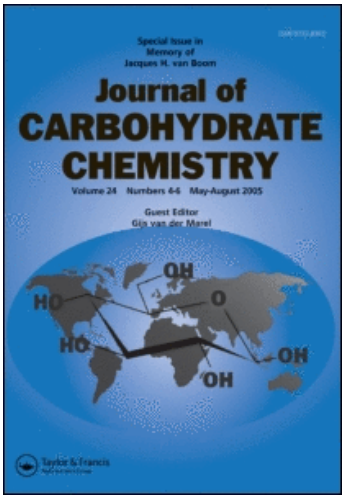

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OLIGOSACCHARIDE STRUCTURAL STUDIES BY ON-LINE HPLC-MS USING FAST ATOM BOMBARDMENT IONIZATION[1]

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ABSTRACT

A moving polyimide belt was used as an HPLC-MS interface for the purification and structural studies of oligosaccharides. The samples, desorbed by FAB in the absence of added liquid matrix, exhibited the following characteristics: a) good signal to noise ratio at low mass; b) a variation in the degree of fragmentation and relative sensitivity with changes in belt speed; c) a diminished sensitivity to signal suppression by contaminants when contrasted to FAB matrix; and d) fragment ions which can provide useful structural information. The moving belt HPLC-MS interface was tested with a series of known standards and a sample of high mannose oligosaccharides isolated from animal urines.

INTRODUCTION

The use of fast atom bombardment (FAB) mass spectrometry (MS) to support biopolymer structural studies has been gaining in popularity over the last few years. This has been due, in large part, to the high sensitivity, opportunity for direct analysis of these very polar materials, ease of instrumental operation, and useful structural information afforded by this technique. Specific applications to carbohydrate research have been particularly fruitful because of the inherent structural complexity of carbohydrates and the lack of alternative analytical techniques. Although the isomeric monosaccharides and their linkages within an oligosaccharide chain cannot

be definitely established by FAB-MS, the mass spectrum can provide molecular weight information and sequence and branching patterns.[2] In order to gain maximum structural information three parameters need to be considered: a) ion extraction mode (positive or negative); b) need for sample derivatization (permethylation, peracetylation, reductive amination); and c) choice of matrix (e.g., thioglycerol, glycerol, triethanolamine) and matrix additives (e.g., NaCl, HCl, TFA, amino-isothiocyanate, tetramethylurea). These operational variables can significantly alter the quantitative as well as qualitative characteristics of FAB mass spectra.[3] In addition, contaminants can have similar effects and may even totally suppress sample desorption and/or ionization. These complications become most pronounced when analyzing trace amounts of sample material isolated from a complex biological matrix. In order to address these latter problems we have evaluated three adjunct techniques in mass spectrometry: a) tandem mass spectrometry (MS-CID-MS);[4] b) off-line HPLC separation of fluorescently labeled carbohydrates followed by FAB-MS;[5] and c) direct liquid injection of sample solutions.[6] We report here an evaluation of an HPLC-FAB-MS with a moving belt interface[7] for the analysis of oligosaccharides.

In the past few years thermospray LC-MS has been successfully used to analyze many different types of polar and thermally labile compounds.[8] The thermospray mass spectra of these compounds generally show abundant molecular-weight related ions with little fragmentation. The use of thermospray LC-MS for the analysis of carbohydrate materials has also been reported;[9-11] however, the spectra of underivatized oligosaccharides have shown extensive fragmentation with very weak molecular-weight related ions. Permethylation has improved the overall sensitivity and increased the relative abundance of molecular-weight related ions.[10] In this paper we wish to report a comparison of FAB mass spectra of carbohydrates desorbed from a polyimide moving belt (FAB-belt) with those desorbed from a liquid matrix (FAB-matrix). Several oligosaccharides and a urine mannosidosis sample have been tested in this application.

RESULTS AND DISCUSSION

Presented in Figure 1 is a schematic diagram of the moving belt interface used in this study. Sample solutions were sprayed directly

