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Electrospray mass spectrometry of neutral and acidic oligosaccharides: methylated cyclodextrins and identification of unknowns derived from fruit material

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ABSTRACT

A qualitative study of the characteristics in the mass spectrometric analysis of various neutral and acidic oligosaccharides using electrospray ionization is reported. Experiments were performed in both positive- and negative-ion modes. In the positive-ion mode molecular mass information for neutral non-derivatized oligosaccharides could be obtained up to M_r 4000. The method was applied to the determination of the degree of methylation of methylated β-cyclodextrins and the identification of unknown oligosaccharides enzymatically derived from pear and apple fruit material.

INTRODUCTION

Oligosaccharides are compounds of major biological importance, either as free carbohydrates or as constituents of glycoconjugates. There is special interest in the characterization of oligosaccharides in a variety of fields such as biochemistry, medicine, plant physiology and pathology and human and animal nutrition. As mass spectrometry is able to offer structural and/or molecular mass information considerable efforts have been made to characterize oligosaccharides by mass spectrometry. The analysis of both derivatized and non-derivatized oligosaccharides by fast atom bombardment mass spectrometry has been reported [1–3]. Addition of small amounts of alkali metal salts to the sample matrix appeared to improve the signal [4,5]. More recently, ^{252}Cf plasma desorption [6], direct chemical ionization [7] and matrix-assisted laser desorption [8] mass spectrometry have been reported to be suitable for the analysis of neutral non-derivatized oligosaccharides of M_r *ca.* 4000, 7000 and 10 000, respectively.

The application of on-line liquid chromatography–mass spectrometry (LC–MS) to the analysis of oligosaccharides has not been extensively described in the literature [9–12]. Until 1991, the use of fast atom bombardment (FAB) directly from a moving belt [11] or the use of an atmospheric-pressure spray system [12] appeared most suitable.

More recently, the use of thermospray [13–16], electrospray [13] and ionspray [17,18] techniques in oligosaccharide analysis has been reported and appear to be serious alternatives in the on-line LC–MS analysis of oligosaccharides. For thermospray, addition of sodium acetate appears to enhance the ion intensity significantly

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[13,14], whereas for electrospray, signal enhancement can be achieved by the addition of sodium acetate or ammonium acetate [13,17,18]. In thermospray, sodium acetate rather than ammonium acetate must be used in order to avoid ammoniolysis of the oligosaccharides to their constituent monomers [14]. The coupling of high-performance anion-exchange chromatography (HPAEC) via either a thermospray [15,16] or an ionspray [18] interface appears to be a very powerful tool in the analysis of complex oligosaccharide mixtures.

In this paper, the characteristics of neutral and acidic oligosaccharides in both positive- and negative-ion electrospray ionization are described. It concerns a qualitative rather than a quantitative study. Initially, various model compounds were studied. The information obtained from this study was used in the qualitative analysis of unknown oligosaccharide samples. In that respect, the characterization of dimethyl-β-cyclodextrin and the analysis of some unknown oligosaccharides isolated from water-unextractable solids from apple and pear fruit material are described.

EXPERIMENTAL

Apparatus

All experiments were performed with a Finnigan MAT (San José, CA, USA) TSQ-70 mass spectrometer, equipped with a 20-kV conversion dynode and a Finnigan MAT electrospray interface.

Sample introduction was performed either by constant infusion or by on-line liquid chromatography. A Model 2400 syringe pump (Harvard Apparatus, Edinbridge, UK) was used for sheath liquid delivery and in all constant infusion experiments. In the latter type of experiments methanol–water (90:10, v/v) or 2-propanol–water (90:10, v/v) was used as a sheath liquid at a flow-rate of 1–2 μl/min.

For solvent delivery in liquid chromatography, a Model 2150 LC pump (LKB, Bromma, Sweden) was used. Sample injection was done with a Rheodyne (Cotati, CA, USA) injection valve, equipped with a 20-μl sample loop.

Chromatography

For the LC separation, a laboratory-packed 150 mm × 2 mm I.D. C_{18} column (5-μm particle size) was used. The mobile phase was 0.1 m*M* aqueous sodium acetate at a flow-rate of 1 ml/min, which was split 1:500 postcolumn to 2 μl/min for electrospray nebulization. In these experiments, methanol–water (80:20, v/v) was used as a sheath liquid at a flow-rate of 2 μl/min.

