Glycoconjugate Journal (1992) **9**:1–12

GLYCOPINION

Editor: RAYMOND A. DWEK

Structure determination occupies a key position in modern glycobiology and has been greatly facilitated in recent years by developments in enzymology, chromatography, NMR and mass spectrometry (MS). Structural analysis of carbohydrates presents greater problems than that of other biomolecules such as oligonucleotides or proteins because of the complexities introduced by branching, linkage and anomericity. Consequently, it has not yet reached the advanced, and sometimes automated, state achieved in these other areas. Nevertheless, considerable progress has been made in recent years, although no single (MS) technique can yet provide all the necessary information.

High sensitivity is an essential requirement for any method of structural analysis as the quantities of oligosaccharide available are generally low. Biological techniques, such as selective cleavages of radiolabelled sugars with specific exoglycosidases, possess the necessary sensitivity and have been the preferred methods to date. However, even though some progress is being made towards automation, these classical sequencing techniques are frequently tedious and time consuming. Physical and chemical techniques, on the other hand, are generally more rapid and may eventually provide the answer to the problem of automating the structural determination of oligosaccharides. Mass spectrometry appears to be particularly promising in this respect as it possesses the ability to yield considerable structural information on very low (pmol–fmol range) concentrations of sample.

This article, by David Harvey, explores the use of both classical and modern mass spectrometry as applied to oligosaccharide analysis and addresses such questions as:

- Will mass spectrometry soon become the most powerful physical analytical technique in oligosaccharide analysis?
- Which mass spectrometric techniques are suitable for on-line coupling to affinity chromatography and capillary electrophoresis and, conversely, which chromatographic techniques are most appropriate for coupling to mass spectrometry?
- When is derivatization necessary?
- Can fragmentation be controlled?
- Can mass spectrometry be used to fingerprint glycoform libraries?
- How sensitive are the different techniques?
- Which technique is best for monosaccharides and which for glycoconjugates?

MINI-REVIEW

The role of mass spectrometry in glycobiology

DAVID J. HARVEY

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

Mass spectrometry originated early in this century with the classic work of scientists such as Aston and Thompson [1], and led to the discovery of stable isotopes, thus allowing the nonintegral masses of the elements to be rationalized. However, it was several decades after the initial discovery before the technique was applied to the structural analysis of organic molecules. Even then, it was largely restricted to analysis of hydrocarbons from petroleum, mainly because of their volatile and thermally stable properties. The coupling of mass spectrometry with gas chromatography (GC) [2], the introduction of chemical derivatization for stabilization of thermally unstable molecules, and the development of efficient data processing systems were the key steps in the extension of the technique to biomolecule analysis. GC/MS proved to be particularly useful and is now a major technique in areas such as lipid and drug metabolite analysis. The coupling of liquid chromatographs and the extension of the mass range accessible by mass

0282-0080

spectrometry have been the more recent advances and the technique is now an indispensable tool for any laboratory engaged in bio-organic structure determination.

Techniques and instrumentation

At first sight, the number of types of mass spectrometer and mass spectrometric techniques is bewildering. However, all techniques rely essentially on the production of ions in the gas phase, the decomposition of these ions to structurally informative fragment ions, and the separation and mass measurement of these ions by a combination of electric and magnetic fields. The complexities arise from the types of inlet system used to introduce the samples, the ionization technique, the method used for ion separation and, to a lesser extent, the method used to detect the ions. Inlet systems and ionization are very much compound-dependent, and methods appropriate to oligosaccharide analysis are discussed below. On the other hand, the system used for ion separation is less so but, as will be evident from the following discussion, certain methods are more appropriately combined with specific methods of ion production.

Types of mass spectrometer

Charged particles move in a magnetic field along circular paths, whose radius is a function of the magnetic field, ion velocity and mass/charge (m/z) ratio. Thus, for singly-charged ions accelerated to a constant velocity, the m/z value is directly proportional to the magnetic field. If the magnet is scanned, a mass spectrum is obtained as a series of ion peaks whose m/z value is a direct function of arrival time at the detector. Masses as high as several kDa can be observed before the instrument resolution becomes limiting. For higher mass analysis and, more particularly for higher resolution, an electrostatic energy filter can also be used, either before or after the magnet in the so-called double-focusing instruments. Mass accuracies to 1 ppm are attainable allowing elemental compositions to be determined. Quadrupole mass filters work by allowing the ions to travel between four rods carrying alternating and direct current fields; only ions of a specific m/z value are stable under a given set of conditions and are transmitted to the detector. The instruments have a linear mass scale, can be scanned very rapidly but only have a limited mass range (about 5 kDa). Time-of-flight instruments, on the other hand, have an almost unlimited mass range as they work simply by timing the arrival of ions produced from a pulsed ion source, placed at the end of an evacuated tube. This time is a function of the m/z value. The ion trap is a device not unlike the quadrupole, in which ions are held in orbits within a donut-shaped electrode positioned between two end caps. By a suitable combination of electric fields, ions can be ejected and detected. Although originally designed with properties similar to those of quadrupoles, recent developments have enabled ions of very high mass to be detected. In addition, resolution can be comparable to that of double-focusing magnetic instruments.

Electron-impact ionization

Electron-Impact (EI) mass spectrometry, the classical technique in which ion production is effected by bombardment of the sample with an energetic beam of electrons, has been applied to the analysis of carbohydrates for nearly 30 years. When combined with gas chromatography, the technique is particularly valuable. Quite obviously, even sugars as small as monosaccharides are too thermally unstable to be volatilized in their native state but, when derivatized, usually in the form of methyl, acetyl or trimethylsilyl (TMS) ethers [3], most molecules are reasonably stable at temperatures below 400°C, allowing sugars as complex as undecasaccharides to be examined by GC/MS [4]. However, most reported work with this technique is concerned with monosaccharide analysis. Methyl ethers, although more difficult to prepare than either of the other two derivatives, are generally preferred, as the mass increment attending derivatization is only 14 units for each derivatized functional group compared with 42 and 72 for the acetyl and TMS derivatives respectively. TMS derivatives, however, confer good GLC characteristics [3, 5] but can produce several GLC peaks for reducing sugars as the result of anomeric isomerization and formation of both furanose and pyranose rings, a situation avoided by the use of alditol acetate derivatives [6]. Nevertheless, these derivatives give very interesting and informative EI mass spectra [7] that can be used to fingerprint monosaccharides (Fig. 1). Major fragment ions appear at relatively low mass such as m/z 73 $\{[Si(CH_3)_3]^+\}$, 147 $\{[(CH_3)_3Si\text{-}O{=}Si(CH_3)_2]^+\}$, 191 $\{[(CH_3)_3Si\text{-}O\text{-}CH{=}O\text{-}Si(CH_3)_3]^+\}$, 204 $\{[(CH_3)_3Si\text{-}O\text{-}CH{=}CH\text{-}O\text{-}Si(CH_3)_3]^+\}$ and 217 $\{[(CH_3)_3\text{-}Si\text{-}O{=}CH\text{-}CH{=}CH\text{-}O\text{-}Si(CH_3)_3]^+\}$ and, although it is often difficult to determine the exact source of a particular fragment, their relative abundances are very diagnostic. Similar ions are present in the spectra of the TMS derivatives of disaccharides [8] and inositols [9]; however, the latter compounds can be distinguished from the hexoses by the presence of an abundant ion (100% relative abundance in five of the eight isomers) at m/z 318 $\{[(CH_3)_3Si\text{-}O\text{-}CH{=}C(OS{:}CH_3)_3\text{-}CH{=}CH\text{-}O\text{-}Si(CH_3)_3]^+\}$. TMS derivatives have also been successfully applied to the analysis of such involatile molecules as sugar phosphates [10, 11]; TMS-phosphate re-arrangement ions dominate the spectra with the abundant ion at m/z 299 $\{[((CH_3)_3SiO)_3P\text{-}OH]^+\}$ being particularly diagnostic. Sulphates, unfortunately, cannot be analysed in this manner as the derivatives are unstable.