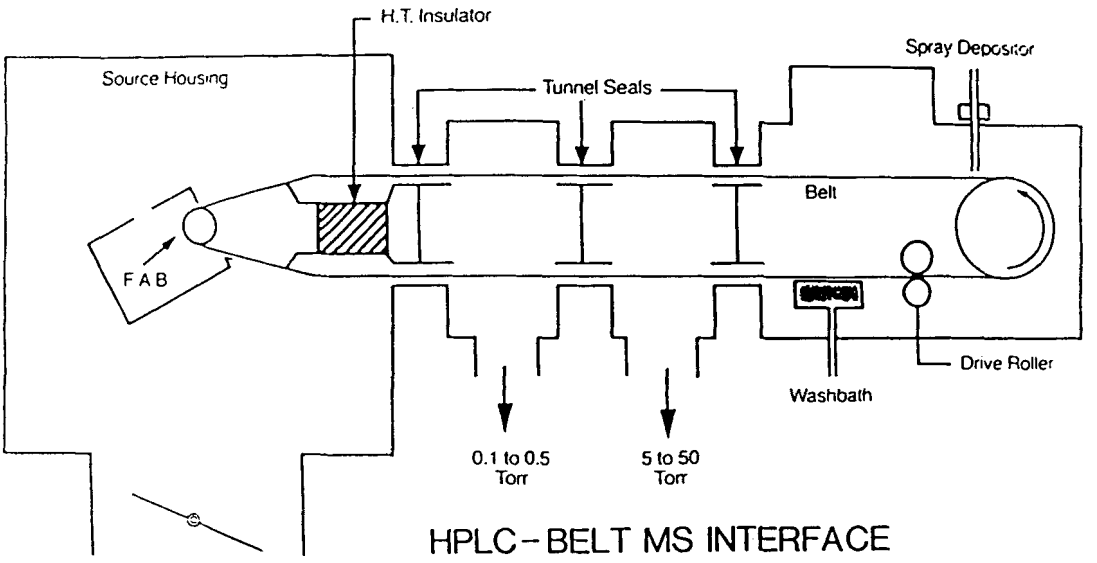

FIG. 1 A schematic diagram of a moving belt HPLC-MS interface.

onto the belt and most of the solvent evaporated as the belt moved through two vacuum-lock chambers. Sample ionization and desorption were initiated by an 8 keV xenon beam focused at the tip of the moving belt probe. Remaining sample and residues were removed from the belt with an aqueous wash bath.

The sensitivity of both FAB-matrix and FAB-belt mass spectrometry of oligosaccharides is dependent on a number of factors (e.g., molecular weight, sample purity, type of matrix, belt speed, mode of ion extraction); therefore, it is difficult to provide an absolute comparison of sensitivity between these two techniques. Under optimum operating conditions, the two techniques appear comparable. Some contrast is observed at the upper and lower mass range of analysis. Small oligosaccharides (below 700 daltons) usually show greater sensitivity with FAB-belt desorption while large oligosaccharides (above 2000 daltons) are somewhat less sensitive. The detection limit for belt desorption of maltohexaose (MW = 990) is about 1 ug. Interestingly, belt desorp-desorption of oligosaccharides with diminished polarity (e.g., peracetylated, partially methylated, or deoxy sugars) show considerable

improvements in sensitivity. As an example, the detection limit for the pentasaccharide Viridopentaose-B, (a partially methylated, deoxy pentasaccharide; MW = 840) is less than 100 ng.

One of the most notable differences between FAB-belt and FAB-matrix mass spectra of carbohydrates is the absence of matrix derived ions in the former case (FIG. 2). Unlike the liquid matrix of conventional FAB, the polyimide moving belt contributes few ions to the mass spectrum. This diminished background is particularly important for the analysis of low molecular weight samples or fragment ions (below 500 daltons) where the liquid matrix derived ions normally dominate the FAB-matrix mass spectrum. Another difference is that while FAB-matrix mass spectra usually exhibit protonated molecular ions $(MH)^+$, desorption from the belt always shows sodiated molecular ions $(M+Na)^+$. When the sample and the solvents were carefully deionized, the abundance of the $(M+Na)^+$ ions was diminished but the protonated molecular ion was not observed. Therefore, it is important to have a trace amount of sodium ions on the belt to maximize sensitivity. The use of tap water in the wash bath proved adequate. Large amounts of salt cause sample signal suppression in both the FAB-matrix and FAB-belt mass spectrometry of oligosaccharides although the latter is less sensitive to this problem. With small amounts of sample direct addition of salt is most frequently met with signal suppression for both FAB-belt or FAB-matrix techniques.

When a mixture of carbohydrates or samples containing contaminants are analyzed by FAB-matrix mass spectrometry, some or all of the molecular ions of the component(s) of interest exhibit signal suppression.[3] We suspect that the uneven distribution of various components in the sample mixture on the matrix surface may be partly responsible for this problem for two reasons: first, surface active materials such as detergents can cause severe signal suppression; second, "suppressed" signals can sometimes be observed just before the matrix is completely evaporated or by using a different matrix material. Since most of the solvent is evaporated before the sample on the belt enters the FAB ion source the surface discrimination between selective components should not occur. The FAB-belt and FAB-matrix spectra of an equimolar mixture of four carbohydrates (stachyose, maltopentaose, maltohexaose, and α-cyclodextrin) are compared in Figure 3. The molecular ion for

