample. To this end, Küster et al. (1997, 1998) have developed a method whereby glycoproteins were separated or isolated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels prior to glycan release by cleavage with PNGase-F within the gel. The glycoprotein was reduced and alkylated either before or after separation to allow the maximum release of the sugars. The glycans were extracted with water and cleaned by passage through a mixed bed resin column of AG-3 (removal of anions), AG-50 (removal of cations), and C18 (removal of organic material) packed into a gel-loader pipette tip. Samples were examined directly by MALDI or HPLC combined with exoglycosidase digestion. The AG-3 resin was found to be essential for the production of MALDI signals, but unfortunately it removed most acidic glycans. Consequently, if sialic or uronic acid-containing compounds were present in the sample, then these compounds had to be methylated, using the method developed by Powell & Harvey (1996) prior to clean-up. After glycan removal, the protein remained intact inside the gel, where it could be cleaved with trypsin to produce peptides whose masses, measured by MALDI, could be used to identify the protein by database matching. Sensitivity was such that only 100 pmol of glycoprotein applied to the gel was sufficient for a complete glycan analysis. The method was used to study the N-linked glycans of GP-120 from the human immunodeficiency virus (Küster et al., 1997) (Fig. 33) and α1-acid glycoprotein from four species (Küster et al., 1998) (Fig. 34). Forty high-mannose, hybrid, and complex glycans were identified from GP-120 by exoglycosidase sequencing, similar to results obtained by Liedtke, Geyer, & Geyer (1997). The study with α1-acid glycoprotein showed that some of the glycans from the cow contained glycolyneuraminic acid; this result might not have been possible had the glycans been released with hydrazine.

![Figure 33](image-url)
FIGURE 34. Positive ion, delayed-extraction, reflectron MALDI-TOF mass spectrum (2,5-DHB) of glycans released in-gel from α1-acid glycoprotein from four species. (From Küster et al., 1998, with permission from Wiley-VCH).
The most sensitive method for N-glycan analysis reported to date is that of Papac et al. (1998). MALDI spectra of sialylated N-linked glycans were obtained from as little as 100 ng of recombinant tissue plasminogen activator. The method relies on the adsorption of up to 50 μg of the glycoprotein onto the polyvinylidene difluoride (PVDF, Immobilon P) membrane of a 96-well MultiScreen IP plate (pore size 0.45 μm), where the glycoprotein was reduced and alkylated before the glycans were released with PNGase-F. Use of the multiScreen plate enabled the solvents and reagents to be removed rapidly by application of gentle suction. It also enabled the glycoproteins to be concentrated from relatively large volumes. Because the amount of glycoprotein could be less than that required to saturate the membrane, any free binding surface was blocked by treatment with polyvinylpyrrolidione (PVP) prior to incubation with PNGase-F. Tris-acetate buffer (10 mM, pH 8.5) rather than the more usual sodium phosphate was used for the incubation in order to avoid any lactone formation from sialic acid residues (Papac, Wong, & Jones, 1996). Examination of tryptic peptides from recombinant-tPA showed the complete removal of all N-linked glycans within three hours. Following release, the glycans were incubated at room temperature for three hours with 150 mM acetic acid to ensure that any of the glycosylamine released by the enzyme was converted into the free sugar. Although this treatment did not cause any significant desialation, subsequent vacuum drying caused a 15% loss of sialic acid. Samples were desalted with AG50W-X8 resin, a process that was found to cause ca. 3% loss of sialic acid. MALDI analysis was performed with THAP/ammonium citrate (negative ion) or with super-DHB. Positive ion glycan profiles from tPA were obtained from 2.5% of 0.1 μg of glycoprotein applied to the membrane, corresponding to glycans released from only 42 fmol of glycoprotein. Glycan profiles qualitatively and quantitatively matched those observed by other methods.

i. Use of bulk, unfraccionated samples. Another approach to the problem of small sample amounts is to examine the glycans in bulk preparations of biological fluids or tissues without any attempt at constitutive protein fractionation. Relatively large amounts of glycans can be recovered in this way, allowing detailed analysis. These compounds can be used as a library to screen glycans subsequently released from individual glycoproteins. Neutral (Chen et al., 1998) and sialylated (Zamze et al., 1998) glycans from whole rat brain have been studied after hydrazine release. Glycan fractionation was by weak anion exchange chromatography (to separate glycans in different charge states), Bio-Gel P4 gel filtration chromatography, and HPLC. Structural elucidation was performed by using sequential exoglycosidase digestions, and MALDI analysis was achieved with a magnetic sector instrument. A large array of structures was found, many with large numbers of sialic acid residues. Some neutral glycans lacked an antenna at the 3-linked core mannose, a structural type that is characteristic of brain-derived glycans.

Similar techniques have been used to investigate heavily fucosylated bi-, tri-, and tetra-antennary glycans from the human parotid gland with sector and TOF instruments (Fig. 35) being used for glycan analysis (Guile et al., 1998). Several specific exoglycosidase arrays and reversed- and normal-phase HPLC were used to separate and identify isomeric fucosylated glycans. PSD and high-energy CID spectra were also used to investigate fucosylated biantennary glycans, and although partially successful, the methods were hampered by the ready loss of fucose residues.

d. Chemical methods as an aid to glycan sequencing

In addition to the use of enzymes, chemical degradation can also yield structural information on N-linked glycans. For example, acetylation specifically cleaves glycans that contain 1→6-linkages and has been used by Vinogradov, Petersen, & Bock (1998) to examine N-linked glycans from Saccharomyces cerevisiae. Many N-linked glycans from fungi, such as those from Saccharomyces species, contain 1→6-polymannose chains substituted with short mannose-containing chains in different linkages. Cleavage of the polynannose chain by acetylation isolated these short chains, with their mannose attachment site and further MALDI analysis showed them to contain up to five mannose residues, whose 1→2 and 1→3 linkages were determined by NMR. Lipniunas et al. (1996) have examined the related high mannose glycans of the ‘‘yeast’’ type obtained from invertase expressed in Pichia pastoris and separated on porous graphite. Structural analysis was performed by MALDI from a mixture of 2,5-DHB and 1-hydroxyisouquinoline and by NMR. The α1→2 and α1→6-linked isomer positions were confirmed by incubation with the specific α1→2-mannosidase from Aspergillus saitoi.

Some unusual structural modifications that can produce resistance to exoglycosidases can occur in glycans obtained from these fungal species, and again chemical degradation may be needed to resolve the problem. Thus, for example, some hydrazine-released N-linked glycans from tPA, expressed in the yeast Pichia pastoris, have been found to be of the high-mannose type that contain up to 18 mannose residues and a branched 3-antenna (Miele et al., 1997). Negative ion MALDI spectra indicated the addition of one phosphate residue to some of these glycans with the Man11-containing compound being the most abundant. Because treatment with alkaline
phosphatase failed to remove phosphate, the presence of an esterified phosphate group was suspected. Mild acid hydrolysis removed one mannose residue to leave a sugar that was sensitive to phosphatase treatment; this experiment confirmed that the glycans were substituted with mannose-α1-phosphate groups.

Periodate oxidation can be used directly for linkage determination (Angel & Nilsson, 1990a; 1990b). This reaction causes oxidative cleavage of the carbon–carbon bond between carbons that bear cis-hydroxy groups with the formation of a di-aldehyde. Reduction of this aldehyde with sodium borodeuteride followed by methylation gives a product whose mass differs from that of the original permethylated oligosaccharide. For hexoses, for example, the presence of 1- and 4-linkages causes cleavage between C-2 and C-3 resulting in a shift of the residue weight of the hexose moiety from 204 to 208 mass units. Hexoses that contain 1- and 3-linkages produce no periodate cleavage product because of the absence of a cis-diol group, whereas linkage at the 1- and 6-positions cause cleavages between C-3 and C-4 and between C-4 and C-5, resulting in the loss of the carbon atom at C-3.

e. Detailed N-linked glycan analysis by use of alkaline degradation
   As an alternative to exoglycosidase sequencing, which removes fragments from the non-reducing end of the glycan, Cancilla, Penn, & Lebrilla (1998) have used the base-induced “peeling” reaction, whereby a strong base causes the successive loss of residues from the reducing end by glycosidic cleavage. A mixture of 10 μL of an aqueous solution that contained 0.1–1 mg/mL of the glycan and 10 μL of 0.01 M sodium hydroxide was heated at 60°C for 3–24 h. The reactions did not proceed to completion due to formation of stable intermediates, and thus sampling was not time-dependent. MALDI spectra were recorded with a FT instrument directly from the reaction mixtures (Fig. 36). Since the basic solutions gave poor spectra, they were acidified to pH 5 and dried on the probe tip. After being redissolved in 1:1 methanol:water, they were mixed with 2,5-DHB and allowed to crystallize. Samples were analyzed by direct MALDI and by CID. Since the “peeling reaction” proceeded only from the reducing end of the molecule, the reaction products provided direct information on sequence and branching. Linkage information was obtained from the cross-ring fragment ions that were particularly abundant from the reducing-terminal residue, as described above. Table 7 lists the cross-ring fragments that are associated with particular linkages. Some other linkage-specific observations were that glycosidic cleavages were not observed for 1→2- or 1→6-linked disaccharides, and that HexNAc residues that contain a 3-linked hexose rarely exhibited cross-ring cleavages.
FIGURE 36. Positive ion MALDI–FTMS spectra of the glycan LNFP-1, (a) before degradation, (b) after 4 h of alkaline degradation (B and C cleavages are due to chemical degradation), (c) after 24 h of alkaline degradation (full sequence and much linkage information is present), and (d) (next page) fragmentation scheme. (Reprinted with permission from Cancilla, Penn, & Lebrilla, Anal Chem 70:663–672, Copyright 1998, American Chemical Society).
Table 8 lists studies in which MALDI mass spectrometry has been used for structural studies on N-linked glycans.