Mass spectrometry

The experiments in the positive-ion mode were performed at a nebulization potential of −3.5 to −4 kV, whereas in the negative-ion mode a nebulization potential of +2.8 to +3.3 kV was used. In the Finnigan MAT electrospray interface used, the counter electrode is at high potential relative to the spray needle, which is grounded. Nitrogen was used as a drying gas at a gas pressure setting of 5; no drying gas heating was applied. The tube lens potential and the nozzle–skimmer potential were optimized before each experiment.

Chemicals

Throughout these experiments, demineralized water was used. Methanol and 2-propanol were purchased from Baker (Deventer, Netherlands). Sodium chloride, sodium acetate, sodium hydroxide, ammonium chloride and ammonium acetate were obtained from Merck (Darmstadt, Germany).

The maltodextrin MD-25 sample was supplied by Roquette (Lille, France), Dimethyl-β-cyclodextrin samples were obtained from Janssen (Tilburg, Netherlands), Wacker (Munich, Germany), Rameb (Budapest, Hungary) and Avebe (Veendam, Netherlands). The monomeric sugars, sucrose and the maltose oligomers were commercially available.

Unsaturated oligomers of galacturonic acid were isolated from an enzyme digest of polygalacturonic acid (Fluka, Buchs, Switzerland) according to the procedure described by Voragen *et al.* [19].

Water-unextractable solids from apple and pear fruit material were subjected to sequential extractions with increasing strength of alkali.

The 1 *M* KOH-extracted pear material and the 4 *M* KOH-extracted apple material were degraded with endo-glucanase IV [20] and subsequently fractionated on a Bio-Gel P2 column by preparative HPAEC with pulsed electrochemical detection.

RESULTS AND DISCUSSION

Initial characterization of the electrospray ionization of oligosaccharides was performed with both neutral and acidic oligosaccharides as model compounds in both positive- and negative-ion modes. The group of neutral oligosaccharides consisted of sucrose ($M_r = 342$), maltotetraose ($M_r = 666$), maltoheptaose ($M_r = 1152$) and a maltodextrin MD-25 mixture. The group of acidic oligosaccharides consisted of glucuronic acid ($M_r = 194$), galacturonic acid ($M_r = 194$), unsaturated digalacturonic acid ($M_r = 352$) and unsaturated trigalacturonic acid ($M_r = 528$). The experiments were performed in the constant infusion mode, unless stated otherwise.

Positive-ion mode

A small amount of alkali metal contamination was present in the liquid phases that were used in these experiments. Further, samples prepared by enzymic degradation of plant cell wall polysaccharides followed by preparative HPAEC are also contaminated with sodium ions. Considering the high sodium affinity of oligosaccharides, the positive-ion mass spectra in most constant infusion experiments are dominated by sodium adduct ions. Obviously, most of the sample contamination can be eliminated by performing on-column reversed-phase LC experiments. However, because the sodium adduct ions of oligosaccharides are so readily formed, it is recommended to work with sodium-containing buffers in order to avoid the generation of mixed spectra. For on-column LC experiments, the addition of 0.1–1 m*M* sodium acetate to the mobile phase was applied to ensure a well defined sodium concentration.

For constant infusion of a 30 μg/ml solution of sucrose or maltotetraose in methanol–water (90:10, v/v), only $[M + Na]^+$ and $[2M + Na]^+$ ions are observed. At higher analyte concentrations the intensity of the $[2M + Na]^+$ peak increases and even other sodiated clusters such as $[3M + Na]^+$ appear in the spectrum. Similar signal intensities are observed for sucrose and maltotetraose.

The mass spectra of acidic oligosaccharides show a high degree of sodium exchange, *i.e.*, in addition to $[M + Na]^+$ also $[M - H + 2Na]^+$ and eventually $[M - (n - 1)H + nNa]^+$ are observed (Fig. 1). The sodium exchange is more extensive at a lower analyte concentration, which can be explained by a more favourable sodium-to-analyte concentration ratio at lower analyte concentrations. Further, the total ion intensities for glucuronic acid and galacturonic acid are similar for 10 and 100 μg/ml solutions. Remarkably, for 10 μg/ml glucuronic acid solution the intensity of the sodiated dimer $[2GlcA + Na]^+$ was found to be higher than for a 100 μg/ml solution. One would expect a more abundant clustering at higher analyte concentrations, as is observed for sucrose.