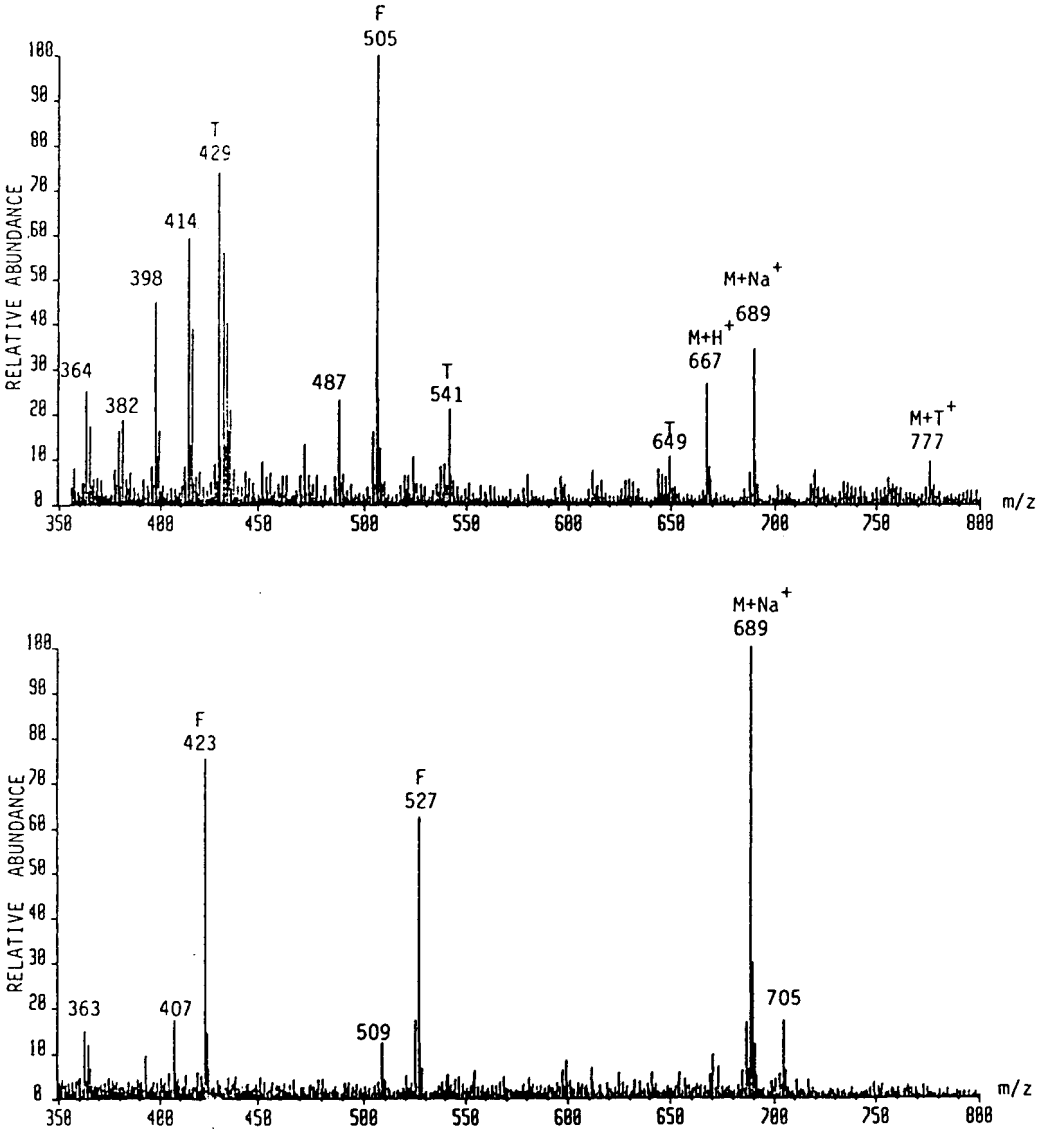

FIG. 2 FAB-matrix mass spectrum of stachyose in thioglycerol (top) compared with FAB-belt mass spectrum of stachyose (bottom). Symbol T represents thioglycerol derived ions, while F represents sample derived fragment ions.