C. O-Linked Glycans

1. Glycan Release

Although O-linked glycans are sometimes released from glycoproteins with hydrazine, as described above, they are more usually released by β-elimination with alkali. However, because the resulting reducing sugars are unstable at high pH, sodium borohydride is added to reduce them to the alditol. This technique has two disadvantages. First, the borohydride also releases some N-linked glycans, and second, the reducing terminus of the sugar is removed, thus preventing labeling with chromophores or other tags. Hydrazine treatment at 65°C under anhydrous conditions partially overcomes these problems because glycans are released with their reducing terminus intact. However an improved method, reported by Cooper, Packer, & Redmond (1994) involved heating at only 45°C for 18 h with 50% aqueous hydrazine that contained 0.2 M triethylamine. The resulting hydrazones were acetylated and incubated in aqueous acetone for 24 h at 55°C to release the free glycan. Another release method, as with N-linked glycans, is to digest the protein with pronase and to recover the glycans attached to their amino acid or to a small peptide. Coddeville et al. (1998) used this method to study glycosylation of the glycoprotein PP3 from bovine milk. Two fractions were obtained and analyzed by MALDI. The larger fraction consisted of N-linked glycans and the smaller fraction contained the three glycans GalNAcα1-, Galβ(1→3)GalNAcα1-, and Galβ(1→4)GlcNAcβ(1→6)Galβ(1→3)GalNAcα1- attached to Thr86.

The analysis of the released glycans usually employs the same methods of exoglycosidase sequencing as used for the N-linked glycans; however, in some cases, the enzymes appear to be less active than with the larger N-linked glycans. In addition, because the sugars are smaller, problems can be encountered during MALDI analysis. These problems arise from two sources. First, interference can occur from matrix ions, and second, the sensitivity of MALDI falls in the region below about m/z 1000, particularly if abundant matrix ions are allowed to hit, and transiently desensitize, the detector. Analysis by PSD, however, is often more fruitful than with the larger, N-linked glycans because there are fewer opportunities for internal fragment ions to form.

Table 7. Fragment ions produced from the reducing terminal of disaccharides.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>-H₂O</th>
<th>₂₀A₂</th>
<th>₂³A₂</th>
<th>₂₀X₁</th>
<th>₂⁴X₂</th>
<th>C₁</th>
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<tbody>
<tr>
<td>β-D-Glc(1→2)D-Glc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-D-Glc(1→3)D-Glc</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>β-D-Glc(1→4)D-Glc</td>
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<td></td>
</tr>
<tr>
<td>β-D-Glc(1→6)D-Glc</td>
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</tr>
<tr>
<td>β-D-GlcNAc(1→4)D-GlcNAc</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>β-D-Gal(1→3)D-GlcNAc</td>
<td></td>
<td></td>
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</tr>
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</table>
## TABLE 8. Studies on N-linked glycans by MALDI mass spectrometry

<table>
<thead>
<tr>
<th>Glycoprotein Type</th>
<th>Glycopeptide</th>
<th>Release</th>
<th>Instrument</th>
<th>Matrix</th>
<th>Other MS</th>
<th>Examined as</th>
<th>Site</th>
<th>Glycans</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2α1-Acid Glucosamine glycoprotein</td>
<td>Human</td>
<td>Glu-C</td>
<td>–</td>
<td>TOF</td>
<td>Sinapinic</td>
<td>FAB</td>
<td>Glycopeptides</td>
<td>Asn-15, 38, 54, 75, 85</td>
<td>Complex</td>
</tr>
<tr>
<td>2α1-Acid Mannosamine glycoprotein</td>
<td>Human, Cow Sheep, Dog</td>
<td>–</td>
<td>PNGase-F in-gel</td>
<td>TOF</td>
<td>2.5-DHB</td>
<td>–</td>
<td>Glycans as Me esters</td>
<td>–</td>
<td>Complex</td>
</tr>
<tr>
<td>2α1-Acid Glucosamine glycoprotein</td>
<td>Human serum</td>
<td>Trypsin</td>
<td>PNGase-F</td>
<td>TOF</td>
<td>2.5-DHB</td>
<td>–</td>
<td>Glycans as 2-AA derivative</td>
<td>–</td>
<td>Complex</td>
</tr>
<tr>
<td>2α1-Acid Glucosamine glycoprotein</td>
<td>Human</td>
<td>–</td>
<td>PNGase-F</td>
<td>TOF</td>
<td>2.5-DHB</td>
<td>–</td>
<td>Glycans</td>
<td>–</td>
<td>Complex</td>
</tr>
<tr>
<td>2α1-Acid Glucosamine glycoprotein</td>
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<td>Trypsin</td>
<td>PNGase-F</td>
<td>TOF</td>
<td>2.4,6-THAP NH₄</td>
<td>Citrate</td>
<td>Glycoprotein</td>
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<td>Complex</td>
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<tr>
<td>2α1-Acid Glucosamine glycoprotein</td>
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<td>–</td>
<td>PNGase-F in-gel</td>
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<td>2.5-DHB</td>
<td>–</td>
<td>Glycans</td>
<td>–</td>
<td>Complex</td>
</tr>
<tr>
<td>2α1-Acid Glucosamine glycoprotein</td>
<td>Human</td>
<td>Glu-C</td>
<td>–</td>
<td>TOF</td>
<td>4-HCCA</td>
<td>ESI MS/MS</td>
<td>Glycopeptides</td>
<td>Asn-15, 38, 54, 75, 85</td>
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<td>CNBr, Lys-C</td>
<td>O-Glyc. PNGase-F</td>
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<td>Asn-72, 90, 109, 155, 337, 586</td>
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MALDI MASS SPECTROMETRY OF CARBOHYDRATES

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2. Applications of MALDI to Different O-Linked Compound Types

a. Mucins

Much of the research that involves the MALDI analysis of O-linked glycans deals with the characterization of sugars from mucins. Up to 80% of the carbohydrate in porcine stomach mucins is O-linked via GalNAc to serine and threonine. The glycans have been shown to vary with the region of the stomach that produces the mucin (Karlsson et al., 1997). In this study, glycans were released by β-elimination, permethylated, and examined by high temperature GC/MS (up to 390°C) and MALDI as lithium adducts. Thirty glycans were identified by both techniques, but another 49 glycans, containing up to 18 residues, were detected by MALDI (Fig. 37). An analysis of O-linked glycans from respiratory mucins of a cystic fibrosis patient, again examined as lithium adducts, has revealed over 60 structures that contain up to 15 residues (Thomsson et al., 1998). Lo-Guidice et al. (1997) have

![FIGURE 37. Positive ion MALDI mass spectra of the permethyl derivatives of O-linked glycans from porcine gastric mucin: (a) low molecular weight region, (b) high molecular weight region. (Reproduced with permission from Karlsson et al., 1997, Biochem J 326:911–917, © the Biochemical Society).](image-url)
released glycans by alkaline borohydride treatment of respiratory mucus from a patient suffering from chronic bronchitis, and have identified eight new monosulfated structures by a combination of MALDI mass spectrometry and NMR. The sulfates, which were 3-linked to galactose or 6-linked to GlcNac, appeared stable under MALDI conditions, and gave abundant [M+2Na−H]+ ions with 2,5-DHB from a 40 pmol sample. A similar analysis of mucins from a human colon carcinoma produced by nude mouse xenografts has revealed the presence of multiple sulfated LewisX determinants (66), which act as selectin ligands (Capon et al., 1997). The presence of these epitopes had previously been missed by conventional experiments that used lectins or monoclonal antibodies.