Linkage analysis using permethylation and GC/MS

Although mass spectrometry has moved on from the days when EI was the only practical way to ionize molecules,

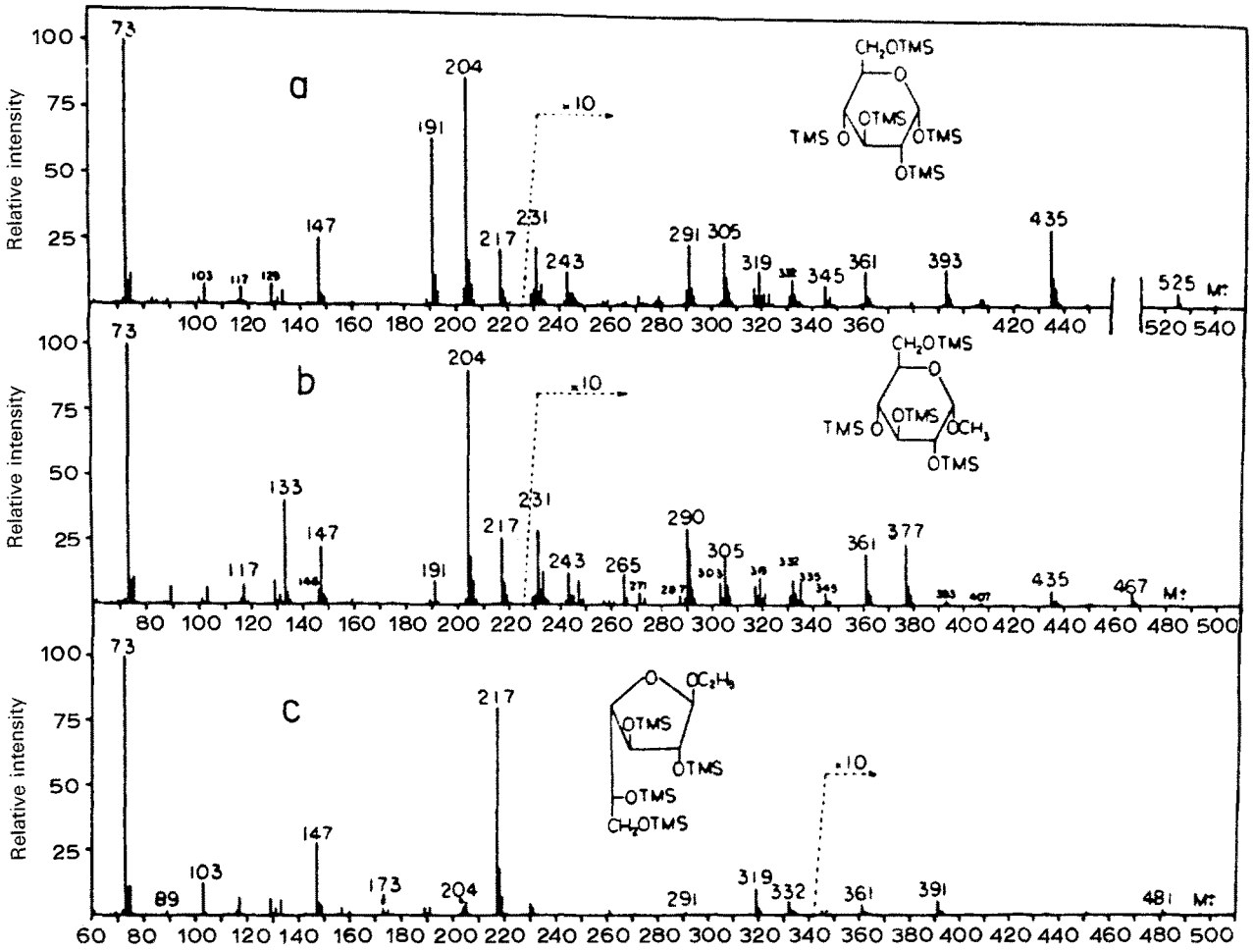

Figure 1. Electron-impact (70 eV) mass spectra of three TMS-sugars showing the abundant, diagnostic fragment ions at low mass. Note the absence of the molecular ion. (From [7]).

this ionization method, combined with GC/MS, still finds a major application in linkage analysis [12]. This usually involves a four-stage derivatization reaction as follows: The oligosaccharide is first permethylated to protect all free hydroxy groups. It is then hydrolysed under acidic conditions to release the monosaccharides which are then reduced to their corresponding alditols. Finally the mixture of alditols is acetylated to derivatize all hydroxy groups generated at the hydrolysis and reduction stages; these comprise the oxygen atoms involved in linkage (Fig. 2). The mixture of permethylated alditol acetates is then examined by GC/MS (Fig. 3), and the compounds identified by both retention time and comparison of their mass spectra with a library of reference compounds. Although widely used, this method is unable to differentiate between all possible linkages. Also, some sugars are decomposed by the acid hydrolysis step and, in addition, the rather complex chemical processing is incompatible with high sensitivity, low nmol quantities are usually needed. With the development of mass spectrometric techniques capable of ionising much larger molecules, we may well see this method becoming less widely used in the near future.

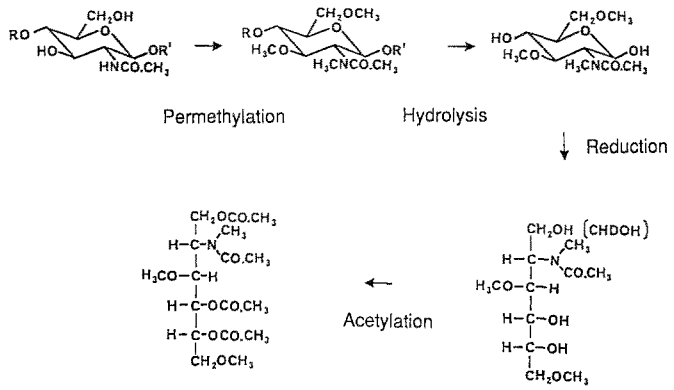

Figure 2. Stages in linkage analysis by GC/MS using permethylated alditol acetates. In the final product, acetate groups occupy the sites involved in bonding whereas methoxy groups are present where the original molecule had free hydroxy moieties. R and R' represent other sugar residues.