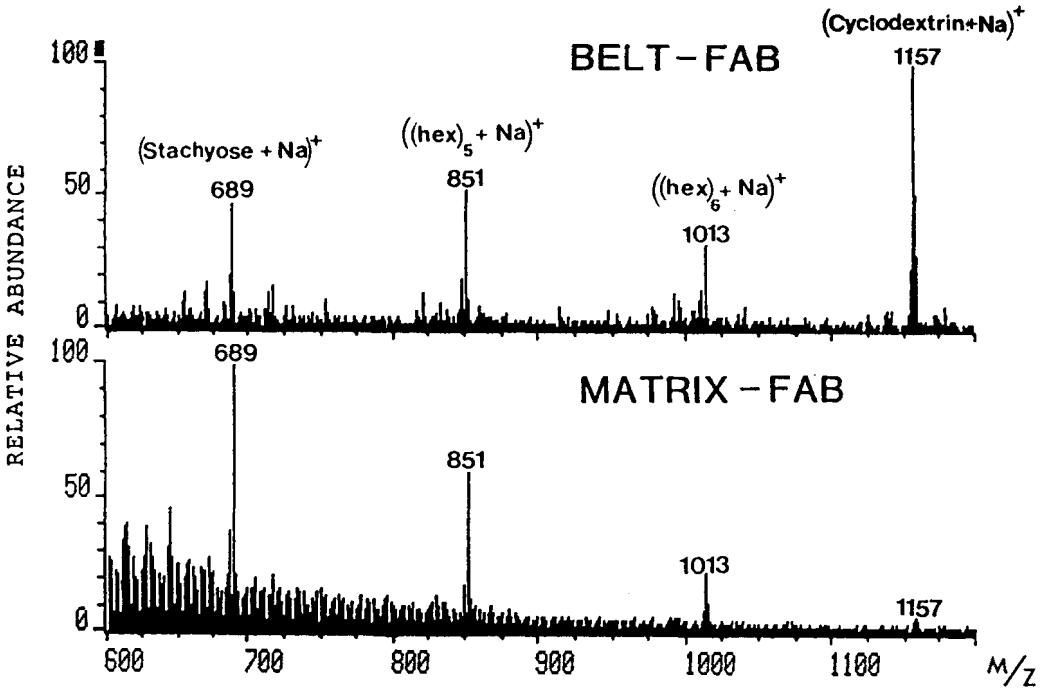

Figure 3. FAB-belt (top) and FAB-matrix (bottom) mass spectra of a mixture of stachyose, maltopentaose, maltohexaose, and β-cyclodextrin.

cyclodextrin is nearly undetectable in the FAB-matrix mass spectrum while all four components can be clearly seen in the FAB-belt mass spectrum. It appears that desorption of carbohydrate mixtures from the belt is less susceptible to signal suppression than the conventional matrix desorption and should be considered as an alternative approach for mixtures of great complexity.

When the carbohydrate sample to be analysed is relatively pure the resulting FAB-belt mass spectrum usually provides unambiguous molecular weight information as well as some structural information. This point is illustrated by comparing FAB-belt mass spectra of three heptasaccharides: α-cyclodextrin, maltoheptaose, and $Man_5GlcNAc_2ol$. The FAB-belt mass spectrum of cyclic heptasaccharide α-cyclodextrin (FIG. 4) shows abundant sodiated molecular ions but no fragment ions can be observed. Since two or more bond cleavages are necessary for the

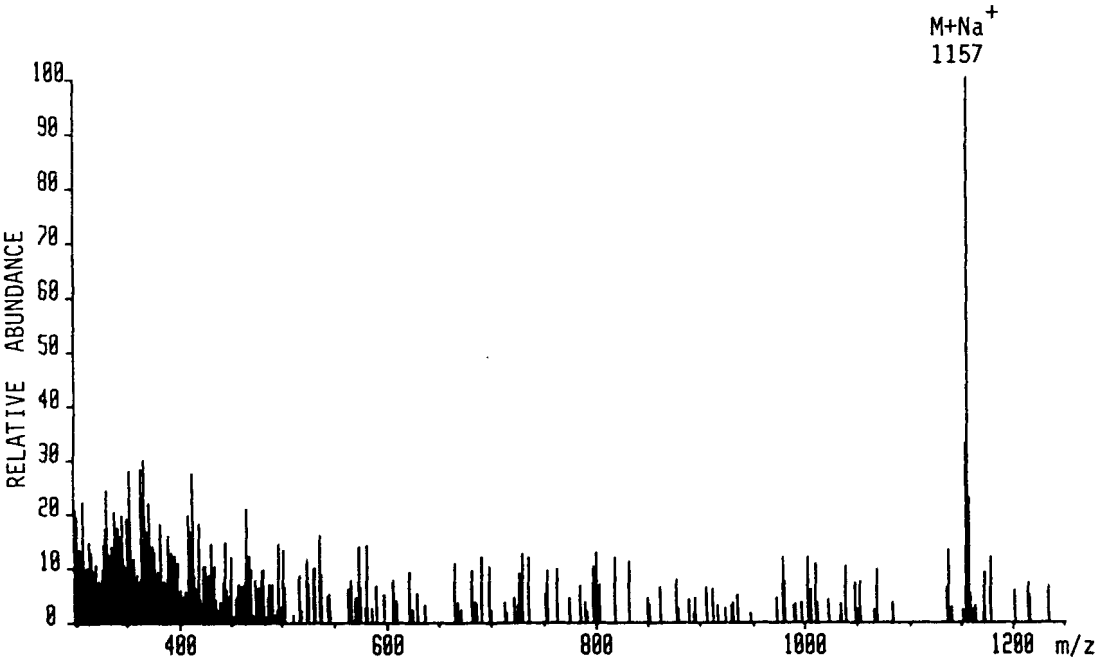

Figure 4. FAB-mass spectrum of β-cyclodextrin.

production of fragment ions from a cyclic structure, it is concluded that multiple bond cleavages do not occur to any detectable extent.