Maes et al. (1997) have identified seven tetra- to octasaccharides, most of them sulfated, from the jelly-coat of amphibian eggs, using similar methods. The glycans gave mainly [M+2K−H]+ ions; the potassium presumably originates from the potassium bicarbonate buffer used to purify the neutral glycans. Neutral O-linked glycans from the jelly-coat of the South African clawed toad (Xenopus laevis) have been released by alkaline borohydride, and have been examined by MALDI (2,5-DHB) interfaced to a Fourier transform mass spectrometer (Tseng et al., 1997). Twelve structures, reported earlier, were confirmed, together with seven new compounds. Several of the fucosylated glycans were observed to fragment by loss of fucose to give an ion that was isobaric with other putative glycans. In order to resolve this problem, the spectrum was re-acquired in the presence of a cesium salt. The resulting cesium adducts, which are not as prone to fragmentation, as described above, produced much less of the [M−Fucose]+ species, showing that this ion was a fragment and not produced by a native glycan.

The interest in the jelly-coats of amphibian eggs stems from the fact that they are the first barriers to be penetrated by fertilizing sperm. Hexa- to dodeca-saccharides have been released by β-elimination from the jelly-coats of the toad Bufo bufo (Morelle & Strecker, 1997) and have been identified by MALDI, methylation GC/MS analysis, and NMR. Structures were of the type shown in 67. Quantities were sufficient for detailed 2-D NMR experiments to be carried out. Experiments on the mucins from Bufo arenarum (Morelle, Cabada, & Strecker, 1998) and Rana dalmatina (Morelle, Guyévant, & Strecker, 1998) have revealed much smaller structures, with the largest having only six sugar residues. Glycans from Rana utricularia, on the other hand, were found to be much larger, with up to nine residues that consist of GlcNac, GalNAc, fucose, galactose, glucuronic acid, and sulfate (Morelle & Strecker, 1998). Sulfated glycans were also found in glycans from Rana temporaria together with 3-deoxy-d-glycero-D-galacto-nonulosonic acid (Kdn) (Maes et al., 1997).

Mueller et al. (1997) have developed a new mass spectrometric strategy for identification of the O-glycosylation sites on mucin-derived glycopeptides. Lactation-associated MUC-1 was isolated from human milk, and was partially deglycosylated with trifluoromethanesulfonic acid to the level of core-GlcNal residues. This procedure enabled the glycoprotein to be cleaved with the Arg-C-specific endopeptidase, clostripain, to yield tandem repeat icosapeptides. These glycopeptides were analyzed by MALDI from 4-HCCA in the linear mode and by PSD. GalNAc was found to be linked to Ser-5, Thr-6, and Thr-14, but additionally to Ser-15 and Thr-19 after in vitro glycosylation. In a comparative electrospray–MS/MS and MALDI–PSD study of decapeptides from MUC-4, each carrying a single GalNAc moiety, it was noted that several of the glycopeptides lost their GalNAc residue under PSD conditions, thus preventing the determination of the glycan linkage position (Alving et al., 1998).

PSD has been shown to be a valuable method for localizing O-glycosylation in MUC-1 glycopeptides, even when the sugars are on adjacent amino acids (Goletz et al., 1997a). A 25-amino acid peptide that contains GalNAc substitutions was used for most of the research, and fragments from peptide and carbohydrate moieties were found. Sugar fragments from disaccharides appeared to be more abundant from the peptide Y-ions than from B-ions from the N-terminus. Acetylation made little difference to the fragmentation pattern (Goletz et al., 1997b). Hanisch et al. (1998), however, report that nanospray MS/MS, using a Q–TOF instrument, gives cleaner fragmentation spectra that contain fewer confusing internal fragment ions.
**b. Glycosaminoglycans**

These highly sulfated glycans, such as heparin, consist of polymers of a uronic-acid glucosamine repeat unit in which the amino or hydroxyl groups contain various degrees of sulfation. Molecular weights can vary from five to over 40 kDa. These glycans are difficult to ionize by MALDI, and they tend to fragment extensively by loss of sulfate. However, excellent spectra have been obtained by Juhasz & Biemann (1994) from a hexasaccharide heparin fragment that contains eight sulfate groups by effectively neutralizing the acidic glycan by formation of a 1:1 non-covalent complex with the basic peptide IRRERNKMAAAKSRNRRRELTDL. The complex gave spectra when ionized by either a UV or an IR laser, with the best signal being observed from sinapinic acid ionized with a UV laser. Further research on this system was conducted with the synthetic peptides (R)6PYRL, (RG)10 and (RG)15 (Juhasz & Biemann, 1995). The non-covalent complex increased in binding strength with the size of the heparin fragment, and hence, the number of sulfate groups. The best results were obtained when the number of basic arginines of the peptide equaled the number of sulfates and were spaced with glycine residues. The best matrix was 3-hydroxypicolinic acid (50) (Fig. 38). The molecular weights revealed the number of sulfate and acetate groups for the smaller heparin fragments but the larger fragments showed a tendency to eliminate sulfate. Larger fragments could be stabilized with the peptide, angiotensin.

To investigate the structures of the heparin-like glycosaminoglycan chains from heparan sulfate proteoglycans, Rhomberg et al. (1998a), from the same laboratory, have developed a buffer system that allows heparinase I to be used to produce short stretches of glycan (2–10 units). These stretches were examined by MALDI, using ion-pairing with (RG)15. The buffer system contained glycerol, which inhibited the crystallization of the matrix unless it was seeded by the addition of crushed matrix crystals (Xiang & Beavis, 1994). Caffeic acid (68) was found to be the best matrix. The ionization efficiency of the glycan–(RG)15 mixture varied with the number of monosaccharide residues, and thus of sulfate groups. Disaccharides with fewer than three sulfates were rarely observed, unless larger molecules were absent. Octa- and hexasaccharides ionized with about twice the efficiency of tetrasaccharides, but larger compounds ionized poorly. Saccharides derived from the reducing terminus of the polymer chains were identified by forming the semicarbazone derivative by reaction with semicarbazide, causing a mass shift of 95.09 mass units. These techniques have recently been applied to the elucidation of heparin depolymerization by heparinase II (Rhomberg et al., 1998b). The enzyme was shown to possess two active sites that cleaved primarily sulfated and unsulfated sites, respectively.

**FIGURE 38.** Positive ion MALDI mass spectrum (3-hydroxypicolinic acid) of a mixture of heparin-derived carbohydrates complexed with the peptide (RG)10. T1 = tetrasaccharide, P1 = pentasaccharide, H1 = hexasaccharide. (From Juhasz = Biemann, 1995, with permission from Elsevier Science Ltd.).
c. Other O-linked glycans

Tryptic peptides from the hinge region of human serum IgA1, with molecular weights in the region of 5000 could not be resolved by MALDI on a linear TOF instrument with 4-HCCA because of the heterogeneity in the several O-linked glycosylation sites (Iwase et al., 1996a). However, after enzymatic removal of sialic acid and galactose residues, clear spectra were obtained. Two populations of glycoproteins were found, one contained five and the other four attached GalNAc residues. The peptides that contained five glycan chains were absent from the serum of a myeloma patient. Experiments with exoglycosidases showed the desialylated molecule to be composed of three components substituted with four Galβ(1 → 3)GalNAc and one GalNAc residue, four Galβ(1 → 3)GalNAc residues, or with three Galβ(1 → 3)-GalNAc and one GalNAc (Iwase et al., 1998). In another experiment, glycans were released with hydrazine, labeled with 2-AP (64), and shown to consist of the above disaccharides substituted with 2,3- and 2,6-sialic acid (Iwase et al., 1996b).

MALDI has been used successfully to identify tyrosine as a novel attachment site for O-linked glycans in the crystalline surface layer glycoprotein from *Thermoanaerobacter thermohydrosulfuricus* LIII-69 (Bock et al., 1994). The mixture of linear glycans, with up to sixty monosaccharide residues, was identified by using NMR, GC/MS, and MALDI mass spectrometry with 2,5-DHB as the matrix. Table 9 lists studies in which MALDI mass spectrometry has been used for structural studies on O-linked glycans.