In-beam EI

Sample volatility is the limiting factor for EI ionization under thermal conditions, but somewhat improved results can be obtained using 'in-beam EI', a technique in which

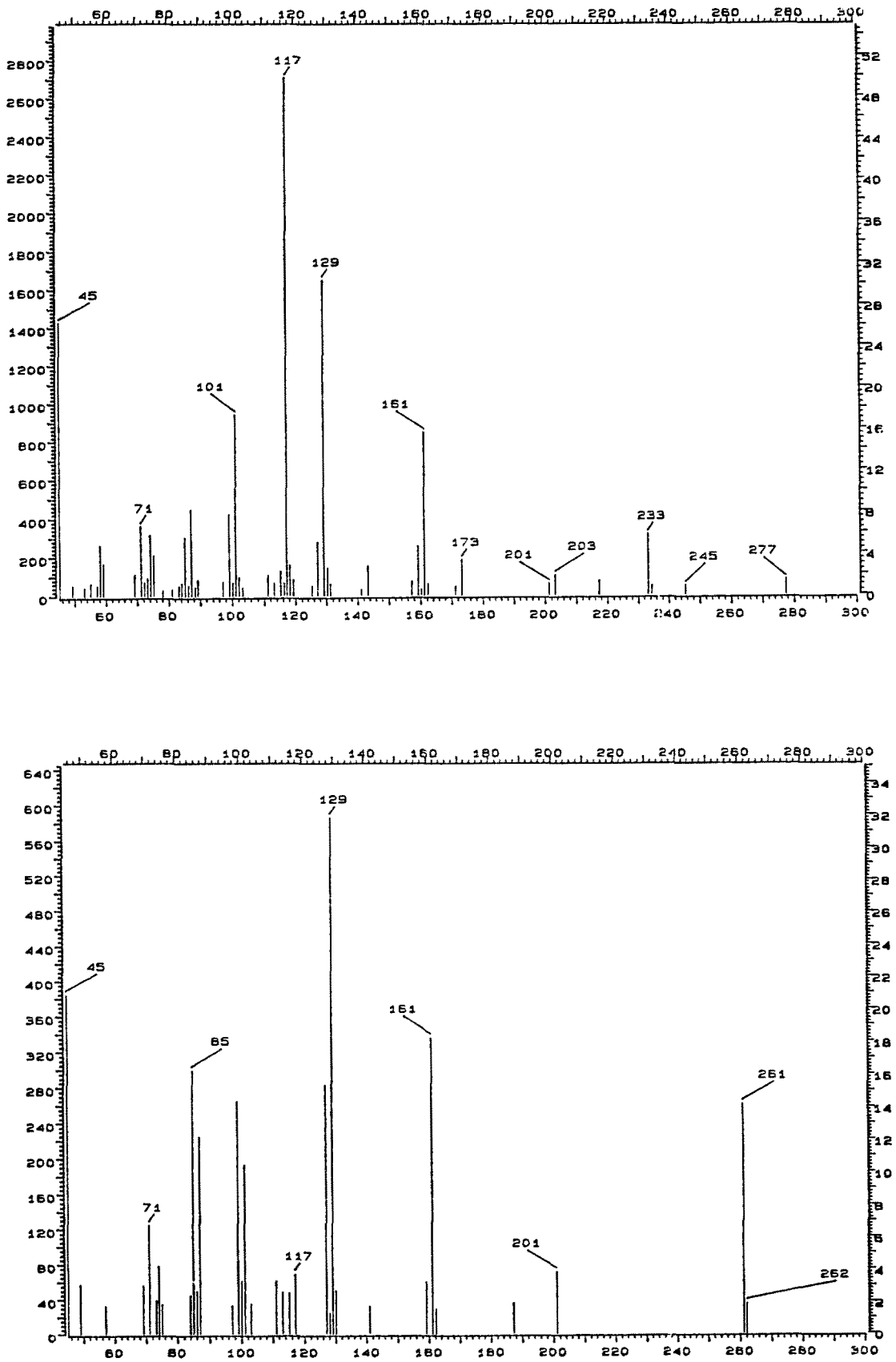

Figure 3. Mass spectra (70 eV) of two typical methylated alditol acetates. Top -1,3,5-tri-O-acetyl-2,4,6-trimethylhexitol and bottom -1,2,3,5-tetra-O-acetyl-4,6-dimethylhexitol.

the sample, on a direct insertion probe, is inserted directly into the beam of ionising electrons. Diagnostic fragment ions of reasonably large sugars (up to 3 kDa [13]) have been obtained in this way. Glycolipids appear to give particularly good results after reduction and permethylation.

Chemical ionization

One of the major disadvantages of EI ionization is that it produces very energetic ions that readily fragment. Although this is valuable for structural studies, it often results in the spectrum containing little, if any, ions at the mass of the molecular ion. This ion, for obvious reasons, is the most important one in the spectrum and its absence from EI spectra is a common feature of the spectra of sugars. To overcome this problem, a number of 'softer' ionization techniques have been developed. Chemical ionization (CI) is probably the most well known. In this method, ionization is effected by collision of sample molecules with a preformed plasma of reactant ions, generated in a high pressure source from gases such as methane, isobutane or ammonia. Fragment ions are sometimes present in the resulting spectra, but the value of the technique lies mainly in enhancing the relative abundance of the molecular ion. This ion is usually produced by hydrogen transfer to give species such as $[M + H]^+$, $[M - H]^-$, commonly referred to as 'pseudo-molecular' ions. Like EI, the method can be used in combination with gas chromatography or in the 'direct' or 'desorption' mode by introducing the sample, deposited on a direct insertion probe, directly into the CI plasma [14]. Ammonia appears to be the most effective reagent gas and sensitivities of the order of three to five times those obtained by fast atom bombardment have been recorded with derivatized sugars with masses up to 3 kDa [15]. The combination of CI with supercritical fluid separation appears particularly attractive for the analysis of mixtures [16].

Fast atom bombardment

One of the most significant events in mass spectrometry over the last decade has been the extension of the accessible mass range and the development of techniques for the ionization of large biomolecules without decomposition. The introduction of fast atom bombardment (FAB) mass spectrometry [17] is generally regarded as marking the start of this trend even though methods such as field desorption (FD) and plasma desorption (PD) had been introduced some years earlier. In FAB-MS, and its many variants, the sample is dissolved in a liquid matrix, usually glycerol, and bombarded with a beam of neutral atoms or ions. This causes desorption of the sample from the liquid surface, usually, as is the case with CI, in the form of $[M + H]^+$ or $[M - H]^-$ ions in the positive and negative modes respectively. In addition, these ions are frequently accompanied by a wealth of structurally diagnostic fragment ions. In the latter respect, the method differs from FD ionization which produces mainly molecular ions with little fragmentation. Unlike the earlier techniques such as EI, charged molecules generally give very good spectra under FAB conditions thus providing the possibility of adding a charge during derivatization to aid the desorption of 'difficult' molecules.

Carbohydrates generally give good FAB spectra [18, 19], with the best results being obtained with derivatized samples. Derivatization, usually by methylation or acetylation, has the additional advantage of greatly improving the sensitivity, often by as much as two orders of magnitude [20]. Detection limits of the order of 1 pmol can be obtained, this usually being governed by the high background arising from concomitant ionization of the matrix. The molecular ion in FAB spectra is usually of relatively high abundance and the major fragment ions, which tend to be formed predominantly by cleavage between the glycosidic units, provide a wealth of sequence information (Fig. 4). Cleavage adjacent to HexNAc residues are particularly abundant. Derivatization is usually necessary for successful sequence determination to prevent ambiguities created by hydrogen migrations from the hydroxy groups. The choice of matrix and ion extraction mode (positive or negative) also appears to be critical and also somewhat compound specific. Matrices such as thioglycerol and triethanolamine have given good results. Information on branching is also present in the spectra, usually in the form of variations in ion abundance. Linkage information is less apparent although this can sometimes be introduced by derivatization techniques such as periodate oxidation [21]. This causes oxidative cleavage when *cis*-hydroxy groups are present, thus introducing mass differences that reflect linkage. Unlike EI mass spectrometry, FAB can be used to analyse sulphates [22] as these compounds give strong signals, particularly in the negative ion mode.

