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REVIEW

Perspectives in the glycosciences – matrix-assisted laser desorption/ionization (MALDI) mass spectrometry of carbohydrates*

David J. Harvey‡, Bernhard Küster and Thomas J.P. Naven

Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford, OX1 3QU, UK

The technique of matrix-assisted laser desorption/ionization (MALDI) mass spectrometry is reviewed with emphasis on the best type of matrix to use with carbohydrates, ways to obtain fragmentation and use of the technique for carbohydrate sequencing.

***Keywords*: mass spectrometry, MALDI, fragmentation, post-source decay**

Introduction

Matrix-assisted laser desorption/ionization (MALDI) emerged in the late 1980s as a technique for ionization of large proteins [1] and was first applied to carbohydrates in 1991 by Mock *et al.* [2]. To obtain a signal, the sample, in solution, is mixed with a suitable matrix, allowed to crystallize on the mass spectrometer target and is irradiated with light from a laser. Normally a UV nitrogen laser (337 nm) is used although spectra may also be obtained by the use of IR lasers. The function of the matrix is to dilute the sample and to absorb the laser energy thus allowing a 'soft' ionization of the sample. Although the ionization process is unclear, some form of chemical ionization appears to be involved as samples invariably ionize in the positive ion mode to give MH^+ ions or the products of alkali metal or ammonia addition. In the negative ion mode, proton abstraction is the dominant process. In the case of neutral sugars, MNa^+ ions predominate with no significant formation of protonated species. Unlike fast atom bombardment (FAB) ionization, which requires derivatization (such as permethylation) to produce strong ion currents, MALDI is capable of producing spectra from large underivatized sugars and has a detection limit of about 500 fmole.

As MALDI is a pulsed ionization technique, it is ideally combined with time-of-flight (TOF) analysers which, in their continuous extraction, linear form, give a reasonably cheap, easy to use, bench-top configuration capable of rapid analysis without the need for specialist operators. However, in this form, ionic resolution is low (typically a few hundred) and mass accuracy, without the presence of an internal standard, is poor (0.1% or worse). Furthermore, the peak broadening which accompanies increased laser powers degrades both resolution and mass accuracy, thus necessitating low laser powers and a weak signal for the best mass measurements. Mass accuracy is much better in the presence of an internal standard but the use of such a standard can considerably lengthen the analysis time as its concentration should be adjusted to give a mass peak of equivalent height to that of the sample.

Advances in TOF technology during recent years, however, have now considerably improved this picture. The recent introduction of delayed extraction (DE) [3], also known as time delay focusing, has considerably improved the resolution of linear instruments. TOF instruments fitted with a reflectron also offer increased resolution. When both DE and a reflectron are used, resolutions of around 4000 and, with external calibration, mass accuracies to 0.005% or better are now routine.

Alternatively, spectra may be recorded on a magnetic sector instrument fitted with an array detector to accommodate the pulsed nature of the ion beam [4]. In addition, magnetic instruments can be operated using slow scanning speeds [5] and spectral accumulation so that eventually ions are captured from all regions of the spectrum. Both of these techniques have the disadvantage that the spectral

‡To whom correspondence should be addressed. Tel: +44 1865 275 750; Fax: +44 1865 275216; E-mail: dh@glycob.ox.ac.uk.
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are built up in stages with the result that there are invariably variations in the ion beam intensity during spectral acquisition. This intensity variation produces distortions to the quantitative profile.

Matrices

3-Amino-4-hydroxybenzoic acid was the first matrix to be used for MALDI analysis of carbohydrates and was introduced by Mock *et al.* in 1991 [2]. This matrix was soon replaced by 2,5-dihydrobenzoic acid (2,5-DHB) [6] which remains the matrix of choice for these compounds [7, 8]. It was originally used dissolved in a 9 : 1 ethanol : water (v:v) mixture but we have found that a more consistent crystallization can be achieved by using either acetonitrile or ethanol as the solvent and then recrystallizing the dried matrix:sample spot from ethanol [7, 8]. In this way, the ring of large crystals with a microcrystalline interior that is obtained with the first crystallization is converted into an even film of small crystals. Sample fractionation between the crystal types during the first crystallization is thought to occur and is supported by work on milk sugars where free sugars were observed to concentrate in the central microcrystalline region [9]. Improved sensitivities can be achieved by purifying the commercial 2,5-DHB by recrystallization before use or by using 'super-DHB', a mixture of 2,5-DHB and 2-hydroxy-5-methoxybenzoic acid (10%) [10].