### Table 9. Studies on O-linked glycans by MALDI mass spectrometry.

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>Type</th>
<th>Glycan preparation</th>
<th>Matrix</th>
<th>Other MS</th>
<th>Examined as</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>z- Amylase</td>
<td>Barley, recombinant</td>
<td>CNBr, Lys-C, Asp-N</td>
<td>4-HCCA</td>
<td></td>
<td>Glycopeptides</td>
<td>Andersen et al., 1994</td>
</tr>
<tr>
<td></td>
<td>from yeast</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromogranin A</td>
<td>Bovine adrenal medulla</td>
<td>Lys-C</td>
<td>4-HCCA</td>
<td></td>
<td>Glycopeptide</td>
<td>Strub et al., 1996</td>
</tr>
<tr>
<td>Chromogranin A</td>
<td>Bovine</td>
<td>Lys-C</td>
<td>4-HCCA</td>
<td>ESI, LC/MS</td>
<td>Glycopeptide</td>
<td>Strub et al., 1997</td>
</tr>
<tr>
<td>Dipterisin</td>
<td><em>Phormia terranovae</em></td>
<td></td>
<td>4-HCCA</td>
<td></td>
<td>Glycoprotein</td>
<td>Uitenweiler-Joseph et al., 1997</td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>Human serum IgA1</td>
<td>Trypsin</td>
<td>4-HCCA</td>
<td></td>
<td>Glycoproteins</td>
<td>Iwase et al., 1996a, 1998</td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>Human serum IgA1</td>
<td>Hydrazine</td>
<td>4-HCCA</td>
<td></td>
<td>Glycans, 2-AP derivative labelled</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-Myc</td>
<td>sf9 Insect cells</td>
<td>Trypsin</td>
<td>4-HCCA 2,5-DHB</td>
<td></td>
<td>Glycopep. labelled with galactose</td>
<td>Chou, Hart &amp; Dang, 1995</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucin</td>
<td>Pig, gastric</td>
<td>β-elimination</td>
<td>2,5-DHB</td>
<td>GC/MS</td>
<td>Glycans, (Per-Me)</td>
<td>Karlsson et al., 1997</td>
</tr>
<tr>
<td>Mucin</td>
<td>Human carcinoma</td>
<td>β-elimination</td>
<td>2,5-DHB</td>
<td></td>
<td>Glycans</td>
<td>Capon et al., 1997</td>
</tr>
<tr>
<td>Mucin</td>
<td>Human epithelial</td>
<td>Asp-N, trypsin</td>
<td>4-HCCA</td>
<td>PSD</td>
<td>Glycopeptides</td>
<td>Goletz et al., 1997a; 1997b</td>
</tr>
<tr>
<td>Mucin</td>
<td>Synthetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucin</td>
<td>Human milk MUC1</td>
<td>Clostripain</td>
<td>4-HCCA</td>
<td></td>
<td>Glycopeptides</td>
<td>Müller et al., 1997</td>
</tr>
<tr>
<td>Mucin</td>
<td>Human respiratory</td>
<td>β-elimination</td>
<td>2,5-DHB</td>
<td>GC/MS</td>
<td>Glycans, (Per-Me)</td>
<td>Thomson et al., 1998</td>
</tr>
<tr>
<td>Mucin</td>
<td>Human respiratory</td>
<td>β-elimination</td>
<td>2,5-DHB, 3-AQ</td>
<td></td>
<td>Glycans</td>
<td>Lo-Guidice et al., 1997</td>
</tr>
<tr>
<td>Mucin</td>
<td><em>Bufo arenarum</em>, egg coat</td>
<td>β-elimination</td>
<td>2,5-DHB</td>
<td></td>
<td>Glycans</td>
<td>Morelle, Cabada &amp; Streck, 1998</td>
</tr>
<tr>
<td>Mucin</td>
<td><em>Bufo bufo</em>, egg coat</td>
<td>β-elimination</td>
<td>2,5-DHB</td>
<td></td>
<td>Glycans</td>
<td>Morelle &amp; Streck, 1997</td>
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<td></td>
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</tr>
<tr>
<td>Mucin</td>
<td><em>Rana dalmatina</em>, egg coat</td>
<td>β-elimination</td>
<td>2,5-DHB</td>
<td></td>
<td>Glycans</td>
<td>Morelle, Guyétant &amp; Streck, 1998</td>
</tr>
<tr>
<td>Mucin</td>
<td><em>Rana temporaria</em>, egg coat</td>
<td>β-elimination</td>
<td>2,5-DHB</td>
<td></td>
<td>Glycans</td>
<td>Maas et al., 1997</td>
</tr>
<tr>
<td>Mucin</td>
<td><em>Rana utricularia</em>, egg coat</td>
<td>β-elimination</td>
<td>2,5-DHB</td>
<td>GC/MS</td>
<td>Glycans</td>
<td>Morelle &amp; Streck, 1998</td>
</tr>
<tr>
<td>Mucin</td>
<td>* Xenopus laevis*, egg coat</td>
<td>β-elimination</td>
<td>2,5-DHB</td>
<td></td>
<td>Glycans</td>
<td>Tseng et al., 1997</td>
</tr>
<tr>
<td>Mucin</td>
<td>Synthetic, MUC4</td>
<td>β-elimination</td>
<td>2,5-DHB</td>
<td>ESI, CID</td>
<td>Glycopeptides</td>
<td>Alving et al., 1998</td>
</tr>
<tr>
<td>Neurofilaments</td>
<td>Rat spinal cord</td>
<td>β-elimination</td>
<td>2,5-DHB, 4-HCCA</td>
<td></td>
<td>Glycans</td>
<td>Dong et al., 1993</td>
</tr>
<tr>
<td>S-layer glycoprotein</td>
<td><em>Thermoanaerobacter thermohydrosulfuricus</em></td>
<td>β-elimination</td>
<td>2,5-DHB</td>
<td>GC/MS</td>
<td>Glycoprotein</td>
<td>Bock et al., 1994</td>
</tr>
<tr>
<td>Tryptase inhibitor</td>
<td>Leech-derived, recombinant <em>(Saccharomyces cerevisiae)</em></td>
<td>β-elimination</td>
<td>Sinapinic acid</td>
<td></td>
<td>Glycopeptides</td>
<td>Bergwerff et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Super-DHB</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

All measurements were made with TOF instruments.
D. GPI Anchors

These complex molecules contain lipid and carbohydrate attached to the protein (Ferguson, 1991), and have rarely been examined intact. The presence of phosphate esters enables the molecule to be split into lipid and glycan portions by heating with HF; each portion can then be analyzed separately. Glycans can be sequenced by the usual exoglycosidases, whereas lipids are usually examined by GC/MS following further degradation.

Meri et al. (1996) have used this approach to determine the glycan portion of the GPI anchor from human urinary CD-59. The anchor was isolated as a tryptic glycopeptide, and was sequenced by exoglycosidase digestion with MALDI detection. The core structure of the glycan was shown to be similar to that from other human GPI-anchored proteins. The sugar portion (saccharides and inositol) of the GPI anchor from calf alkaline phosphatase has been obtained by digestion of the glycoprotein with pronase and dephosphorylation with HF. It has been sequenced by a combination of HPLC, exoglycosidase digestion, chromic acid oxidation, and MALDI analysis of the peracetyl derivatives (Armesto et al., 1996). Six glycan structures were found, differing in the number of mannose and GalNAc residues, and two were additionally substituted with palmitoyl or myristoyl chains. Ralton & McConville (1998) have carried out extensive investigations into the biosynthesis of these anchor molecules from *Leishmania mexicana*. The glycan is assembled on a specific phosphatidylinositol (1-C18:0-alkyl,2-stearoyl) and the stearoyl group is replaced by myristoyl or lauroyl chains. Analysis of these glycolipophosphatidylinositol species was by MALDI from 4-HCCA and by GC/MS.

E. Identification of New Biochemical Pathways for Glycoprotein Glycosylation by Use of MALDI

Recent analyses that involve MALDI mass spectrometry have revealed several new metabolic pathways in the formation of N- and O-linked glycans. Thus, for example, Raju, Ray, & Stanley (1995) used a combination of techniques to demonstrate the presence of an additional β1→6-linked GlcNAc residue in the core pentasaccharide of N-linked glycans produced by a mutant CHO cell line. The kinetics of UDP-N-acetylglucosylamine-1-phosphotransferase have been studied by Bao et al. (1996). MALDI from 2,5-DHB was used to identify the product that formed with OMe-mannoside.

A combination of MALDI mass spectrometry, HPLC, and enzymology has revealed alternative pathways for glycan processing in the yeast *Schizosaccharomyces pombe* (Ziegler, Gemmill, & Trimble, 1994). The smallest glycan detected was (Man)₉(GlcNAc)₂, the des-gluco precursor to the normal N-linked glycans. These data suggest the absence of the mannosidases that are responsible for trimming this glycan prior to elaboration into hybrid and complex sugars. Instead, glycans with up to 13 hexoses were found by MALDI; the additional hexoses were either mannose or galactose. These compounds are lower members of a series of much larger N-linked glycans, known as galactomannans. Those galactomannans from *S. pombe* have an average composition of (Gal)₅₂(Man)₆₄(GlcNAc)₂ (Gemmill & Trimble, 1996), and have been found with up to six pyruvic acid molecules acetal-linked to C-4 and C-6 of galactose. This linkage appears to be only the second report of this sugar modification in eucaryotes. The glycans were removed by digestion with endo-H and subjected to methanolation in order to identify the individual constituent monosaccharide types. The dimethyl–pyruvate–galactose was identified by MALDI MS from 2,5-DHB. In the linear mode, this modified monosaccharide gave the MN+ ion, but in the reflector mode a fragment ion that represents the loss of the methylpyruvate group was present.