Flowing-FAB

For the analysis of oligosaccharide mixtures it is preferable to separate the constituents either off-line or on-line, prior to analysis, or to use MS/MS techniques. Otherwise, it will not be possible to determine the relationship between the several molecular ions and their respective fragments. On-line examination has been achieved using a direct coupling of liquid chromatographs to the FAB probe and permethylated carbohydrates have been examined at the 10 pmol level [23]. A potential advantage of this method of sample introduction is that a very much lower concentration of FAB matrix can be used thus giving a considerably improved signal/noise (S/N) ratio.

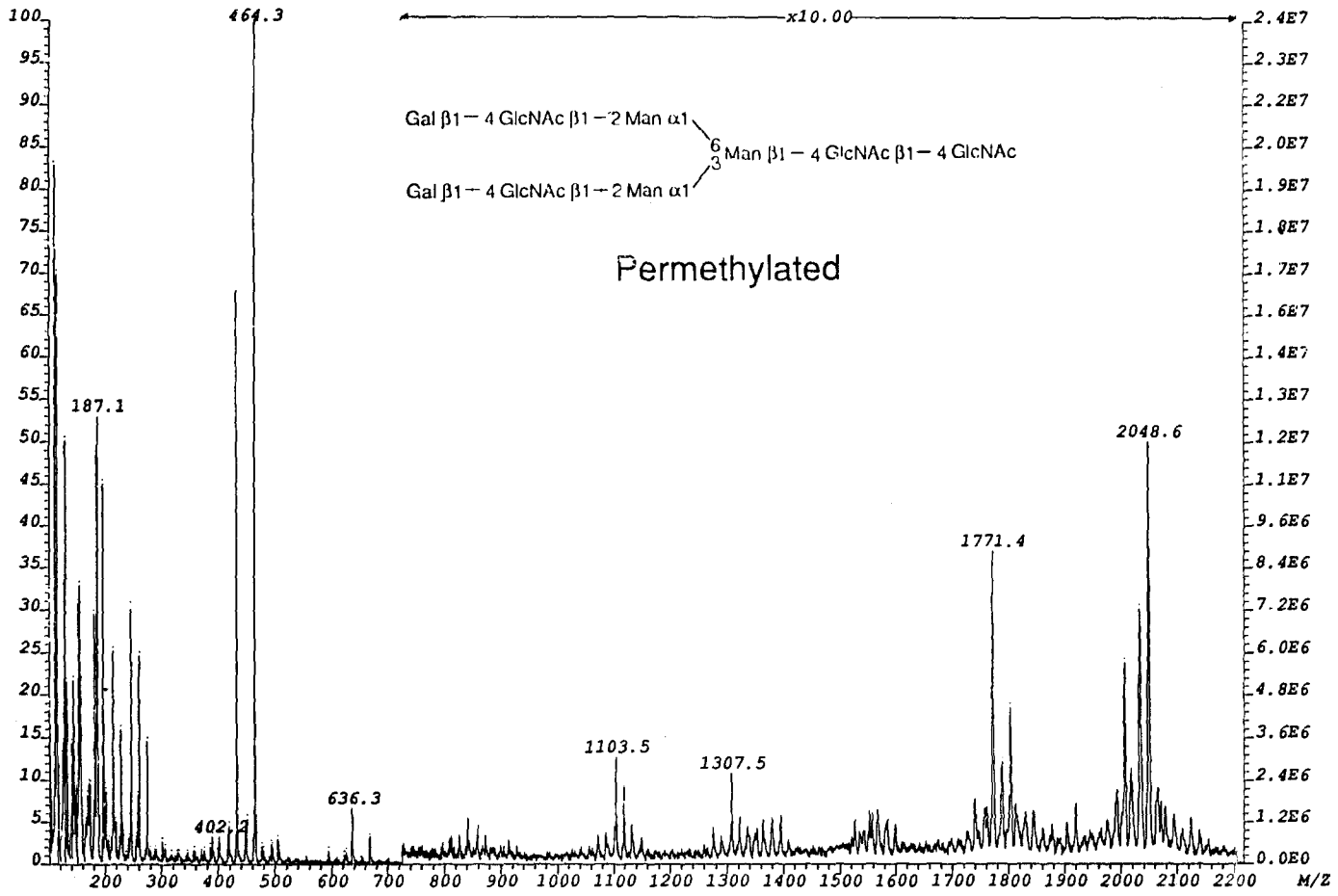

Figure 4. FAB Mass spectrum of a permethylated oligosaccharide showing the protonated molecular ion and extensive fragmentation. The abundant ion at *m/z* 1771 is the result of loss of the GlcNAc from the reducing terminus and the ion at *m/z* 464 represents one of the Gal-GlcNAc fragments from the other end of the molecule. Both fragments are formed by cleavage adjacent to a GlcNAc residue. The spectrum was obtained from 100 pmol of sugar suspended in a 1:1 glycerol:thioglycerol matrix and bombarded with caesium ions.

MS/MS techniques

To obtain definitive information from a mixed FAB spectrum, various MS/MS techniques can be used. Here, a multianalyser mass spectrometer is used, firstly to select one of the parent ions and, secondly to analyse fragment ions generated specifically from this ion following collisional activation in a gas collision cell. This not only ensures that all detected fragment ions arise only from the selected parent ion, but the method also produces a much 'cleaner' spectrum as the substantial background signal from the ionized matrix is also eliminated. Several MS/MS techniques are possible, the simplest of which involves scanning two of the fields of a double-focussing mass spectrometer in various ways, depending on what information is required. The main disadvantage of this approach is that either daughter or parent ion resolution is low, depending on the scan function used. To achieve good resolution of both types of ion, a further analyser, either quadrupole or magnetic can be used to examine the daughter ions of a parent ion selected with the first stage of the instrument. The advantages presented by these techniques for mixture analysis are considerable even though the instrumentation is expensive. Sugars appear to give very informative MS/MS data using collisional activation of the selected parent ions (Fig. 5) [24, 25] and compounds with masses of up to 3 kDa have been examined. However, this by no means appears to be the upper limit.

Another major advantage of MS/MS methods appears to be the increased ease with which positional isomerization and linkage can be determined compared with use of simple FAB mass spectrometry [26]. This appears to be related to bond stability with the more hindered bonds fragmenting more readily [27]. Kovacik *et al.* [28], for example, have reported that, although no linkage information is available in the FAB spectra of a series of xylans, this information is readily apparent from the MS/MS data following collisional activation. 1–4-Linkages have been identified in the FAB/MS/MS spectra of acetylated oligosaccharides by