Several other compounds have been investigated as suitable matrices for oligosaccharides. Most isomeric dihydroxybenzoic acids produce signals, but with considerably reduced intensity to that produced by the 2,5-dihydroxy isomer [11]. The reason for this is not clear although it may be speculated that, because 2,5-DHB is the only isomer capable of photochemical decarboxylation to give the stable *p*-benzoquinone, this photochemical reaction is responsible for the ionization. Other matrices such as α-cyano-4-hydroxycinnamic acid and esculetin (6,7-dihydroxycoumarin) also ionize sugars but generally produce weaker signals. Sinapinic acid appears to be ineffective. 3-Aminoquinoline has recently been reported to give superior results to 2,5-DHB for the ionization of plant insulins [12]. In particular, the spectral peaks produced from this matrix appeared sharper than those produced by 2,5-DHB and the baseline was lower. However, to date, the general applicability of this matrix for oligosaccharide analysis has not been determined. Another matrix which has proved to be very satisfactory is 2,5-DHB containing 25% 1-hydroxyisoquinoline [13]. This matrix provided both high resolution and sensitivity, reduced the relative abundance of matrix ions and was remarkably tolerant to the presence of buffers. Recently we have found 2-(*p*-hydroxyphenylazo)benzoic acid (HABA) to be a good matrix, providing sharp peaks at comparatively low laser power.

In order to overcome problems with quantification, mentioned above, with samples requiring long acquisition times, Kolli and Orlando [5] have developed a liquid matrix consisting of a mixture of α-cyano-4-hydroxycinnamic acid and 3-aminoquinoline. Long-lasting ion currents with picomole sensitivity were reported.

Sample preparation

Although MALDI is comparatively tolerant to the presence of contaminants such as buffer salts and detergents [14], these compounds can degrade the strength and quality of the signal. Carbohydrates, however, appear to be more affected by the presence of these contaminating substances than proteins. Consequently, a number of techniques have been used to remove these contaminants thereby considerably improving the quality of the spectra. Drop dialysis using a 500 Da cut-off membrane is a convenient technique for removing salts, whereas a Nafion membrane [15], in its hydrogen form, can be used to remove both salts and protein/peptide contaminants. An on-target desalting technique has recently been reported by Rouse and Vath [16] which involve the mixing of the sample:matrix solution with ion-exchange beads on the laser target, allowing crystallization to occur and then removing the beads with air and a microspatula.

Quantification

The matrices 2,5-DHB and HABA do not show a saturation effect with increasing amounts of sample, unlike 3-amino-4-hydroxybenzoic acid, which does [11]. Thus, the height of the peak in the spectrum is directly proportional to the amount of the sample loaded onto the target. Unlike peptides which show large variations in peak height as a function of structure and proton affinity, most carbohydrates give similar peak heights as the result of an equivalent ionization method (formation of MNa^+ ions) [17]. The exceptions to this are large sugars where a slight fall in peak height with increasing mass is observed, and small sugars with masses less than about 1000 Da where a drop in peak height is observed with reducing molecular weight when spectra are recorded with linear TOF instruments. This signal suppression is thought to be due to detector saturation by matrix peaks and is not seen when spectra are recorded with a magnetic sector instrument.

Maximum signal strengths are seen with single compounds. With mixtures, the ion current is split between the components and weaker signals are observed.

Effects of derivatization

Although not necessary for the MALDI process, permethylation can be used to enhance signal strength by about an order of magnitude. However, complete permethylation is seldom achieved and most investigators prefer to work with the native sugars.

Reducing terminal derivatization has been attempted for increasing signal strength. Naven and Harvey [18] have synthesized several amine-containing derivatives by reductive amination and found increases in sensitivity of about 10-fold. Takao *et al.* [19] have produced derivatives by reductive amination with 4-aminobenzoic acid 2-(diethylaminoethyl) ester and reported a 400-fold increase in sensitivity. MH^+ Rather than MNa^+ was the major ion. However, the popular 2-aminobenzamide (2-AB) derivatives [20] which are used to introduce a fluorophore for HPLC detection, have little or no effect on sensitivity. The introduction of a constitutive cationic charge has been investigated with the aim of avoiding the need to ionize the sugar during the MALDI process. The best results were obtained by reacting the sugars with Girard's reagent T [18]. This method also offers the advantage, over reductive amination, that sample clean-up is not necessary prior to MALDI analysis because sodium salts are not formed. Sensitivity increases were reported to be in the region of 10-fold. Ashton *et al.* [21] have reacted sugars with 1-phenyl-3-methylpyrazoline-5-one to give a product containing two pyrazoline molecules and have used the derivatives for detection of carbohydrates in humanized IgG antibodies expressed in CHO cells. Increases in sensitivity of up to 100-fold by use of a charged derivative in MALDI were recently reported by Whittal *et al.* [22] for examination of oligosaccharides in serum. Derivatization was achieved with tetramethylrhodamine with linkage through a methoxycarbonyloctyl chain [23].