The FAB-belt mass spectrum of the linear heptasaccharide maltoheptaose (FIG. 5) shows a sodiated molecular ion and a series of fragment ions at intervals of 162 daltons. These fragments can be attributed to single bond cleavage, more or less at random, at various glycosidic linkage positions along the oligomer chain. In contrast to the results obtained for this linear oligosaccharide, the FAB-belt mass spectrum of a doubly branched heptasaccharide $Man_5GlcNAc_2ol$ (FIG. 6) shows an interrupted series of fragment ions with deletions occurring at each of the two branch points. These results suggest that double cleavages do not occur in FAB-belt desorption, a result consistent with those obtained for the cyclic glycan (e.g., molecular-weight related ion only, FIG. 4). Conventional FAB-matrix ionization seems to involve greater energies[12] where double cleavages are frequent. The broader ion energy spread characteristic in FAB-matrix[13] spectra makes this technique unreliable for the determination of oligomer branching.

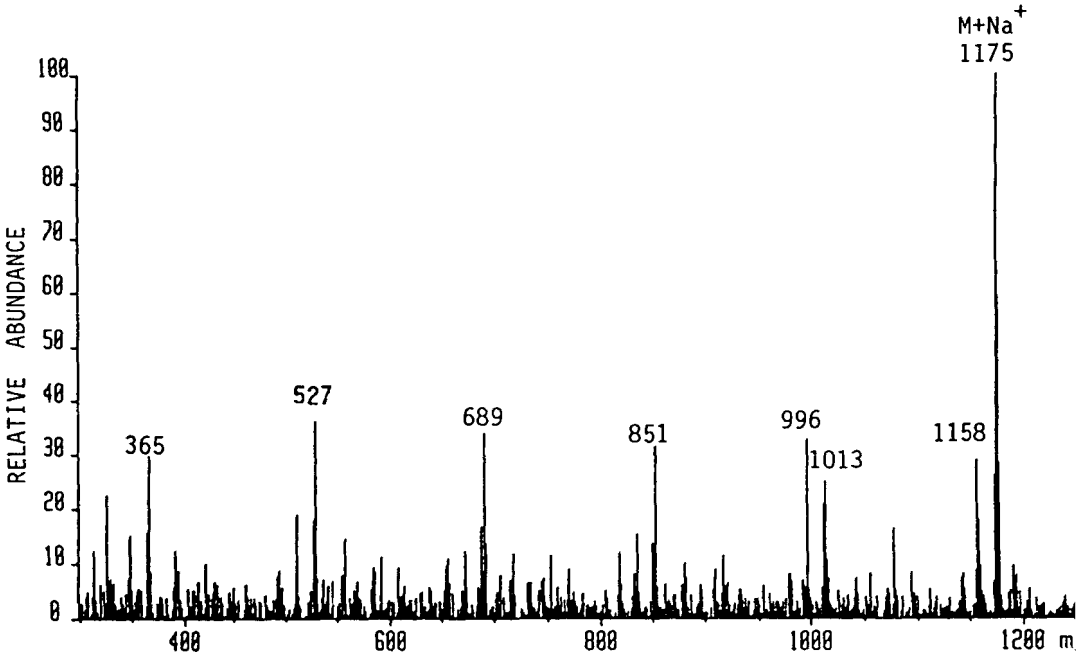

FIG.5 FAB-belt mass spectrum of maltoheptaose

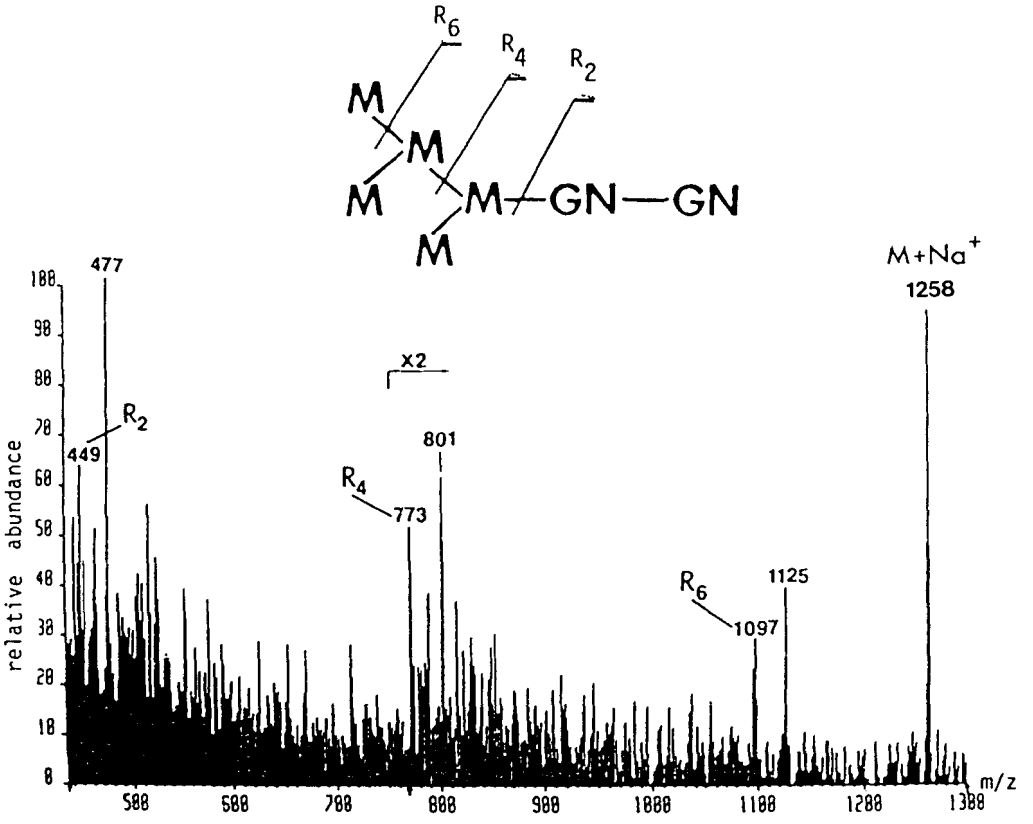

Figure 6. FAB-belt mass spectrum of $Man_5GlcNAc_2ol$.

An interesting and maybe useful feature of FAB-belt-MS is that the abundance of the fragment ions relative to those of the sodiated molecular ions can be varied by adjusting the belt speed. This effect can be demonstrated with the pentasaccharide, viridopentaose B, recorded at three belt speeds (FIG. 7). It is uncertain what factors lead to this observation, but, we speculate that at the slower speeds the non-conducting belt surface resides in the flux of the primary beam longer and acquires a greater surface charge as a result of sputtering. Samples desorbed through this charged surface are likely to have greater internal energies and exhibit greater fragmentation.