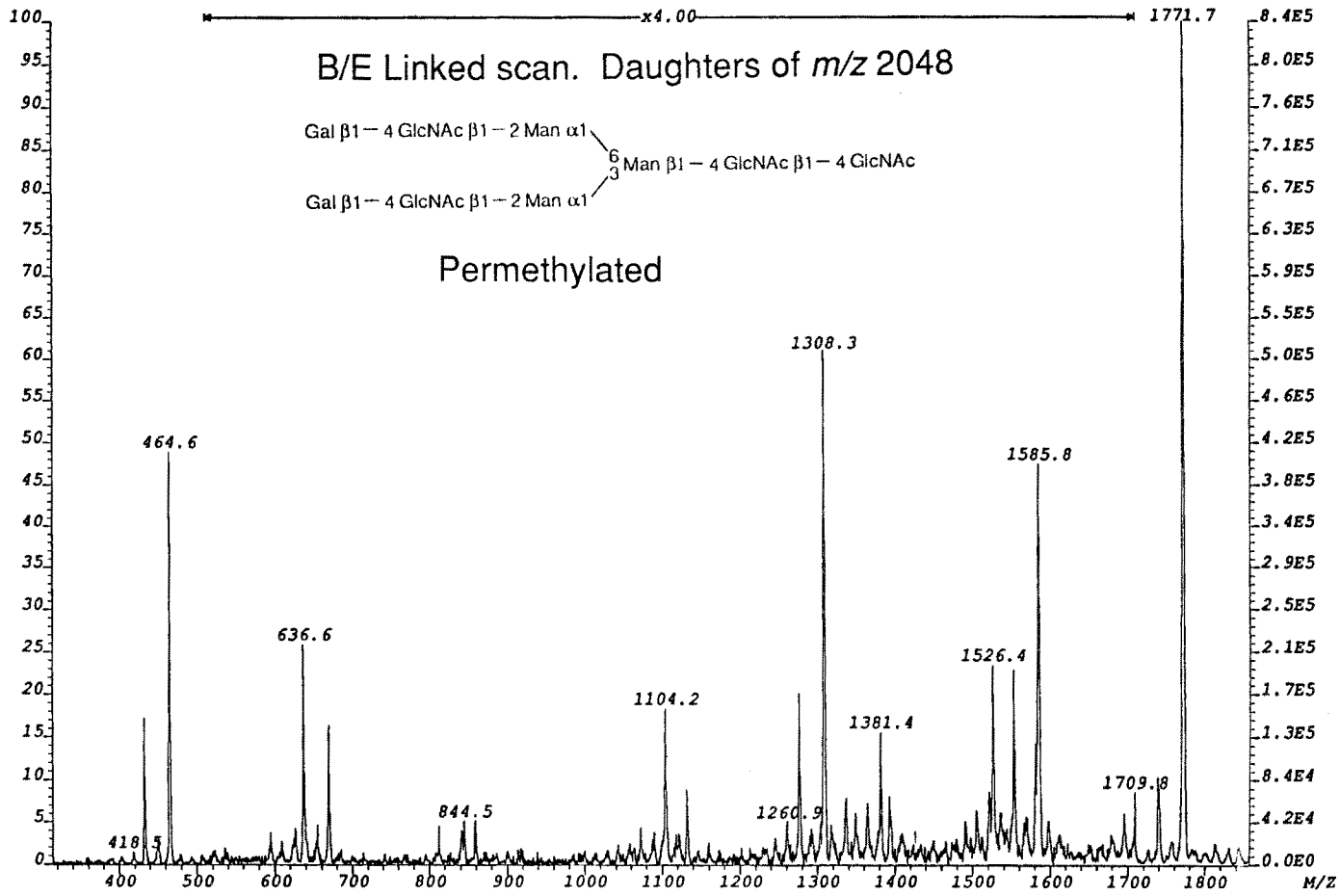

Figure 5. B/E linked-scan spectrum of the permethylated oligosaccharide shown in Fig. 4. The magnetic and electrostatic fields of a double focussing mass spectrometer were scanned with a constant ratio so that the spectrum contains only daughter ions of the parent ion.

observing changes in the relative abundances of fragment ions in the region of the spectrum containing disaccharide residues [29]. Similar results have been obtained with studies on isomeric alpha-D-galactopyranose sulphates; whereas no significant differences could be observed in the FAB spectra, the 2, 3, 4, and 6-monosulphates could be easily distinguished in the FAB/MS/MS spectra using electric sector scanning [30].

Array or focal-plane detectors

Detection limits for FAB mass spectrometry are around 1 pmol for derivatized oligosaccharides using a scanning mass spectrometer with a conventional 'point' detector. As this detector records only a minute section of the spectrum at any one moment of a scan, most of the ions produced in the ion source are never detected. To overcome this limitation, focal plane or array detectors, capable of simultaneously capturing and integrating the ions over a considerable fraction of the spectrum have recently been developed. This has resulted in an increase in sensitivity of over 100-fold provided that sensitivity is not limited by high backgrounds such as are present in FAB spectra. A further increase can be obtained with derivatized oligosaccharides ionised under FAB conditions by utilizing the observation that the temporal distribution of ions immediately following firing of the FAB gun is heavily biased towards the sample rather than the matrix. The resulting increase in signal:noise ratio captured with a focal plane detector during the first few milliseconds after firing the gun, thus achieves an even better detection limit; the high amol range has been achieved with peptides.

Ion yield under FAB conditions falls off considerably with increasing mass and about 10 kDa appears to be the upper usable mass for the method. For extension of the mass range beyond 10 kDa, other ionization methods must be used.

Plasma desorption

Two of these ionization techniques, plasma and laser desorption, utilize the high mass range of a time-of-flight mass spectrometer with a pulsed ion source. These easily overcome the mass limitation inherent in the other

techniques and extend the usable mass range by nearly two orders of magnitude. In plasma desorption mass spectrometry, the sample is loaded onto a target and bombarded with fission fragments from ^{252}Cf. This element decomposes by the simultaneous ejection of particles in diametrically opposite directions. One of these bombards the target and the other is used to trigger the timing circuits. Successive spectra are accumulated until a satisfactory S/N ratio is obtained; a process that can, unfortunately, take several hours. Nevertheless, some spectacular results have been obtained, particularly with polymer-coated targets [31]. Thus, the molecular weights and degrees of sulphation of di-, tetra- and hexameric fragments of hararin have been determined using tridodecylmethylammonium chloride-coated targets [32].

Methods using lasers

Lasers may be used in mass spectrometry, either to desorb ions or to fragment ions formed by other techniques. A potential advantage of laser-induced fragmentation is that the laser frequency can be changed in order to induce specific fragmentations. In carbohydrate research, CO_2 lasers working at 10.6 μm have been used to effect simultaneous desorption and fragmentation of small oligosaccharides [33]. Under these conditions underivatized molecules tend to fragment by ring cleavages involving fragmentation of two or more bonds in contrast to their behaviour under most other ionization conditions where glycosidic cleavages predominate.

Matrix-assisted laser desorption

A more recent development is matrix assisted laser desorption mass spectrometry. For this technique, the biomolecule is co-crystallized with a large excess of a UV absorbing compound, introduced into the mass spectrometer and irradiated with a short pulse of UV light. Energy is absorbed by the matrix and transferred to the sample causing formation of ions that are again analysed with a time-of-flight mass spectrometer. The technique works extremely well for peptides, proteins [34–38], glycoproteins and carbohydrates [39, 40] producing mass accuracies of the order of 0.1% or better. The spectra of proteins and glycoproteins are generally characterized by abundant $[M + H]^+$ ions, together with weaker dimeric and doubly-charged ions. Adduct formation with sodium, potassium, or photodecomposition fragments from the matrix can cause peak broadening and a reduction in mass accuracy, but these adducts can be minimized by reducing the concentration of alkali metals in the target and by using a matrix such as sinapinic acid [41], which does not form adducts to the same extent as earlier matrices such as nicotinic acid.

Carbohydrates are best examined using a substituted benzoic acid as the matrix. 3-amino-4-hydroxy- [39] and, in particular, 2,3-dihydroxy-benzoic acids [40] are particularly effective. Although no derivatization is necessary, an increase in sensitivity by a little over an order of magnitude can be achieved following permethylation. Thus, unmodified sugars can be detected at the 1–10 pmol range and derivatized sugars down to about 100 fmol. The high sensitivity of the technique is the result of the integrating nature of the detection system and to the ability of the instrument to combine successive spectra produced by multiple laser firings. Spectra are dominated by $[M + Na]^+$ ions rather than by the $[M + H]^+$ ions present in those from proteins and, unlike the protein spectra, contain no multiply-charged or multimeric ions. Adduct formation is also less of a problem than with proteins, and fragment ions have not been observed (Fig. 6). The method is thus ideal for identifying components of mixtures. Even underivatized sugars containing sialic acid can be examined, although with a somewhat decreased detection limit. An additional sodium atom is generally introduced for each carboxylic acid group present.