Use of MALDI for carbohydrate profiling and sequence determination

The ability of MALDI to produce single MNa^+ ions whose abundance reflects the quantity of sugar on the target makes the technique ideal for mixture analysis. Rapid profiles can be obtained from sugars released from glycoproteins as shown by several authors [7, 24]. However, as only mass measurement is involved, it is not possible to separate isomers; this must be done by chromatography. Because of the comparatively small number of isobaric monosaccharides comprising the majority of N- and O-linked sugars, the mass measurement leads directly to a composition in terms of these isobaric monosaccharides.

Further structural information can then be obtained by using the MALDI mass spectrometer as a detection system for conventional sequencing techniques using exoglycosidases. This technique was first demonstrated by Sutton *et al.* [25] for the glycoprotein recombinant human tissue inhibitor of metalloproteinase. Tryptic peptides were obtained from the glycoprotein and examined by MALDI using α-cyano-4-hydroxycinnamic acid as the matrix. Although this matrix tends to produce stronger signals with glycopeptides than does 2,5-DHB with native sugars, the glycopeptides had significantly higher masses than the sugars themselves, a property which could lead to problems on mass spectrometers with low resolution, Sugars released from human IgG [7] and glycoproteins from the human parotid gland [26] have also been investigated in this way using 2,5-DHB as the matrix. The use of MALDI for this analysis has the advantages over gel filtration or HPLC-based detection systems of speed, higher resolution, the production of an isobaric monosaccharide composition, and the ability to handle samples without derivatization. Resolution of constituents is also considerably enhanced providing that they are of different mass.

Several methods have recently appeared for performing sequential exoglycosidase digestions on the micro-scale specifically for MALDI analysis. In the method reported by Küster *et al.* [27] the enzyme digestion was performed on the mass spectrometer target with sample clean-up being achieved by use of drop dialysis and Nafion membrane treatment. Sequencing of N-linked sugars could be achieved at the 100 pmole level. Microdigestion and on-target clean-up was used by Rouse and Vath [16] for sequencing at the 120 pmole level.

Anionic sugars

Sialylated sugars generally give weak positive ion signals but relatively strong spectra in the negative ion mode where the $[M-H]^-$ ion dominates the spectrum [7]. Depending on the composition of the sample, variable amounts of sodium salt formation can occur giving multiple peaks for polysialylated sugars. 2,5-DHB has so far proved to be the most satisfactory matrix but HABA (unpublished) and 2,4,6-trihydroxyacetophenone are also satisfactory [28]. 'Hotter' matrices such as α-cyano-4-hydroxycinnamic acid catalyse considerable loss of sialic acid. This loss is also apparent with 2,5-DHB at higher laser powers and particularly with spectra recorded with magnetic sector instruments. Powell *et al.* [29] have overcome these problems by forming methyl ester derivatives of sialic acids with methyl iodide (uncatalysed). The sialic acid was first converted into its sodium salt by passage down a short AG-50 ion exchange column which had previously been equilibrated with sodium hydroxide. The salt was dried, dissolved in dry dimethylsulfoxide (DMSO), and reacted with methyl iodide for 2 h at room temperature. After removal of the methyl iodide with a stream of nitrogen the sample, in DMSO, was applied to the MALDI target and, after evaporation of the solvent, was resuspended in the MALDI matrix (2,5-DHB). The resulting spectra gave strong positive ion signals, did not contain peaks produced by sodium salts and did not show loss of sialic acid.