The mass chromatograms for the analysis of a maltodextrin MD-25 sample using a reversed-phase C_{18} column and a 10^{-4} *M* aqueous sodium acetate mobile phase are shown in Fig. 2. Methanol–water (80:20, v/v) was used as the sheath liquid for electrospray nebulization. The relative response (area/μmol) in electrospray ionization is plotted as a function of the degree of polymerization (*DP*) in Fig. 3 and compared with earlier data on the response in thermospray ionization [14]. The degree of polymerization indicates the

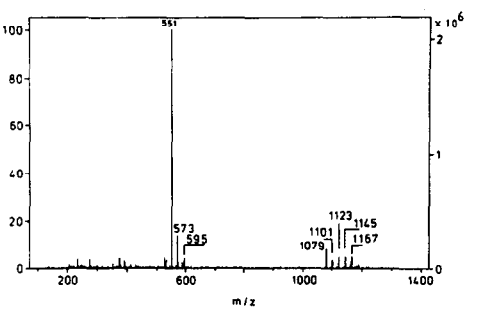

Fig. 1. Positive-ion electrospray mass spectrum of unsaturated trigalacturonic acid ($M_r = 528$), obtained by constant infusion of a 10 μg/ml solution in 2-propanol–water (90:10, v/v) at a flow-rate of 1 μl/min.

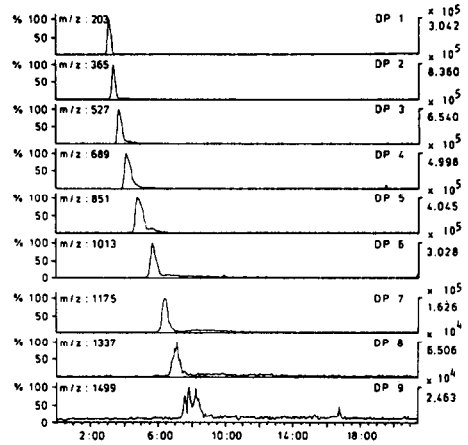

Fig. 2. Mass chromatograms for the LC–electrospray MS analysis of a maltodextrin MD-25 mixture. Multiple-ion detection in the positive-ion mode. Conditions: 150 mm × 2 mm I.D. C_{18} column, 10^{-4} *M* aqueous sodium acetate as a mobile phase at 1 ml/min, postcolumn splitting to 2 μl/min into the mass spectrometer, injection volume 20 μl, sheath liquid methanol–water (80:20, v/v) at 2 μl/min. After splitting, 0.5 μg of sample is consumed in the mass spectrometer.

number of sugar monomers present in the oligosaccharide. As higher responses are observed in electrospray ionization than in thermospray for the higher *DP* oligomers, it can be concluded that the former is the more appropriate for the analysis of the larger oligosaccharides.

The positive-ion mass spectrum of a maltodextrin MD-25 sample (1 mg/ml) obtained via constant infusion for 5 min at 1 μl/min is shown in Fig. 4. Singly charged sodiated species $[M + Na]^+$ are observed for the oligomers with *DP* = 1–11 and doubly charged disodiated species $[M + 2Na]^{2+}$ for the *DP* 6–23 oligomers. The molecular mass of the *DP* 23 oligomer (3744) further emphasizes the advantage of electrospray over thermospray ionization in the LC–MS analysis of oligosaccharides. An additional advantage of electrospray is the low flow-rate, which permits constant infusion of sample for several minutes with the consumption of only a small amount of sample. This is especially important in the characterization of unknowns in samples obtained from enzymic degradation of polysaccharides where the sample amounts are limited.

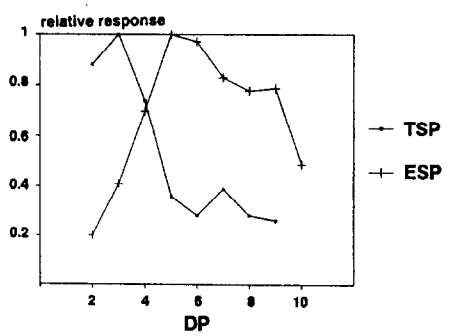

Fig. 3. Relative response measured as peak area per amount injected (mol) in LC–thermospray MS and LC–electrospray MS as a function of the degree of polymerization (*DP*). The injected amount of each oligomer was calculated from the mass percentages of each oligomer, obtained from the separation of MD-25 on an Aminex HPX-22H stationary phase with a refractive index detector [14].