As this method is extremely rapid (two to three minutes is all that is required to prepare the sample and obtain a spectrum), it provides an ideal technique for the rapid screening of oligosaccharides released from glycoform mixtures (Fig. 7). Furthermore, because of the relatively few monosaccharide types (hexose, *N*-acetyl-hexose, fucose etc.) usually present in oligosaccharides, a mass measurement to 0.1% accuracy can lead directly to the composition. Although no information is present relating to sequence, branching or linkage, previous knowledge that the compound is, say, an *N*-linked sugar, limits the number of possibilities to such an extent that the probable structure can often be predicted with reasonable accuracy.

Unlike techniques such as GC/MS, the dynamic range is relatively low and there is little correlation between peak height and sample concentration. Nevertheless, quantitative measurements can be made if an internal standard of similar structure is included in the matrix. Although the absolute peak heights bear little relationship to sample concentration, the peak ratio appears to reflect concentration with a considerable degree of accuracy.

Electrospray ionization

Unfortunately, the low resolution of a simple time-of-flight mass spectrometer limits the use of matrix-assisted laser desorption for glycoform analysis to masses below about 10 kDa. Although this generally presents no problems for many native oligosaccharides, the technique is less attractive for mixtures of glycopeptides and glycoproteins whose molecular weights are often well in excess of this. More complex instrumentation including, for example, a time-of-flight mass spectrometer fitted with a reflectron, can improve the situation, but a more attractive solution is to use yet another of the new mass spectrometric techniques, electrospray ionization.

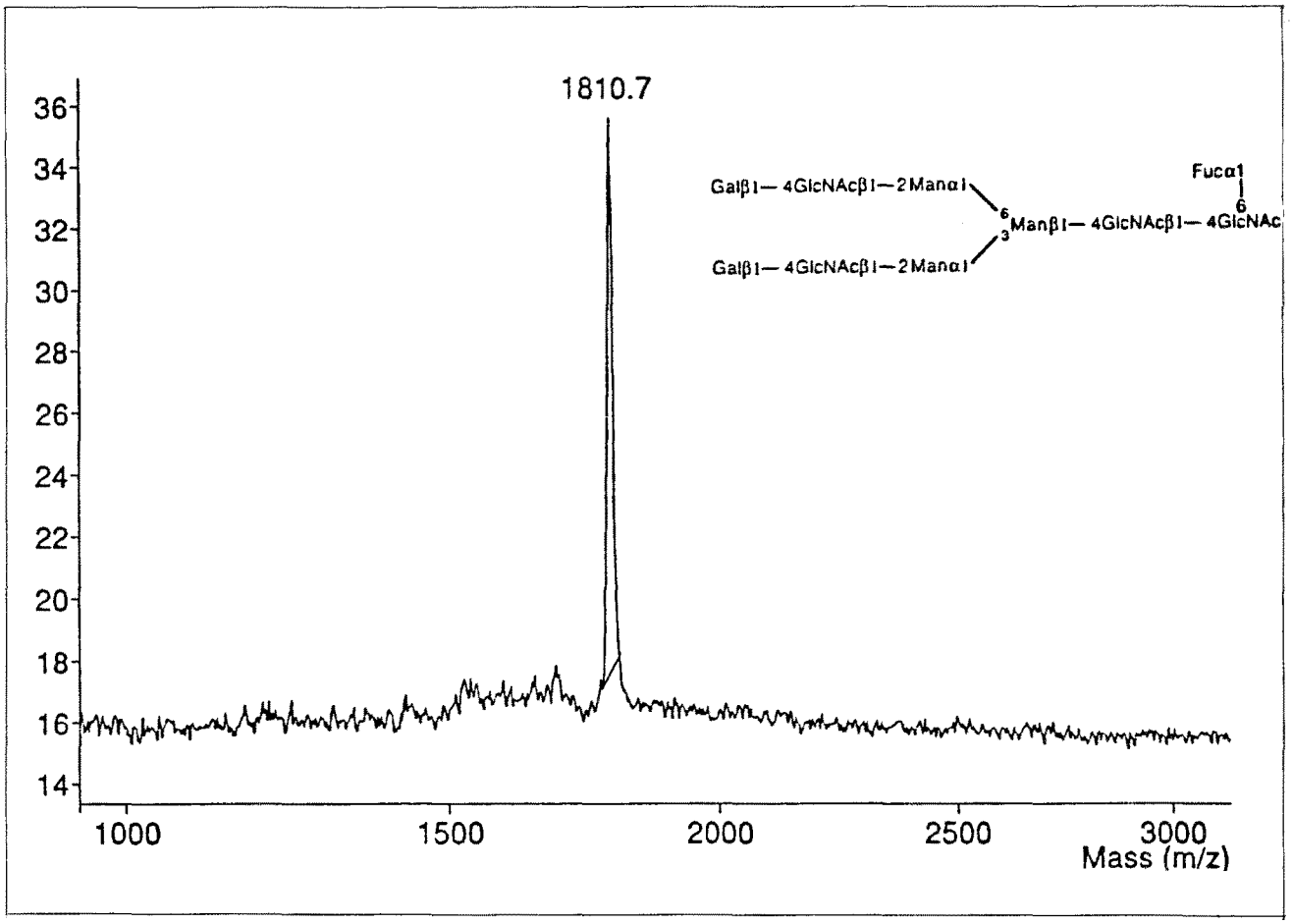

Figure 6. Matrix-assisted laser desorption mass spectrum of an oligosaccharide. The spectrum was obtained from 10 pmol of sugar using 3-amino-4-hydroxy-benzoic acid as the matrix, a laser frequency of 337 nm and a pulse width of 3 nsec.

Although generally regarded as new, electrospray ionization has, in fact, been around for a considerable time. However, the fact that it produces a range of multiply-charged ions of the type $[M + nH]^{n+}$ was, for many years regarded as a nuisance when mass spectrometry dealt primarily with the fragmentation of singly-charged ions, and the technique was largely ignored. However, the efficient production of multiply-charged ions provides a means of attaining high mass on even a modest instrument by considerably reducing the m/z ratio. For example, it is possible to protonate proteins, on average, at about one site for every 1 to 2 kDa in mass and, thus, the necessary mass range that the instrument must possess is only of the order of 2000 for even the larger peptides, well within the capability of even the lower performance instruments such as quadrupoles.

To obtain the spectrum, the sample is sprayed into an atmospheric pressure ion source from a needle carrying a high potential, usually against a countercurrent of an inert gas to desolvate the ions. The resulting spectrum consists of a range of ions of differing charge states from which the mass of the molecule can be calculated with a knowledge of the mass of any pair of ions. In practice, a deconvolution algorithm is generally applied using the mass spectrometer data system and a mass spectrum is extracted. As with laser desorption, mass accuracies are generally better than 0.1%, but the higher resolution of quadrupole and magnetic sector instruments, compared with their time-of-flight counterparts, considerably extends the useful range of the technique. Unfortunately, it does not appear to work as well as laser desorption for underivatized oligosaccharides, as these compounds are not protonated as efficiently. The successful analysis of cyclodextrins, however, provides an example of its applicability [42]. For glycopeptides, on the other hand, it appears superior to matrix-assisted laser desorption. For example, the 5 glycoforms of ribonuclease-B (ca 15 kDa) have recently been measured to better than ± 1 Da with no difficulty (Fig. 8) whereas with laser desorption, resolution at this mass, is limiting.