Fragmentation

In order to make full use of MALDI mass spectrometry for structural analysis, fragment ions as well as molecular ions

should be examined. Although fragment ions are rarely observed from neutral sugars when examined with a linear TOF system, fragmentation does occur and can be observed directly if ion retention in the ion source is long enough for fragmentation to occur prior to ion extraction. Such fragmentation can, therefore, be observed with TOF instruments fitted with delayed extraction ion sources. Fragmentation which occurs in the flight tube as the result of post-source decay (PSD) and which cannot be observed with a linear TOF instrument, can, nevertheless be recorded with an instrument fitted with a reflectron. Alternatively, fragmentation can be induced by collisions with a gas held in a collision cell and observed either with a reflectron or with an orthogonal-TOF analyser.

Fragmentation can occur either between the constituent sugar rings or across the rings. The former, glycosidic, fragmentation usually predominates at low energy and can produce ions that retain the charge on either the reducing or non-reducing fragment. In addition, fragmentation can occur from both ends of the molecule to produce the so-called 'internal' fragment ions. Fragmentation occurs with transfer of a hydrogen atom in order to ensure elimination of a neutral molecule. In the nomenclature proposed by Domon and Costello [30], the ions produced by glycosidic cleavage and which retain the charge on the reducing terminus are labelled as Y or Z ions depending on whether the cleavage results in retention or loss of the linking oxygen atom respectively. The complementary ions retaining charge at the non-reducing terminus are labelled as C and B. The cross-ring cleavages are named X and A and follow the above scheme with superscript numbers to indicate the bonds cleaved.

In-source decay (ISD) fragmentation

Increasing the laser power on a linear TOF instrument considerably broadens the peaks and is not conducive to efficient fragment ion detection. However, with the increased energy resolution provided by a magnetic sector instrument, this in-source fragmentation can be observed to give ions of up to about 10% of the relative abundance of the molecular ion [7, 31]. However, interference with matrix ions below about m/z 500 restricts the use of this technique to masses above this value. Most ions are the products of fast, low energy processes and consist mainly of ions produced by glycosidic cleavage. With N-linked sugars, a B-type cleavage of the chitobiose core with loss of the reducing-terminal GlcNAc is usually the dominant ion. B-cleavages adjacent to non-reducing GlcNAc residues of the antennae probably also produce such ions, as in fast atom bombardment (FAB) spectra but, in our experiments, are not observed because of matrix ion interference. Some sequence information on underivatized sugars can be gained from Y-ions but the main problem stems from the relatively high abundance of internal glycosidic cleavage products which yield ions of the same mass from various areas of the molecule. In order to differentiate these ions, permethylation should be performed [32] to introduce a mass difference between the two ion types.

A few cross-ring cleavages are observed in ISD spectra but are mainly confined to the reducing terminal ring where two abundant ions, ^{0,2}A and ^{2,4}A are produced. The mass of the ^{2,4}A ion allows the linkage of a core fucose residue (3 or 6) to be established.

Although the technique can give abundant fragments, the spectra are somewhat dependent on the condition of the ion source and, with a clean source, may not be observed. Also, because ion gating is not used, examined compounds should be pure rather than components of mixtures.

Delayed extraction (DE) (time-delay focusing) fragmentation

The ability of delayed extraction to retain the ions in the ion source for a few hundred nsec prior to acceleration enables some of them to fragment and to be focused with a linear TOF instrument. In general, the ions are similar to those seen in ISD spectra (unpublished data) and their relative abundance increases with the delay (we have observed them at delay times of up to 3 μsec). As with ISD spectra, the absence of ion gating restricts the use of this fragmentation mode to single compounds.

Post-source decay (PSD) spectra

Fragment ions in these spectra are formed between the ion source and the reflectron and are subsequently focused by the reflectron. They were first reported from glycopeptides by Huberty *et al.* [33] and from native sugars by Spengler *et al.* [34] who found a range of glycosidic, internal and some cross-ring ions. Similar spectra have since been reported by others [16, 31, 35]. Spectra that we have recorded [31 and unpublished data] appear to consist mainly of glycosidic and internal fragment ions. There appears to be an inverse correlation between the relative abundance of these ions and the delay time in DE instruments because, clearly, only molecular ions that have survived the acceleration process will fragment between the ion source and reflectron and will subsequently be observed in the PSD spectra; fragment ions leaving the ion source will be gated out. With a given ratio between the acceleration and reflectron voltages, only ions from a limited mass range will be focused at the detector and, thus, in order to acquire a complete spectrum, the ratio of the voltages must be altered in steps and the complete spectrum assembled from the individual segments. This stepwise scanning method presents problems for accurate reproduction of the spectra as the ion currents may well differ between segments. On the other hand, PSD is better than DE or ISD for mixture analysis as parent ion selection is involved, albeit with a rather broad ($\sim$20 Da) mass window.