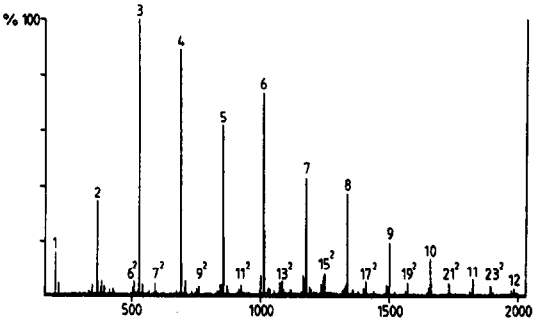

Fig. 4. Positive-ion electrospray mass spectrum of a 1 mg/ml maltodextrin MD-25 sample obtained by constant infusion at 1 μl/min in $3 \cdot 10^{-4}$ *M* sodium acetate in methanol–water (40:60, v/v) for 5 min. *DP* values are given on the peaks; DP^2 values indicate doubly charged ions.

Negative-ion mode

Constant infusion in the negative-ion mode of a solution of 30 μg/ml sucrose in methanol–water (90:10, v/v) results in $[M - H]^-$ and $[2M - H]^-$ ions, as illustrated in Fig. 5a. Addition of 10^{-4} *M* sodium chloride results in the formation of both deprotonated $[M - H]^-$ and chloridated $[M + Cl]^-$ molecules and clusters. By further increasing the chloride concentration to 10^{-3} *M*, the negative-ion spectrum almost entirely consists of chloridated molecules (Fig. 5b). The total ion intensity of sucrose with or without sodium chloride addition does not differ significantly. Additionally, the use of either ammonium or sodium chloride does not have much

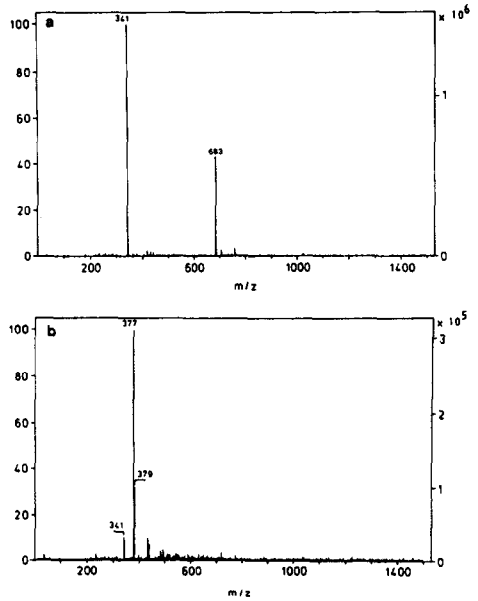

Fig. 5. Negative-ion electrospray mass spectra of sucrose ($M_r = 342$) obtained by constant infusion of a 30 μg/ml solution in (a) methanol–water (90:10, v/v) and (b) methanol–water (90:10, v/v) containing 10^{-3} *M* NH_4Cl. Flow-rate, 1 μl/min. For further explanation, see text.

influence on the total ion intensity. However, sodium acetate results in a more pronounced deprotonation than ammonium acetate. The latter can be explained by the acidity of the NH_4^+ ions, which prevents deprotonation. In addition, the acidic pH of the ammonium acetate solution might also play a role.

Maltotetraose and maltoheptaose show similar behaviour. However, the total ion intensity in the negative-ion mode for these compounds is about ten times lower than in the positive-ion mode.

For the acidic oligosaccharides the experiments were performed in 2-propanol–water (90:10, v/v) because with methanol stable ionization conditions appeared difficult to achieve. Again, glucuronic acid and galacturonic acid show a ten times better total ion current in the positive- than in the negative-ion mode. The addition of acetate or hydroxide results in a fourfold signal improvement but, in contrast to the neutral oligosaccharides, the addition of chloride hardly affects the spectrum. The addition of sodium hydroxide or sodium acetate induces the formation of the various cluster ions of the uronic acid: $[2M - H]^-$, $[2M + Na - 2H]^-$ and $[3M + 2Na - 3H]^-$.

Similarly to the positive-ion mode, the total ion intensity of 10 μg/ml glucuronic acid solution is comparable to that of a 100 μg/ml solution, while more extensive sodium exchange and stronger ion cluster formation occur at the lower analyte concentration also.