The mechanism of action of hen egg white lysozyme has been investigated with the hexasaccharide GlcNAc₆ (Hadfield et al., 1994). The sugar was incubated with the protein for 14 days, after which the products were analyzed with MALDI. As expected, cleavage was mainly between GlcNAcs 4 and 5. A mutant enzyme, in which the key residue, Asp-52, was replaced by a serine residue, produced a more random set of products.

The substrate specificity of a secretory variant of human fucosyltransferase III from BHK-21 cells has been shown to be the O-4 position of GlcNAc in a Galβ(1→3)GlcNAc motif when present in small oligosaccharides, glycolipids or glycoproteins. The corresponding Galβ(1→4)GlcNAc motif was not fucosylated. Glycans from the test glycoprotein, asialofetuin, were released by automated hydrazinolysis, and were purified by HPLC prior to MALDI analysis from 2,5-DHB (Costa et al., 1997b).

F. Studies on N- and O-linked Glycosylation in Disease

MALDI provides a very convenient method for rapidly obtaining profiles of glycans in normal and disease states. Alterations in normal glycosylation patterns have been associated with a number of diseases, notably rheumatoid arthritis (Roitt et al., 1990), glycoprotein deficiency syndrome (discussed above), and solid tissue diseases such as gastrointestinal polyps and carcinomas. Solid tumors are often examined by microscopy, following embedding in paraffin wax for archival purposes. MALDI has recently been used to show that N-linked glycans
remain intact under these conditions, and can be examined after some time in storage (Dwek et al., 1996).

Pohl et al. (1997) used MALDI to characterize glycans from β-trace protein from human cerebrospinal fluid (CSF) collected from normal individuals and from patients who suffer from carbohydrate-deficient glycoprotein syndrome of the type that involves phosphomannomutase (PMM) and N-acetyl-glucosaminyltransferase II deficiencies. The latter enzyme adds GlcNAc to the 6-arm during biosynthesis of complex N-linked glycans, and thus the disease would be expected to be characterized by complex glycans that lack this antenna. The results showed that, normal and PMM-deficiency patients had very similar glycan profiles of mainly biantennary sugars, whereas the GlcNAc-II deficiency patients produced mainly monoantennary glycans with only three compounds in common. A further comparison of β-trace protein from CSF, blood, and urine (Hoffmann, Nimtz, & Conradt, 1997) has shown that, whereas the CSF-derived material displayed a typical “brain-type” profile, the glycoprotein from urine and blood contained longer and more heavily sialylated carbohydrates. Furthermore, the levels of the glycoprotein in the serum of patients with renal disease greatly exceeded those levels from control individuals; this observation holds out the prospect of the level of glycoprotein being a useful marker for renal dysfunction.

The protein c-Myc is a zipper phosphoprotein that regulates gene expression. Mutations of this protein are associated with various types of tumor in several species. It has recently been shown that a major phosphorylation site, Thr-58, can also be occupied by O-linked GlcNAc, with major implications for the regulation of this protein. The attachment site was identified in the tryptic peptide by enzymatically labeling the GlcNAc with 3H-galactose, with major implications for the regulation of this protein. The study also provided the first direct evidence of the specificity of the enzyme. PSD spectra recorded using 5-chloro-2-benzothiazole (69) from the glycopeptides of Streptococcus sanguis, also showed major ions from the fragmentation of the peptide chain of the MNa+ ion and from a Y-type glycosidic cleavage of the disaccharide unit (Beranova-Giorgianni, Desiderio, & Pabst, 1998).

Peptidoglycans from the spores of Bacillus subtilis have also been cleaved with muramidase into fragments of 1–3 kDa in size (Popham et al., 1996). HPLC and negative ion MALDI analysis of these fragments have shown the chains to consist of alternating GlcNAc and muramic acid residues with each alternate muramic acid in the lactone form. Muramic acid peptide chains contained 1–4 amino acids (Xu et al., 1997b), following cleavage with muramidase. Sub-picomole detection of monomer to pentamer units was reported with 5-chloro-2-benzothiazole (69) as the matrix in the positive and negative ion modes. PSD studies of the monomer provided sequence information on the peptide chain that was complemented by reaction with lysostaphin, an enzyme that cleaves peptide chains at glycine bonds. The study also provided the first direct evidence of the specificity of the enzyme. PSD spectra recorded using 5-chloro-2-benzothiazole (69) from the glycopeptides of Streptococcus sanguis, also showed major ions from the fragmentation of the peptide chain of the MNa+ ion and from a Y-type glycosidic cleavage of the disaccharide unit (Beranova-Giorgianni, Desiderio, & Pabst, 1998).

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2. Glycans from Archaea

Natronococcus occultus is an archaean that lives at 35–40°C at a pH of 9.5–10, and must possess a base-stable cell wall. Its structure, which is unique among the known archaeal cell walls, has been elucidated, using partial acid hydrolysis, chromatography, GC/MS, and MALDI (Niemetz et al., 1997). It consists of a polyglutamic acid chain linked via amide bonds to short carbohydrate chains that contain D-amino acids. The study also provided the first direct evidence of the specificity of the enzyme. PSD spectra recorded using 5-chloro-2-benzothiazole (69) from the glycopeptides of Streptococcus sanguis, also showed major ions from the fragmentation of the peptide chain of the MNa+ ion and from a Y-type glycosidic cleavage of the disaccharide unit (Beranova-Giorgianni, Desiderio, & Pabst, 1998).

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IX. USE OF MALDI TO STUDY PROTEIN GLYCATION

Protein glycation refers to the non-enzymatic reaction between sugars and proteins, and is of particular importance in the pathology of diabetes, where proteins can be exposed to high levels of circulating sugars. The main reaction is between hexoses and the ε-amino group of lysine to form an Amadori complex (Scheme 6). Various oxidations and dehydrations follow (Maillard, 1912), leading eventually to protein cross-linking and tissue damage, which produce the well-characterized
diabetes complications of retinopathy, neuropathy and nephropathy. The accuracy with which MALDI can measure the mass of large proteins has been utilized to monitor these glycation reactions, in vitro and in vivo. Bovine serum albumin (66.4 kDa) was shown in 1993 (Lapolla et al., 1993) to react with glucose in a time- and concentration-dependent fashion to add a maximum of 51 glucose residues. Spectra were obtained with a linear TOF instrument from sinapinic acid. Bovine pancreatic ribonuclease also showed reactivity (Lapolla et al., 1994), with up to four glucose residues being added over six days. Fructose also reacted, but further reactions increased the complexity of the products (Lapolla et al., 1994). Cross-linked reaction products were observed for lysozyme, following a 14 day incubation with glucose (Lapolla et al., 1996a). In vivo measurements of human serum albumin on well and badly controlled diabetic patients showed a clear increase in molecular weight in both groups with respect to controls, with more glycation being apparent in the badly controlled patients (Lapolla et al., 1995). Similar results have been found for IgG, again measured by using sinapinic acid as the matrix. The molecule showed increases in mass by 512–1565 and 827–4270 Da in the two patient groups, respectively, over the mass of 148,267 Da measured for the pure glycoprotein (Lapolla et al., 1997a). Much of the earlier work on the use of MALDI to study protein glycation has been briefly reviewed (Traldi et al., 1997; Lapolla et al., 1996b).

Another well-studied glycation reaction is that between hemoglobin (HbA1c, 16 kDa) and glucose. Here, the glucose reacts with the amino group of valine at the N-terminus of the β-chain, and the glycation product accumulates during red blood cell life. Although other methods can be used to monitor this product, only MALDI provides information on the exact nature of the glycation products. Spectra were recorded from sinapinic acid (Lapolla et al., 1996c). Further studies (Lapolla et al., 1997b), using 4-HCCA, enabled glycation of the α- and β-chains of hemoglobin to be detected together with products of ‘oxidative stress’. The study also showed that the commonly used HPLC method for detecting HBA1c levels should be related to the total pool of glycated and oxidized α- and β-globins and not simply to glycated β-globin. The oxidized structures were thought to be hydroxy-methylfurann derivatives, although the question arose as to whether the ions seen at [M+97 to 99]+ were in fact due to sulfate or phosphate. By using a delayed-extraction instrument, improved resolution enabled correct assignment of masses as [M+98]+, consistent with sulfate, which was proposed to arise from the heparin used to prevent the blood samples from coagulating. However, this possibility was offset by use of fluoride–oxalate sample tubes to collect the samples (Resemann et al., 1998). In a study of groups of healthy and diabetic patients, Lapolla et al. (1999) showed that MALDI could not only be used to evaluate the glycation level, but also to give an indication of oxidative stress.