The problem of differentiating glycosidic from internal cleavage ions in PSD spectra has recently been investigated by Lemoine *et al.* [36]. Permethylation was found to be unsatisfactory because of the weak nature of the resulting spectra. Reduction in glycosidic ion intensity can be ascribed to the absence of hydroxylic hydrogen atoms which have been shown to be the main source of the migrating hydrogens in the formation of these ions [37]. Peracetylation was more satisfactory, producing spectra with both B and Y ion series present, with the B-ions dominating. However, only one mass unit differentiated acetylated hexose from acetylated hexosamines thus complicating the identification of HexNAc groups in the original sugar. In order to overcome this problem, perdeuterioacetylation was recommended. Reducing-terminal derivatization by reductive amination as aminophenyl or 2-AB derivatives again produced spectra containing only MNa^+ ions and glycosidic cleavages although the fragmentation pattern was somewhat simpler than that observed with the free sugars. Increasing the basicity of the derivative enabled MH^+ ions to be observed from the crystalline ring of 2,5-DHB in spite of the fact that the amorphous centre of the target still yielded MNa^+ ions. Fragmentation of the MH^+ species yielded mainly Y ions and gave the most informative spectra.

Although PSD spectra consist mainly of glycosidic fragment ions, some linkage information has, nevertheless, been obtained from the relative abundance of these ions. Thus, Rouse *et al.* [38] were able to distinguish isomers of triantennary N-linked sugars by a difference in ratio between the ions produced by loss of either of the two antennae.

Fragmentation by high energy collisional activation

High energy (800 eV) fragmentation spectra from MALDI-generated parent ions have been obtained by ionization on a magnetic sector mass spectrometer with product ions being observed with an orthogonal-TOF analyser [31]. Because of the pulsed nature of the ion source it was important to synchronize the push-out pulse of the TOF analyser with the laser pulse so that this pulse coincided with the arrival time of the ions at the TOF analyser. This method of fragmentation offers several advantages over the other modes: firstly, parent ion selection can be performed under high resolution conditions thus making the method ideal for mixture analysis; secondly, as no interference from matrix ions is observed, fragment ions can be observed over the entire mass range; thirdly, the high energy collisions induce much cross-ring fragmentation which yields information on branching and linkage. Glycosidic and internal fragments are also present but, often, the cross-ring cleavages and, in particular, the $^{1,5}X$ cleavages predominate. Fragmentation patterns from N-linked sugars are similar to those observed under high energy collisions from FAB spectra [39–41] but unlike the latter case, no derivatization is needed to produce a strong signal. In common with FAB and FAB–collision-induced decomposition (CID) spectra, B-cleavages adjacent to HexNAc residues are abundant. Thus, neutral bi-, tri- and tetraantennary N-linked sugars yield an ion at m/z 388 ($[Gal\text{-}GlcNAc\text{-}Na]^+$) as the most abundant fragment.

Derivatization or the presence of a peptide fragment at the reducing terminus alters the fragmentation [42]. The most complete series of B and Y ions is observed from the native sugars whereas cross-ring cleavages are most abundant in the spectra of the glycopeptides. The least informative spectra are observed from derivatives such as those incorporating 2-AB and which are formed by reductive amination. The open ring of the reducing-terminal sugar, for example, prevents formation of the $^{0,2}A$ and $^{2,4}A$ ions which allow core fucose isomers to be differentiated.

The smallest amount of material that can be fragmented and observed with the orthogonal-TOF has not been determined although strong spectra have been obtained from about 10 pmole of sample loaded onto the target. A possible disadvantage of the technique is that ion yields from each laser shot are low and spectra must be acquired from the accumulated fragments of many laser shots. Spectral acquisition times are limited by the firing rate of the laser, typically 10–20 shots/sec giving a spectral acquisition time of 10–20 mins.

Conclusions

MALDI mass spectrometry has been shown to be an excellent technique for analysis of underivatized sugars. Modern instruments can achieve high resolution and a routine mass accuracy of better than 0.005%. Fragmentation methods are now becoming available for more detailed examination of the structure of complex sugars and in the next few years the method should become one of the major tools for carbohydrate analysis.

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