In contrast to glucuronic acid, but as normally expected, a 100 μg/ml unsaturated digalacturonic acid solution shows stronger cluster formation than 10 μg/ml solution. Again, the spectrum of the 10 μg/ml unsaturated digalacturonic acid solution shows a strong sodium exchange, which in principle gives information about the number of acidic groups in the molecule. On the other hand, from signal-to-noise point of view, the sodium exchange and cluster formation are undesirable. In order to avoid sodium exchange for acidic oligosaccharides the sodium concentration in solution has to be considerably lower than the analyte concentration, which is obviously difficult to achieve, especially at low analyte concentrations. For a 10 μg/ml unsaturated trigalacturonic acid solution the ion intensity in the negative-ion mode is comparable to that in the positive-ion mode.

Dimethyl-β-cyclodextrin

Cyclodextrins are an interesting class of compounds that are used in analytical chemistry, *e.g.*, as mobile-phase additives and for fluorescence enhancement. Further, they are under investigation in pharmaceutical technology as metabolically inert drug carriers and also in food technology. Within the class of cyclodextrins, methylated cyclodextrins form a special group of interest because of their high solubility in both water and oil. Their inclusion behaviour is significantly different from that of the parent cyclodextrins. Dimethyl-β-cyclodextrin [heptakis-(2,6-di-O-methyl)-β-cyclodextrin, $(Me)_{14}$β-CD] is expected to be the most useful host molecule for inclusion complexes in pharmaceutical applications. However, considerable difficulties arise with the selective methylation of the hydroxyl groups of β-cyclodextrins and for pharmaceutical applications the purity of

$(Me)_{14}\beta$-CD is of extreme importance. Koizumi *et al.* [21] described a laborious procedure for the analysis of $(Me)_{14}\beta$-CD samples. In our laboratory, it was observed that $(Me)_{14}\beta$-CD samples obtained from various commercial sources show different elution profiles in size-exclusion LC [22]. Therefore, mass spectrometric characterization of the methylated cyclodextrins by means of constant infusion to the electrospray system was attempted. The singly charged sodiated molecule of $(Me)_{14}\beta$-CD is expected at m/z 1354 and the doubly charged disodiated molecule at m/z 688.5.

Constant infusion in the positive-ion mode of a 14 μg/ml $(Me)_{14}\beta$-CD sample in methanol–water (80:20, v/v) at 1 μl/min resulted in strong $[M + Na]^+$ and $[M + 2Na]^{2+}$ peaks, as illustrated in Fig. 6a. The spectrum was acquired at a tube lens potential of 200 V. Significantly different optima were found for the nozzle–skimmer potential for the singly charged ion (200 V) and the doubly charged ion (10 V). The difference cannot be readily explained. From the mass spectrum in Fig. 6a, it can be concluded that the methylation of the $(Me)_{14}\beta$-CD sample is inhomogeneous: $(Me)_n\beta$-CD with $n = 13$–17 at m/z 1340, 1354, 1368, 1382 and 1396, respectively, are found to be present in this particular sample. The mass spectrum of $(Me)_{14}\beta$-CD from another commercial source (see Fig. 6b) shows even more inhomogeneity, which in this instance is primarily due to insufficient methylation, *i.e.*, $(Me)_n\beta$-CD with $n = 9$–15, *e.g.*, m/z 1284 corresponds to $n = 9$ and m/z 1326 to $n = 12$. From these results it may be concluded that electrospray ionization can be used as a rapid method for characterizing different $(Me)_{14}\beta$-CD batches in terms of methylation non-uniformity.