β-2-Microglobulin (β2M) is a 99-amino acid globular cell-surface protein whose concentration is highly elevated in the blood of hemodialysis patients. Glycation of β2M leads to so-called ‘advanced glycosylated end-products’ (β2M-AGEs), which have been linked with bone and joint destruction. β2M, when incubated for one month with glucose, was found by MALDI analysis from 4-HCCA to incorporate up to seven glucose residues, correlating well with the seven basic amino acids in its sequence (Tang et al., 1996). β2M and β2M-AGE were both detected in spiked but not in normal bovine serum. It was noted that the signal from β2M-AGE was lower than that from β2M because glucose reacts with the α-amino group of the N-terminal isoleucine residue; the N-terminus is the proposed major protonation site of proteins in MALDI MS (Olumee et al., 1995). Sinapinic acid was used for measurements in human serum because of its ability to produce a stronger signal in the more complicated protein profile. It was found possible to quantify β2M-AGE in serum, using β2M as the internal standard.

**SCHEME 6.** Reaction between reducing carbohydrates and proteins.
standard. Because the $\beta_2$M-AGE peak consisted of several components, a deconvolution algorithm was applied to extract the data from each glycation adduct. It was found that these data were more reliable than measurements made on the undeconvoluted peak.

Protein glycation is of major significance for long-lived proteins such as collagen and lens proteins. Major intrinsic protein (MIP) is expressed exclusively in lens fiber cells, where it is important for maintaining lens clarity. Glycation, therefore, could contribute to cataract development. Swamy-Mruthinti & Schey (1997) have incubated calf lens MIP with 1 M sucrose for five days and demonstrated glycation at Lys-238, 259 and possibly Lys-288, all from the C-terminal peptide, which was cleaved with cyanogen bromide (CNBr). MALDI analysis of the peptide (MW $\approx 8797.1$) from 4-HCCA revealed glycated forms with one, two, and three glucose residues. The glucose residues were located on their respective Lys residues by MALDI following digestion with trypsin.

**X. ANALYSIS OF GLYCOLIPIDS WITH MALDI MASS SPECTROMETRY**

**A. Sphingolipids**

Sphingolipids are relatively small molecules and are normally examined intact. However, it is often difficult to determine the distribution of carbon atoms between the acyl and sphingosine chains of the ceramide portion of the molecule, and consequently techniques such as GC/MS often accompany MALDI analysis. The glycan chains can be examined by exoglycosidase sequencing, as described above.

1. **Methodology**

The first report of the analysis of gangliosides by MALDI mass spectrometry appeared in 1992 (Juhasz & Costello, 1992). The best matrices were found to be 2,5-DHB, 1,5-diaminonaphthalene (70), 4-hydrizinobenzoic acid (71), and 6-aza-2-thiothymine (72). Although the compounds gave negative and positive ion spectra, better results were obtained in the negative ion mode. These spectra showed improved resolution, a better signal:noise ratio, less alkali salt formation, and less fragmentation than their positive ion counterparts. Spectra were recorded on a linear TOF instrument, which gave comparatively poor resolution as the result of extensive fragmentation from this compound (Fig. 39). Loss of sialic acid and CO$_2$ (decarboxylation) dominated the fragmentation profile. There was some indication that the relative ratios of the ions that result from sialic acid loss could be used to differentiate structural isomers that contained two single sialic acid residues from those in which the sialic acids were linked (see Fig. 39). Spectra could be obtained from 10–20 pmol of sample, but increases in sensitivity by two orders of magnitude (to 100 fmol) were recorded following permethylation. These derivatives also produced more highly resolved spectra with reduced fragmentation, with the best spectra being recorded from 2-thiohydantoin (72) in the positive ion mode. M$\text{Na}^+$ ions were the most abundant.

Harvey (1995a) studied a range of sphingolipids from sphingosine to gangliosides, using a magnetic sector mass spectrometer fitted with an array detector. This instrument overcame the problems seen with gangliosides on linear TOF instruments as the result of metastable decay, and produced sharp, well-resolved ion peaks (Fig. 40). The ability to operate the laser at higher power provided considerable diagnostic fragmentation. Neutral glycosphingolipids gave the strongest signals from 2,5-DHB, 4-HCCA, and esculetin (6,7-hydroxy-coumarin, 73). Acidic compounds that contain sialic acid were best examined from 2,5-DHB (Fig. 40) because matrices such as 4-HCCA caused extensive fragmentation by the loss of sialic acid and CO$_2$ (Fig. 41). Loss of the acyl amide from the ceramide moiety was prominent in the spectra recorded from matrices other than 2,5-DHB that preferred to induce cleavage between the glycan and the ceramide. Other fragment ions were mainly the result of glycosidic cleavages, revealing the structure of the glycan chain.

Sugiyama et al. (1997) also found that 2,5-DHB produced less sialic acid loss than 4-HCCA and that fragmentation could be minimized by the formation of the methyl esters. Penn et al. (1997b) obtained similar results.
from these two matrices with a Fourier transform mass spectrometer, but with much improved resolution (Fig. 42). They were able to stabilize the molecules somewhat by doping the sample with CsCl to form the Cs adducts. They also noticed that the di-sialylated ganglioside GD1a (see Figs. 40 and 41 for structure) appeared to form a fragment ion of composition [(NeuNAc)$_2$-2H$^+$Na$^+$], which they postulated was the result of an initial hydrogen bonding within the molecule. The use of the FT instrument enabled resolutions as high as 706,000 to be obtained (Fig. 43). Other matrices that have proved useful for ganglioside analysis are HABA (32) (Juhasz, Costello, & Biemann, 1993) and 5-chloro-2-mercaptobenzothiazole (33) (Xu et al., 1997a).

A method for deacylating galactosyl ceramide (74) and its sulfate (sulfatide) involving basic hydrolysis in a microwave oven, has been developed by Taketomi et al. (1996a). This method enables the chain length of the sphingosine (75) to be examined by MALDI, and that of the acyl groups to be obtained by difference. The technique has been used to show that porcine lysocerebroside contains only 18:1 sphingosine, whereas equine sphingolipids contain a mixture of 18:1, 18:0, 20:0, and 18-and 20-phytosphingosines. Equine sulfatides contained additional 16:1, 17:1, and 20:1 sphingosines. The sulfatides gave strong spectra in the negative ion mode because of the presence of the sulfate group. Larger glycolipids were also deacylated by this microwave-assisted basic hydrolysis reaction (Taketomi et al., 1996b), which appears to remove the long-chain acyl group from the sphingosine in preference to the acetyl group of N-acetylamino-sugars. The sialic acid groups of gangliosides were stable under these conditions, although considerable deacylation occurred (Taketomi et al., 1997). 4-HCCA was the preferred matrix for all of the resulting lyso-compounds.

**FIGURE 39.** Positive ion MALDI mass spectra (2,5-DHB) of the gangliosides GD1a and GD1b recorded with a linear TOF mass spectrometer. The latter compound has its two sialic acids linked. (From Juhasz & Costello, 1992, with permission from Elsevier Science Ltd.)
2. Applications

Three neutral glycosphingolipids, Manβ(1 → 4)Glcβ1 → Cer, Glcβ1 → Cer, and GlcNAcβ(1 → 3)Manβ(1 → 4)Glcβ1 → Cer have been identified from the nematode Caenorhabditis elegans (Gerdt et al., 1997). The glycan moiety was identified by MALDI analysis (2,5-DHB) coupled with exoglycosidase digestion, whereas the ceramide was identified by GC/MS. The fatty acid portion was dominated by 2-hydroxy fatty acids, and the sphingosine had a 17:1 structure with iso- (ω-1) and anteiso- (ω-2) methyl branches. Two acidic glycosphingolipids from the porcine parasitic nematode, Ascaris suum, have also been examined by a combination of chemical hydrolysis and derivatization, combined with MALDI (2,5-DHB), LSIMS and GC/MS (Lochnit et al., 1998a). One sphingosine was shown to have the unusual inositol phosphate-containing structure, Galβ1 → InsP → 1ceramide, and the other had 3-sulfogalactosylcerebroside. Zwitterionic sphingolipids containing phosho-
Choline and phosphoethanolamine have also been found in this parasitic worm, and those sphingolipids were characterized by similar methods (Lochnit et al., 1998b). Both compounds gave positive ion MALDI spectra from 2,5-DHB in the linear mode, but were found to have lost choline and ethanolamine, respectively, by PSD when examined in the reflectron mode. The cleavage of phosphate ester bonds in phospholipids, particularly when recorded from 4-HCCA, has been noted before (Harvey, 1995b). The parasite also contains mono- to tri-saccharyl-ceramides and methyl-branched sphingosine chains (Lochnit et al., 1997).