The upper mass range capability of FAB-belt-MS of carbohydrates has not been fully explored. We have analysed oligosaccharides up to 3000 daltons without difficulty; however, when a series of glucose polymers (2000-4000 Da) were compared with FAB-matrix samples desorbed from the belt, they exhibited some mass discrimination. Further research on modifying belt surfaces may be very fruitful in this regard.

Direct coupling of HPLC and FAB-MS offers many advantages for the studies of oligosaccharides. Most oligosaccharides do not contain a suitable chromophore for sensitive UV or fluorescent detection of the sample in the HPLC eluent. FAB-MS offers a sensitive, structurally informative, and universal detector for oligosaccharides that extends to relatively high mass and requires no prior sample derivatization. To further evaluate the merits of this HPLC-FAB-MS interface, we applied this device to study an oligosaccharide mixture obtained from sheep urine intoxicated with the plant alkaloid swansonine (induced mannosidosis). Figure 8 shows the profile of the total-ion-current (TIC) obtained from analysis of this mixture. Examination of selected spectra within this profile provided easy identification of the expected N-linked mannose oligomers, $(Man)_nGlcNAc_2$ (where, n = 2,3,4,5, and 6). Component resolution following HPLC-FAB-MS analysis appears to be somewhat degraded when compared with an HPLC-UV trace of the same sample after first coupling with 2-aminopyridine.[5] However, the ability to "recover" this information by using the data system to generate selective mass chromatograms combined with the possiblity of carrying out MS-MS analysis on non-isobaric, co-eluting components more than compensates for this apparent loss.