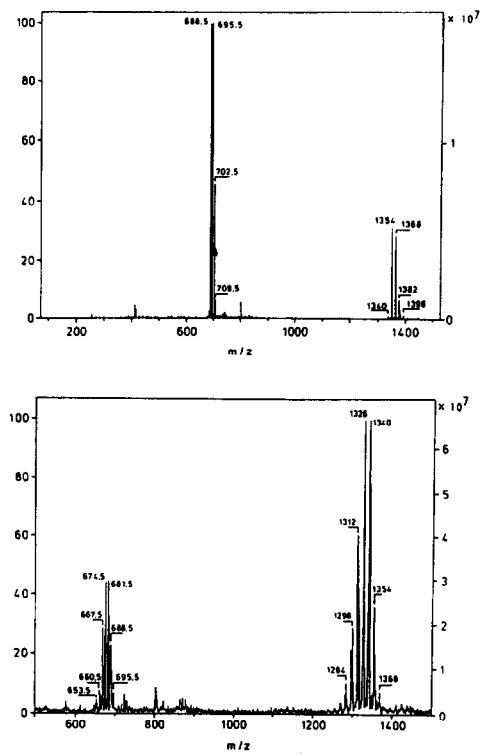

Fig. 6. Positive-ion electrospray mass spectra of 14 μg/ml solutions of dimethyl-β-cyclodextrin ($M_r = 1331$) samples obtained from two different commercial sources. Solvent: methanol–water (80:20, v/v) at 1 μl/min. For further explanation, see text.

As expected, no spectrum of $(Me)_{14}\beta$-CD could be obtained in the negative-ion mode, because no free hydroxyl groups are available for deprotonation at the outside of the molecule.

Analysis of unknown oligosaccharides

There is considerable interest in the rapid characterization of oligosaccharides obtained by the chemical and/or enzymic degradation of glycoproteins and plant cell wall polysaccharides. Many of these oligosaccharides can be satisfactorily separated by HPAEC but their elution behaviour is unpredictable. For example, fucose-containing oligosaccharides have a tendency to elute faster than oligomers of similar *DP* lacking fucose [23], whereas arabinose and uronic acid show an opposite effect. For mass spectrometric analysis, in most instances FAB-MS and FAB-MS–MS are used. However, as electrospray was found to be a good alternative to FAB, various unknown oligosaccharide samples from enzymatic degradation of plant cell wall polysaccharides were analysed by constant infusion electrospray mass spectrometry in the positive-ion mode. Although the spectra obtained lack structural information, rapid molecular mass determination proved to be extremely useful, as is illustrated by the following examples.

Fractionation of the 1 *M* KOH-extracted pear

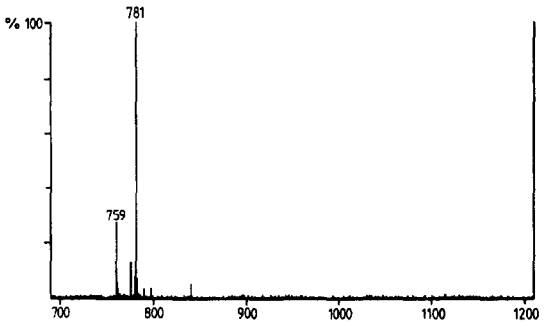

Fig. 7. Positive-ion electrospray mass spectrum of an unknown oligosaccharide after enzymatic digestion of a pear 1 *M* KOH extract. For further explanation, see text.

digest on Bio-Gel P2 showed some oligomeric products around $K_{av} = 0.15$ with a xylose-to-uronic acid ratio of *ca.* 5:1. In the colorimetric assay used, distinction between GalA, GluA and 4-O-methyl-GlcA was not possible. One oligomer was purified from this pool using preparative HPAEC. Sugar analysis of this fraction revealed the presence of some 4-O-methyl-GluA [24]. However, quantification was not possible as no proper standards were available.

The electrospray positive-ion mass spectrum of this unknown oligosaccharide derived from pear fruit tissue is shown in Fig. 7. Two major peaks are observed at *m/z* 759 and 781. The mass difference of 22 indicates the presence of one acidic sugar unit in the molecule. Therefore, the peak at *m/z* 759 is assigned to be the sodiated molecule $[M + Na]^+$, resulting in a molecular mass of 736. Plant cell wall polysaccharides are expected to contain hexoses, particularly glucose, mannose and galactose ($M_r = 180$), pentoses, particularly xylose and arabinose ($M_r = 150$), deoxyhexoses, particularly fucose and rhamnose ($M_r = 164$), hexuronic acids, *e.g.*, glucuronic or galacturonic acid ($M_r = 194$) and/or O-methylhexuronic acids ($M_r = 208$). A short algorithm, developed in our laboratory, was used to calculate all possible combinations of these monomeric units, leading to a molecular mass of 736. The five possible compositions that were found for this sugar oligomer are summarized in Table I. Additional information from sugar analysis rules out all possibilities except one: $(pent)_4(4\text{-O-methyl-GluA})_1$. Hence this method not only easily distinguishes between hexuronic acids and 4-O-methylhexuronic acids, but also permits the determination of the pentose-to-4-O-methyluronic acid ratio. In order to determine the precise configuration of the unknown oligosaccharide, it is necessary to perform additional experiments, *e.g.*, with tandem mass spectrometry [25]. Based on the work of Chanda *et al.* [26], Labavitch and Greve [27] suggested the presence of glucuronoxylan oligomers in a similar Bio-Gel P2 pool. However, no attempts were made to characterize this fraction further. Debeire *et al.* [28] also reported similar fragments derived from larchwood on enzymic treatment.