Glycosphingolipids from bovine erythrocytes, mouse kidney, and fetal calf brain have been characterized by Perreault et al. (1997), using a combination of MALDI and LSIMS. Acetyl derivatives, prepared by gas phase acylation, could be detected at the low femtomole level by MALDI, but only at the subnanomole level by LSIMS. MALDI–PSD spectra gave sequence information on the glycan chains, with B- and Y-ions being the most abundant (Fig. 44). However, CID had to be used to obtain information on the carbon distribution between the two chains of the ceramide portion.

a. Glycolipids from bacteria

i. Lipopolysaccharides (LPS) and derived glycans. MALDI has been used successfully by a number of laboratories for the examination of these compounds. The various parts of the molecules are usually studied independently on account of their size. They are typically extracted using hot phenol and water, and purified by column chromatography or microcentrifugation. Mild acid hydrolysis is used to separate the lipid A portion,
and hydrolysis with TFA has been used in order to isolate the carbohydrate repeat unit.

**ii. O-Specific chains.** Several strains of *Hafnia alvei* have been studied with this general procedure, combined with FAB mass spectrometry, CID, NMR, and methylation linkage analysis. MALDI analysis from 2,5-DHB of the chain of *H. alvei* strain 32 gave a series of ions up to a mass of 16 kDa and an average repeat unit mass of 919 Da. The linear repeat unit was found to consist of one each of rhamnose (10), galactose (6), galacturonic acid, GlcNAc (8), and GalNAc. The mass difference between the measured and calculated masses, assuming that only these monosaccharides were present, was found to be due to partial acetylation, as confirmed by MALDI analysis of the deacetylated sugar, which gave the correct mass difference of 890 Da (Jachymek et al., 1996). Similar results were obtained from strain 1192, where the partially acetylated hexose repeat unit was found to consist of three

**FIGURE 42.** Positive ion MALDI mass spectra of the ganglioside G_{D1a} recorded with a FT mass spectrometer: (a) 2,5-DHB, (b) 4-HCCA. (Reprinted by permission of I.M. Publications from “Direct comparison of matrix-assisted laser desorption/ionization and electrospray ionization in the analysis of gangliosides by Fourier transform mass spectrometry” by Penn et al., European Mass Spectrom 3:67–79, Copyright I.M. Publications 1997).

**FIGURE 43.** High resolution MALDI mass spectrum of the \([M-(2 \times NeuNAc)+Na]^+\) ion from the ganglioside G_{D1a} recorded with a FT instrument in the heterodyne mode. (Reprinted by permission of I.M. Publications from “Direct comparison of matrix-assisted laser desorption/ionization and electrospray ionization in the analysis of gangliosides by Fourier transform mass spectrometry” by Penn et al., European Mass Spectrom 3:67–79, Copyright I.M. Publications 1997).
rhamnose residues (10), one ribose (1), one glucuronic acid (9), and one GlcNAc (8).

The negative ion MALDI spectrum from 2,5-DHB again gave peaks to about 16 kDa (Jachymek et al., 1995). Strain 1206 was found to have a pentasaccharide repeat substituted with d-allothreonine (76) (Petersson et al., 1997a), giving masses to 16 kDa (Fig. 45) whereas strain 1209 had smaller chains that consisted of 8–11 pentasaccharide repeats with masses to 12 kDa (Niedziela et al., 1996). Even smaller were the chains in strains 774, PCM 1194, and PCM 1201, which gave masses only up to about 8 kDa (Petersson et al., 1997b). Strains 774 and PCM 1194 had similar chains, with a d-hydroxybutyryl group substituted at the 4-position of one of the two GalNAc residues.

\[
\text{H} \quad \begin{array}{c}
\text{H}_2\text{N} - \overset{\text{C}}{\text{C}} - \text{COOH} \\
\text{H} - \overset{\text{C}}{\text{O}} - \text{OH} \\
\text{CH}_3
\end{array}
\]

76, Allo-Threonine
A somewhat different approach to the analysis of these carbohydrates was adopted by Rahman, Guard-Petter, and Carlson (1997) in a study of O-antigens from *Salmonella enteritidis*. LPS was again extracted with phenol:methanol:chloroform, but was divided into low and high molecular weight fractions by preparative gel electrophoresis. Glycan chains were released by mild acid hydrolysis and were cleaved with the bacteriophage P22, which contains endorhamnosidase activity. Glycan chains were examined by FAB, MALDI, MS/MS, and NMR, and were found to have two repeating units that contained mannose (5), rhamnose (10), galactose (6), glucose (4), and tyvelose (15). MALDI in this study was performed on the monomers.

**iii. Lipid A.** The lipid A portion of LPS has proved to be difficult to analyze on account of its two phosphate groups and high hydrocarbon content. Much of the earlier work was performed with laser desorption mass spectrometry (without a matrix) (see, for example, Seydel et al., 1984; Allmaier et al., 1990; Kulshin et al., 1992; Moran, Lindner, & Walsh, 1997). Kaltashov et al. (1997) overcame the problem of phosphate substitution for lipid A released from *Rhodobacter*, by methylation of the phosphates with diazomethane. MALDI mass spectra were obtained with a reflectron instrument from 2,5-DHB and with an ion trap instrument, using a laser frequency of 226 nm and nicotinic acid (20) as the matrix. The ion trap spectrum contained a prominent B-type glycosidic cleavage ion that was not seen in the reflectron spectrum. However, the ion was seen in the linear spectrum, indicating that it was formed by a rapid ISD cleavage.

Two other ions seen in the ion trap spectrum correspond to cross-ring fragments minus a phosphate moiety; the lengthy two-stage fragmentation reaction probably contributed to the absence of these ions from the normal spectra. The structures of the five fatty acids were established by CID as dodeca- and tetradecanoic acids substituted with one hydroxy or keto group or with a double bond (18).

Deacylation of the esterified hydroxy fatty acids also appears to improve the signal quality of lipid-A-type molecules. This form of the molecule has been used by White et al. (1997) to study enzymatic attachment of Kdo (13) to lipid A from *Haemophilus influenzae*. Metabolite identification (lipid A+Kdo) was achieved by negative ion MALDI mass spectrometry from 2,5-DHB, where strong signals around 1.6 kDa were obtained.

**iv. Intact R-LPS.** The core structure of LPS from *Erwinia carotovora*, a plant pathogen that causes soft rot in vegetables, has been elucidated, with the aid of NMR, FAB, and GC/MS, as a phosphated decasaccharide that contains glucose (4), galactose (6), GlcNAc (8), L-glycero-d-mannoheptose, and Kdo (13) (Fukuoka et al., 1997). Negative ion MALDI spectra were obtained from the intact LPS (Fig. 46) and deacylated molecules from 2,4,6-THAP (44). Ions in the spectrum of the native molecule from 2.5–3.6 kDa reflected heterogeneity in the core (three oligosaccharides were present) and the seven acyl chains. L-glycero-d-mannoheptose has also been found in LPS from *Klebsiella pneumoniae* (Susskind et al., 1998).
Lipooligosaccharides from *Haemophilus influenzae*, *H. ducreyi*, and *Salmonella typhimurium* have been ionized from 2,5-DHB that contains 1-HIQ following removal of the O-linked fatty acids with hydrazine to render them water soluble (Gibson et al., 1997). Under continuous extraction conditions, broad peaks with abundant salt adducts were produced, together with “prompt” (ISD) fragments due to loss of phosphoric acid. More informative spectra were obtained with delayed extraction, particularly in the negative ion mode. At high laser powers several in-source fragments produced by glycosidic cleavages were seen (Fig. 47). Both ISD and PSD yielded fragment ions that gave information on the location of the phosphate and ethanolamine substitution in the lipid-A moiety.

vi. *Free glycans.* These compounds, being free, are much easier to extract than the glycolipids, such as LPS. Nohata, Azuma, & Kurane (1996) have used partial acid hydrolysis to elucidate the structure of a neutral polysaccharide from *Alcangenes latus*, and they found a repeat unit of -2-α-d-Manp(1 → 3)α-L-Fucp-1. MALDI from 2,5-DHB showed polymers with up to 20 repeat units.

b. Glycolipids from mycobacteria

Phenolic glycolipids are located at the surface of mycobacteria, which are organisms that cause diseases such as leprosy (*Mycobacterium leprae*) and tuberculosis (*M. tuberculosis*). They consist of a *p*-alkyl-substituted phenol that is linked, depending on the species, with from one to four partially methylated sugar units. The alkyl chain is typically methyl-branched with about 30 carbon atoms and is substituted with two mycocerosate ester groups that contain 26–34 carbon atoms each (77). These molecules have been ionized by FAB mass spectrometry and by MALDI, using 2,5-DHB as the matrix (Hartmann et al., 1994). Later work (unpublished), using delayed extraction MALDI mass spectrometry from 2,5-DHB or HABA, gave improved resolution and sensitivity to that provided by FAB, and clearly showed heterogeneity in the alkyl chains.