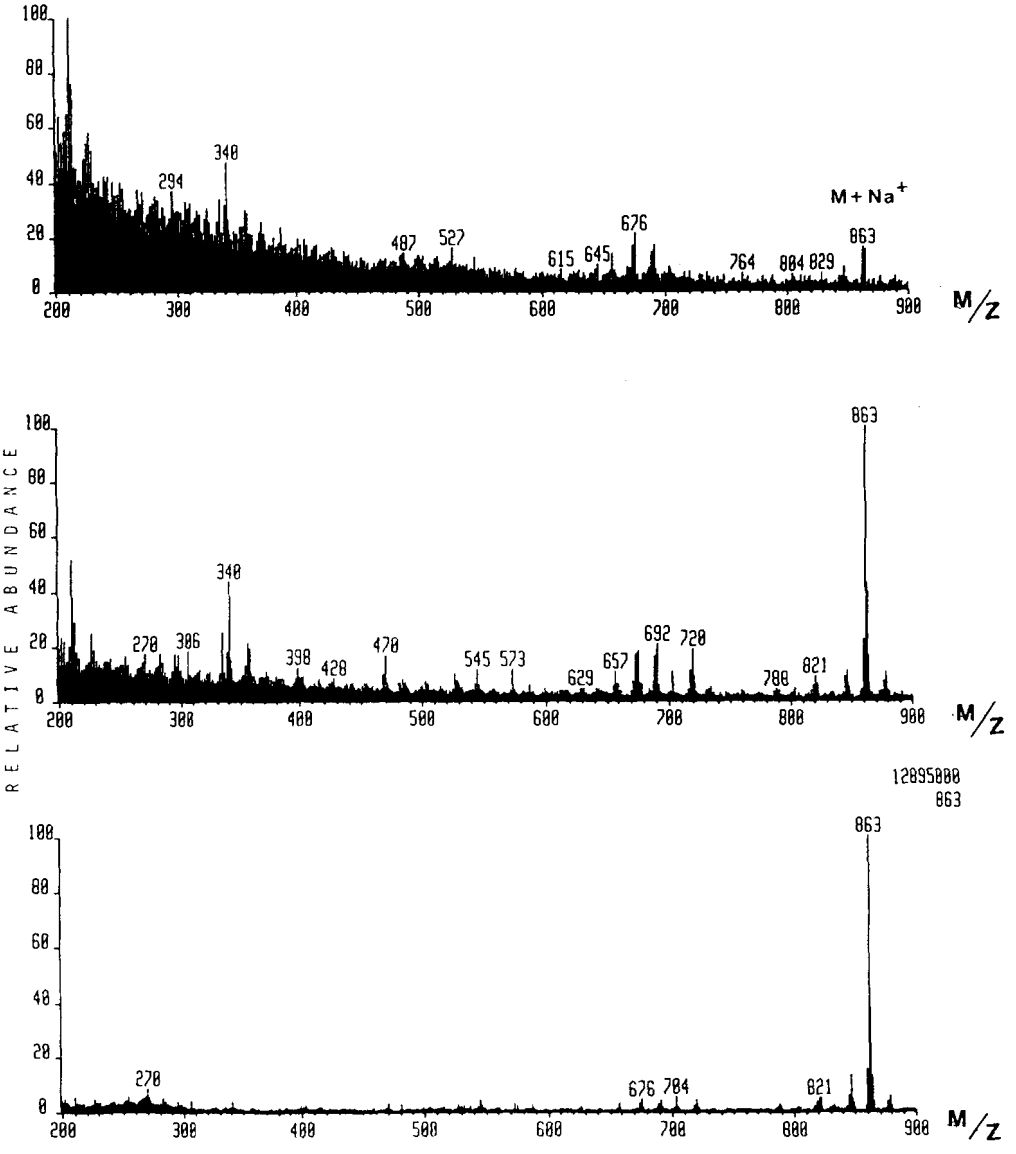

Figure 7. FAB-belt mass spectra of viridopentaose B at increasing belt speeds of 0.5 (top), 1.0 (center), and 3.0 (bottom) cm/sec.