Two other advantages of electrospray are relevant. Firstly, it is possible to induce fragmentation by variation of the potential on certain ion source elements and, secondly,

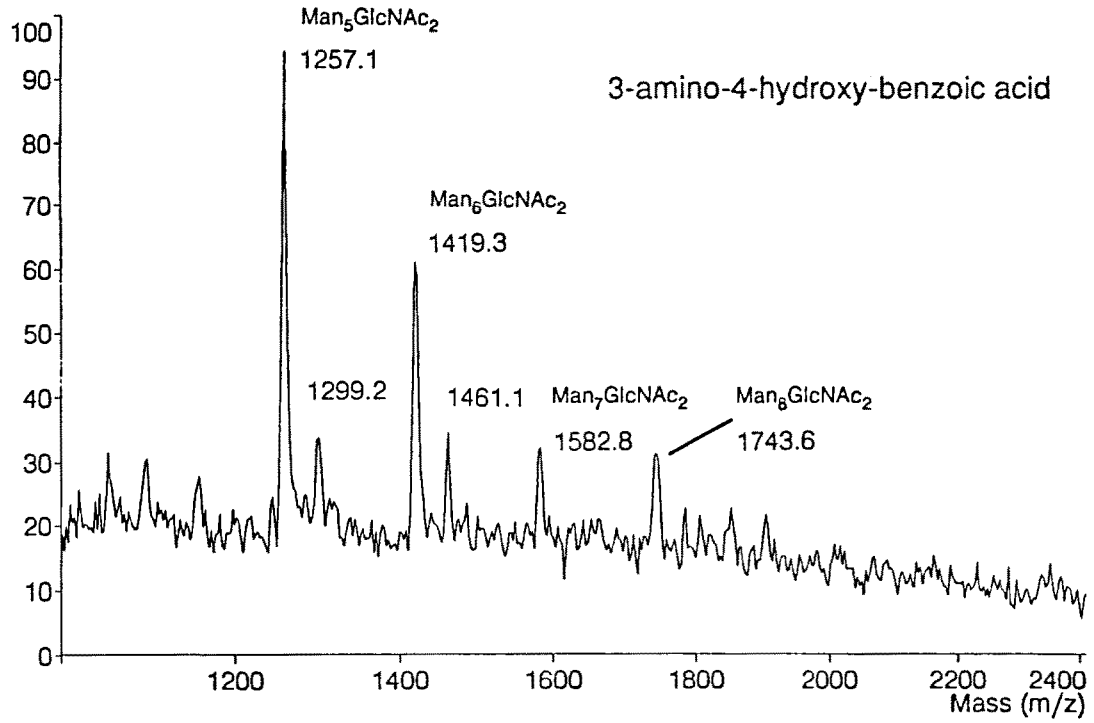

Figure 7. Matrix-assisted laser desorption mass spectrum of the oligosaccharides (man-5 to man-9) released from ribonuclease by hydrazineolysis and reacetylated. Experimental conditions were the same as in Fig. 6.

it is relatively easily coupled to liquid chromatographic techniques such as HPLC and capillary electrophoresis. The ability to induce fragmentation greatly extends the usefulness of the technique and, when combined with HPLC, provides a method as powerful as flow-FAB whose spectra usually contain a considerable number of fragment ions [43]. LC/MS techniques, therefore, have the potential of producing analytical methodologies for large biomolecules at least as powerful as GC/MS.

Conclusions

Where, therefore, do these techniques fit in the modern glycobiology laboratory? For the analysis of single glycopeptides with masses of several tens of kDaltons, either laser desorption or electrospray are ideal. However for mixtures in this mass range, electrospray appears to offer the advantage of higher resolution and, of course, the ability to be coupled to liquid chromatographic techniques. For molecular weight determination of released oligosaccharides, laser desorption is undoubtedly superior as no derivatization is necessary and mixtures are easily resolved. However, no information on sequence, branching or linkage is produced. For this information, FAB mass spectrometry appears to be the answer with suitable chemical manipulation to direct fragmentation into modes revealing details of branching and linkage. MS/MS techniques appear to offer exceptional promise for the determination of linkage. Mixtures can also be handled by MS/MS techniques and sensitivity considerations addressed by use of array detectors. Finally, until MS/MS techniques have been perfected for linkage determination, GC/MS of permethylated alditol acetates still appears to be the most satisfactory.

Many of the major problems in applying mass spectrometry to carbohydrate analysis are being rapidly solved. We now have methods for obtaining molecular ions from most molecules but, as yet, fragmentation of these ions does not provide all of the required information for structural identification. Chemical manipulation prior to ionization may be the answer, but for automated analysis, this should preferably be kept to a minimum. For mixture analysis, some form of chromatographic inlet offers the distinct advantage of avoiding the production of mixed spectra and ensuring that sensitivity is maximized. With MS/MS techniques, much of the ion current is not used if the mass spectrometer is used to perform the compound separation. For confirmation of glycosylation patterns, the simplicity of matrix-assisted laser desorption has much to offer. Now that the release of oligosaccharides has all but been automated, this may well provide a routine method for such tasks as purity checking, particularly in an industrial environment.