![Phenolic glycolipid from *Mycobacterium kansasii*](image)

77, Phenolic glycolipid from *Mycobacterium kansasii*

vi. Capsular polysaccharides (K-antigens). Analysis of these compounds generally follows methods established for LPS, namely extraction with hot phenol–water and partial acid hydrolysis. Methylation analysis and NMR are used extensively, with MALDI being used for an increasing number of analyses. Using such methods, for example, Forsberg & Reuhs (1997) have determined that the capsular polysaccharide from the nitrogen fixing bacterium *Rhizobium fredii* has a disaccharide repeat unit that contains mannose and Kdo. Degradation of the polysaccharide from *Erwinia stewartii*, the causative agent of wilt in maize, has been achieved by acid hydrolysis and degradation with lithium in ethylenediamine, which removed side-chains that consist of d-Glc-pβ-(1 → 6)-d-Galpz-(1 → 4)-d-GlcApβ. Further analysis relied heavily on GC/MS methylation analysis, NMR, and MALDI. The heptasaccharide repeat unit contained galactose, glucose, and glucuronic acid, and was partly analyzed by permethylation followed by reduction with LiB(Et)₃D to convert the glucuronic acid into deuterium-labeled glucose (Yang, Gray, & Montgomery, 1996b).

c. Other glycolipids

A unique series of glucose polymers esterified with a long-chain fatty acid has recently been found in brain associated with Alzheimer paired helical filaments.
FIGURE 46. Negative ion MALDI mass spectrum (2,4,6-THAP) of native LPS from Erwinia carotovora FERM P-7576. (From Fukuoka et al., 1997, with permission from European Journal of Biochemistry).
FIGURE 47. Negative ion, delayed extraction, linear MALDI mass spectrum of LOS from Haemophilus influenzae strain 276.4. (From Gibson et al., 1997 with permission from the American Society for Mass Spectrometry and Elsevier Science Inc.).
XI. APPLICATIONS OF MALDI MASS SPECTROMETRY TO MONITOR THE PRODUCTS OF CHEMICAL SYNTHESIS

The speed and convenience of MALDI has been used to advantage by several laboratories to monitor products of chemical synthesis that involve carbohydrates. Such products include chitosan oligomers (1 → 4-linked 2-amino-2-deoxy-β-D-glucose) (analyzed as peracetyl derivatives from 2,5-DHB) (Akiyama, Kawazu, & Kobayashi, 1995), biantennary dodecasaccharides of the O-linked type (Niemiä et al., 1995a), pentasaccharide core structures of Chlamydia lipopolysaccharides (Kosma et al., 1994), and synthetic N-linked glycopeptides (Wong et al., 1993; 1994). The use of glycosyl transferases is the most common method for synthesizing specific isomers. Niemelä et al. (1995b), for example, have used an α1 → 3-L-fucosyltransferase from human milk to synthesize biantennary glycans with one fucose residue on a GlcNAc moiety of one of the antennae. Helin et al. (1995) have used enzyme transferase reactions to synthesize large N-linked glycans with the largest compound being an octa-antennary sugar with a measured mass of 6409.5 (from 2,5-DHB). Tetra-antennary octadecameric sugars that carry distal zα(1 → 3)galactose or β(1 → 3)GlcNAc residues, thought to be characteristic of the sperm-binding saccharides of mouse eggs, have been synthesized by Seppo et al. (1995). Hog gastric mucosyl microsomes, which possess β1,6-N-acetylglucosaminyltransferase activity, have been used to synthesize novel branched pentasaccharides of the lacto–globo type. The products were analyzed by MALDI from 2,5-DHB (Natunen et al., 1997).

XII. USE OF MALDI MASS SPECTROMETRY TO MEASURE PROTEIN–CARBOHYDRATE COMPLEXES

The ability of MALDI to measure increases in molecular weight of proteins, when they are conjugated to other molecules, has been utilized in a number of laboratories for studies on protein–protein interactions and of drug binding to proteins. In the carbohydrate field, MALDI has been used to monitor lactose (molecular weight 342) binding to human serum albumin (mass, 66,480). Mass shifts of 7030 and 7880 in two experiments showed a loading value of 21–24 molecules of lactose (Siegel et al., 1993). Loading values for conjugates to monoclonal antibodies measured by MALDI have been shown to parallel those values found by other techniques. (Siegel et al., 1991) Tanaka et al. (1997) have shown that up to 15 steroidal alkaloid glycosides become attached to BSA in an immunostaining technique for solamargine.

XIII. CONCLUSIONS

Over the last decade, MALDI mass spectrometry has proved itself to be a rapid and convenient technique for the analysis of many types of carbohydrate and carbohydrate-containing compounds. Its ability to produce ions from native compounds is attractive in that it avoids the necessity for the derivatization that is required with techniques such as FAB mass spectrometry, thus allowing it to be used for the rapid reaction monitoring and profiling. MALDI is ideally combined, in this context, with exoglycosidase sequencing for rapid determination of glycan chain structure. Although a relatively large number of matrices have been investigated for carbohydrate analysis, only a few have found general acceptance. These matrices include 2,5-DHB, THAP, arabinosazone, and 2,5-DHB combined with either 1-HIQ or 2-hydroxy-5-methoxybenzoic acid. For the analysis of glycopeptides and other glycoconjugates, other matrices such as 4-HCCA are often more appropriate. Although much structural information can be obtained from PSD spectra, the problem of abundant and relatively non-specific internal cleavage ions will probably mean that this method for obtaining fragmentation spectra will not be as useful as techniques, such as CID, which produce more linkage-specific cross-ring fragments. Although MALDI is now well-established in the carbohydrate field, there is still room for development, particularly in the analysis of very small amounts of material. Work during the coming years is likely to see considerable improvements in this area, together with many more applications of the technique to ever more complex compounds.

ABBREVIATIONS:

2-AA 2-aminobenzoic acid
2-AB 2-aminobenzenamid
2-AP 2-aminopyridine
ABEE 4-aminobenzoic acid ethyl ester
AGE advanced glycosylated end-product
AGP α1-acid glycoprotein
AMAC 2-aminoacridone
APTS 1-aminopyrene-3,6,8-trisulfonate
Arg arginine
Asn asparagine
Asp aspartic acid
ATPase adenosine triphosphatase
BOC butoxycarbonyl
BSA bovine serum albumin
CE capillary electrophoresis
Cer ceramide
CHO Chinese hamster ovary
CMBT 5-chloro-2-mercaptobenzothiazole
CNBr cyanogen bromide
CID collision-induced decomposition
CSF cerebrospinal fluid
CZE capillary zone electrophoresis
Da Dalton
HABA 2-(p-hydroxyphenylazo)benzoic acid
DHB dihydroxyacetophenone
DMB diaminomethylenebenzene
DMSO dimethylsulfoxide
Endo-H endoglycosidase-H
Epo erythropoetin
FAB fast atom bombardment
Fuc fucose
FT Fourier transform
FWHM full-width half-maximum
Gal galactose
GC/MS gas chromatography/mass spectrometry
Glc glucose
GlcnAc N-acetylglucosamine
Km Michaelis constant
LC/MS liquid chromatography/mass spectrometry
LPS lipopolysaccharide
Lys lysine
MALDI matrix-assisted laser desorption/ionization
Man mannose
MIP major intrinsic protein
MurNAc N-acetylmuramic acid
MW molecular weight
MS/MS mass spectrometry/mass spectrometry
NeuNAc N-acetylenuraminic acid
NMR nuclear magnetic resonance
NP-40 nonylphenoxo polyethoxy ethanol
PAGE polyacrylamide gel electrophoresis
PD plasma desorption
PMMA phosphomannomutase
PMP 1-phenyl-3-methyl-5-pyrazolone
PNGase protein-N-glycosidase
ppm parts per million
PSD post-source decay
PVDF polyvinylidene difluoride
PVP polyvinylpyrrolidone
RAAM reagent array analysis method
Ser serine
SDS sodium dodecyl sulfate
TFA trifluoroacetic acid
THAP 2,4,6-trihydroxyacetophenone
Thr threonine
TIMP tissue inhibitor of metalloproteinases
TMAPA trimethyl-(p-aminophenyl)amino
TMR tetramethylrhodamine
TOF time-of-flight
tPA tissue plasminogen activator
uPA urokinase plasminogen activator
UV ultraviolet

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