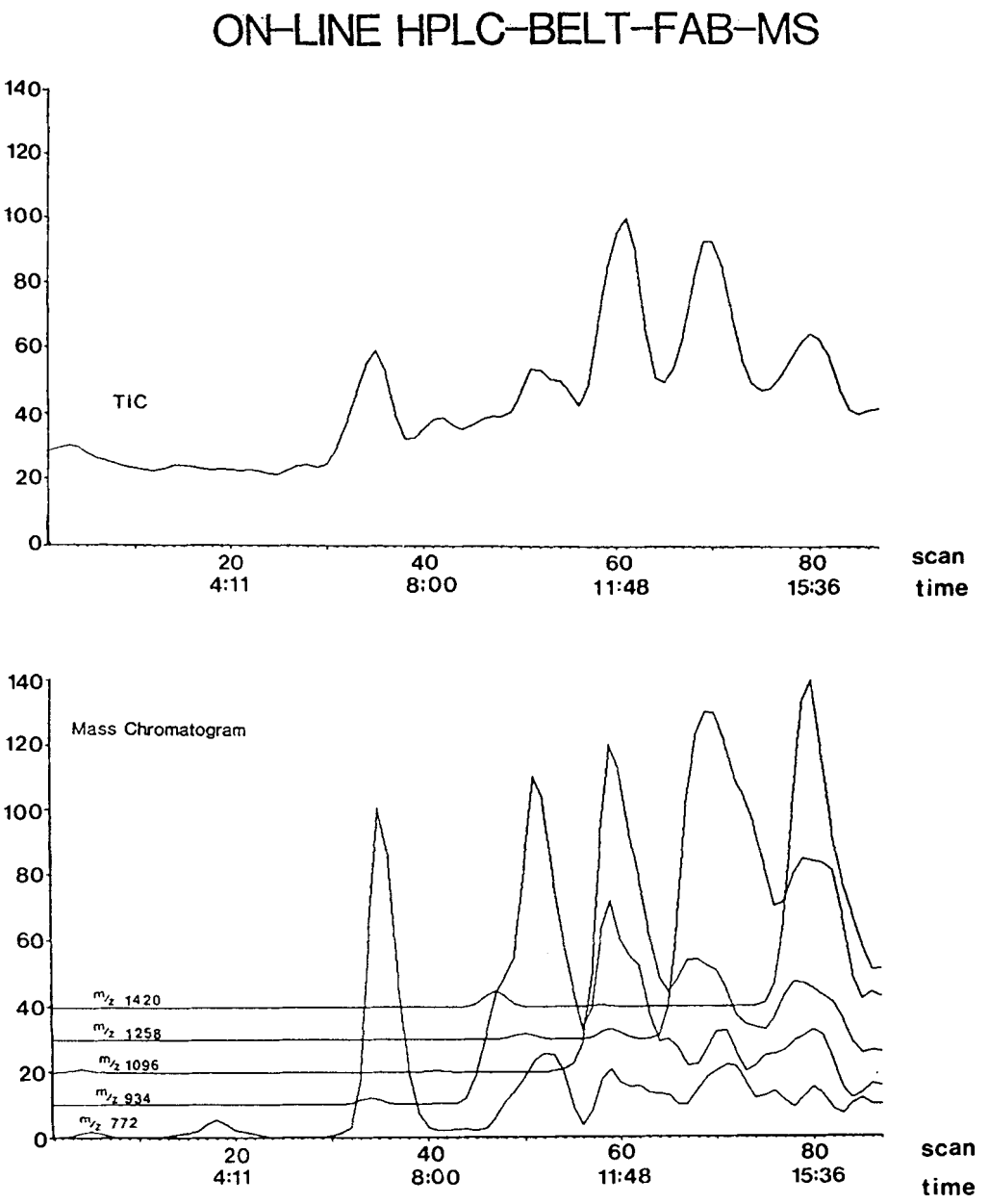

Figure 8. Total-ionization-current (TIC) profile obtained from on-line HPLC-FAB-MS of a mixture of oligosaccharides isolated from sheep urine, (top). Plot of selected mass chromatograms for $Man_nGlcNAc_2$, where n = 2 - 6, (bottom).

Although this HPLC-FAB-MS interface has numerous advantages, it is not without problems and these are possed by the relatively hydrophobic polyimide belt. First, some oligosaccharides, especially at higher sample loading, may not be completely removed in the wash bath and "ghosting" occurs with belt recycling. The addition of an organic modifier such as methanol or acetonitrile to the wash bath water can partially remedy this problem; however, this approach is self-limiting since many oligosaccharides are not very soluble in organic solutions. A second problem is the efficient and uniform transfer of HPLC effluent to the hydrophobic belt surface. The fine droplets from the nebulizer tend to aggregate to form larger puddles at irregular intervals. Considerable attention must be devoted to solvent flow rate, belt speed, and the spray nozzle temperature for uniform sample deposition. Droplet aggregation markedly diminishes sensitivity and makes it difficult to obtain a stable secondary ion flux and resultant total ion profile. These parameters are sensitive to solvent composition and if gradient elution is being used, the nozzle temperature needs to be readjusted accordingly. Clearly, a focused study on factors effecting sample deposition (e.g., belt composition), or alternative methods of sample delivery to the primary FAB beam would be important areas for further development.[14]

EXPERIMENTAL SECTION

All solvents were HPLC grade and obtained from Burdick and Jackson (Muskegon, MI 49442). Chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI 53201) or directly isolated from natural sources. The moving belt interface and the mass spectrometer (VG-ZAB-SE) were manurfactured by VG Analytical, Ltd. (Manchester, U.K.). Samples were desorbed by bombardment with a neutral xenon beam accelerated at 6 - 8 kV. The belt speed was varied at either 1, 2 or 3 cm/sec; sample solutions were sprayed onto the belt by a nebulizer with the nozzle temperature adjusted to give a fine aerosol (120-220 ^{o}C depending on solvent flow rate, composition). Typically, 20 ug of oligosaccharide dissolved in about 50 ul of solvent was injected and delivered to the nebulizer at a flow rate of 0.4 ml/min. Samples can also be applied to

the belt by a frit contact which appears to provide better sensitivity and more uniform sample deposition.[7] Samples (1-10 ug) dissolved in 50% aqueous acetonitrile (10-100 uL) were placed in a Waters (Milford, MA 01757), model UK6 injector and delivered to the nebulizer with a Waters M6000A pump at a flow rate of 0.2-0.5 cm^3/min. A standard FAB ion source was used for both FAB-belt and FAB-matrix experiments. All mass spectra were recorded in the positive ion mode with ion accelerating voltage set at 8 kV.

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