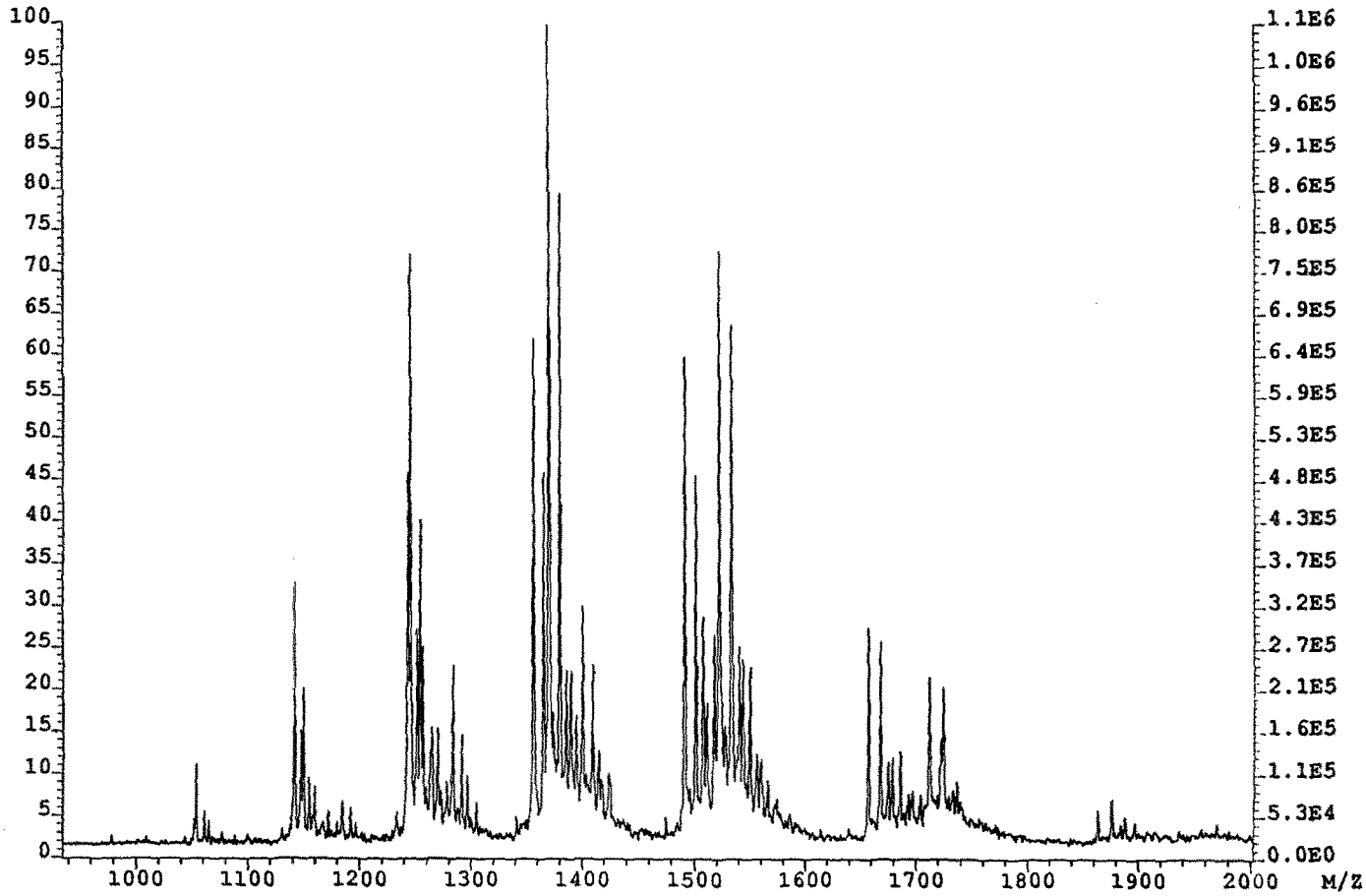

Figure 8. Electrospray mass spectrum of a mixture of ribonuclease A and B obtained at a concentration of 10^{-6} molar. Each group of peaks represents a different charge state. The largest peaks arise from ribonuclease A and its phosphate (and/or sulphate adduct) in its plus 10 charge state. These are superimposed on those of the ribonuclease B peaks from the plus 11 state.

References

1. Majer JR (1977) *The Mass Spectrometer*, London: Wykeham.
2. McFadden WH (1973) *Techniques of Combined Gas Chromatography/Mass Spectrometry: Application in Organic Analysis*, New York: Wiley.
3. Sweeley CC, Bentley R, Makita M, Wells WW (1963) *J Am Chem Soc* **85**:2497–507.
4. Karlsson H, Carlstedt I, Hansson GC (1989) *Anal Biochem* **182**:438–46.
5. Kakehi K, Honda S (1989) In *Analysis of Carbohydrates by GLC and MS* (Biermann CJ, McGinnis GD eds) pp 43–85. Boca Raton: CRC Press.
6. Fox A, Morgan SL, Gilbert J (1989) In *Analysis of Carbohydrates by GLC and MS* (Biermann CJ, McGinnis GD eds) pp 87–117. Boca Raton: CRC Press.
7. DeJongh DC, Radford T, Hribar JD, Hanessian S, Bieber M, Dawson G, Sweeley CC (1969) *J Am Chem Soc* **91**:1728–40.
8. Karkkainen J (1969) *Carbohydrate Res* **11**:247–56.
9. Sherman WR, Eilers NC, Goodwin SL (1970) *Org Mass Spectrom* **3**:829–40.
10. Zinbo M, Sherman WR (1970) *J Am Chem Soc* **92**:2105–14.
11. Harvey DJ, Horning MG (1973) *J Chromatogr* **76**:51–62.
12. Carpita NC, Shea EM (1989) In *Analysis of Carbohydrates by GLC and MS* (Biermann CJ, McGinnis GD eds) pp 157–216. Boca Raton: CRC Press.
13. Breimer ME, Hansson GC, Karlsson K-A, Leffler H, Pimlott W, Samuelsson BE (1981) *FEBS Letters* **124**:299–303.
14. Reinhold VN (1986) In *Mass Spectrometry in Biomedical Research* (Gaskell SJ ed) pp 181–213. Chichester: Wiley.
15. Reinhold VN, Carr SA (1982) *Anal Chem* **54**:499–503.
16. Merritt MV, Sheeley DM, Reinhold VN (1991) *Anal Biochem* **193**:24–34.
17. Barber M, Bordoli RS, Sedgwick RD, Tyler AN (1981) *Nature* **293**:270–5.
18. Dell A, Ballou CE (1983) *Carbohydrate Res* **10**:50–6.
19. Miller KJ, Kennedy E, Reinhold VN *Science* **231**:48–51.
20. Forsberg LS, Dell A, Walton DJ, Ballou CE (1982) *J Biol Chem* **257**:3555–63.
21. Angel A-S, Nilsson B (1990) *Biomed Environ Mass Spectrom* **19**:721–30.
22. Dell A, Morris HR, Greer F, Redfern JM, Rogers ME, Weisshaar G, Hiyama J, Renwick AGC (1991) *Carbohydrate Res* **209**:33–50.
23. Barr JR, Anumula KR, Vettese MB, Taylor PB, Carr SA (1991) *Anal Biochem* **192**:181–92.

24. Carr SA, Reinhold VN, Green BN and Haas JR (1985) *Biomed Mass Spectrom* **12**:288–95.
25. Orlando R, Bush CA, Fenselau C (1990) *Biomed Environ Mass Spectrom* **19**:747–54.
26. Laine RA (1989) *Methods Enzymol* **179**:157–64.
27. Laine RA, Pamidimukkala KM, French AD, Hall RW, Abbas SA, Jain RK, Matta KL (1988) *J Am Chem Soc* **110**:6931–9.
28. Kovacik V, Petrakova E, Hirsch J, Mihalov V, Heerma W, Versluis C (1988) *Biomed Environ Mass Spectrom* **17**:455–8.
29. Dornon B, Mueller DR, Richter WJ (1990) *Biomed Environ Mass Spectrom* **19**:390–2.
30. Heerma W, Versluis C, Kulik W, Ruiz Contreras R, Kamerling JP (1988) *Biomed Environ Mass Spectrom* **17**:257–63.
31. Macfarlane RD (1988) *Trends Anal Chem* **7**:179–83.
32. McNeal CJ, Macfarlane RD (1986) *Biochem Biophys Res Commun* **139**:18–24.
33. Martin WB, Silly L, Murphy CM, Raley TJ, Cotter RJ, Bean MF (1989) *J Mass Spectrom Ion Processes* **92**:243–65.
34. Karas M, Hillenkamp F (1988) *Anal Chem* **60**:2299–301.
35. Beavis R, Chait BT (1989) *Rapid Commun Mass Spectrom* **3**:233–7.
36. Beavis R, Chait BT (1989) *Rapid Commun Mass Spectrom* **3**:436–9.
37. Beavis R, Chait BT (1990) *Anal Chem* **62**:1836–40.
38. Beavis R, Chait BT (1990) *Proc Acad Sci USA* **87**:6873–7.
39. Mock KK, Davey M, Cottrell JS (1991) *Biochem Biophys Res Commun* **177**:644–51.
40. Stahl B, Steup M, Karas M, Hillenkamp F (1991) *Anal Chem* **63**:1463–6.
41. Beavis R, Chait BT (1989) *Rapid Commun Mass Spectrom* **3**:432–5.
42. Huang EC, Henion JC (1990) *Rapid Commun Mass Spectrom* **4**:467–71.
43. Hemling ME, Roberts GD, Johnson W, Carr SA, Covey TR (1990) *Biomed Environ Mass Spectrom* **19**:677–91.
44. Carr SA, Reinhold VN (1984) *Biomed Mass Spectrom* **11**:633–42.

Letters or comments relating to this article would be received with interest by Pauline Rudd, Assistant to the Special Advisory Editor, R. A. Dwek.