The *DP* of an unknown oligomer fractionated from the 4 *M* KOH-extracted apple digest was estimated to be larger than 10 (based on the Bio-Gel P2 elution pattern). The electrospray mass spectrum of this oligosaccharide is shown in Fig. 8. The base peak in the spectrum is at *m/z* 958. Because of its even mass, the peak cannot be attributed to a singly charged monosodiated oligosaccharide. Assuming that it is due to a doubly charged disodiated molecule leads to a molecular mass of 1870. The absence of a peak

TABLE I

POSSIBLE SUGAR COMPOSITIONS FOR $M_r = 736$ (*CF.*, FIG. 7)

Hexose	Pentose	Deoxyhexose	Hexuronic acid	4-O-Methylhexuronic acid
1	2	2	0	0
0	3	1	1	0
0	4	0	0	1
0	0	0	3	1
1	0	0	1	2

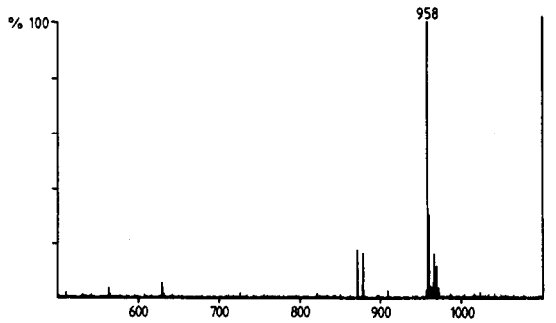

Fig. 8. Positive-ion electrospray mass spectrum of an unknown oligosaccharide obtained after enzymatic digestion of an apple 4 *M* KOH extract. For further explanation, see text.

at *m/z* 969 (or 947) excludes the presence of an acidic group in the molecule. Using the computer algorithm it was found that there is only one possible composition without acidic sugars, as is illustrated in Table II. Sugar composition analysis confirmed the absence of uronic acids. Actually, the oligomer was found to contain glucose, xylose, galactose and fucose in a ratio of 3:1:1:1. Therefore, from the possible compositions in Table II, $(hex)_8(pent)_2(deoxy)_2$ ($DP = 12$) can be selected as the correct configuration. Other workers have reported xyloglucan oligomers [29,30], but a dodecamer has never been described before. Considering the homology in xyloglucan structure, this oligomer probably has a backbone of six glucose residues and contains two Xyl–Gal–Fuc side-chains. More experiments are needed to determine the exact location of these side-chains.

CONCLUSIONS

Electrospray can be used as an ionization technique for the mass spectrometric analysis of oligosaccharides. The generation of sodiated molecules in the positive-ion mode and either deprotonated or chloridated molecules in the negative-ion mode is to be preferred. Electrospray ionization provides a better response than thermospray ionization for the larger oligosaccharides. Molecular mass information for oligosaccharides up to at least $M_r = 4000$ can be obtained. Spectra of acidic oligosaccharides in both the positive- and negative-ion modes are characterized by sodium exchange, which gives information on the number of acidic groups in the molecule. The better response found for small neutral and acidic oligosaccharides in the positive- than in the negative-ion mode becomes less pronounced for the larger oligosaccharides. Constant infusion of dimethyl-β-cyclodextrins appears to be an accurate and non-laborious method for the determination of the degree of methylation. Further, electrospray appears to be a powerful tool in the characterization of unknown oligosaccharide samples. The molecular mass determined for oligosaccharides allows the calculation of possible sugar compositions in terms of the number of, *e.g.*, pentose, hexose, deoxyhexose and uronic acids. Combination of these data with information concerning the origin of the polysaccharide and the extraction procedure applied generally provides useful information in identifying unknowns, in helping

TABLE II

POSSIBLE SUGAR COMPOSITIONS FOR $M_r = 1870$ (*CF.*, FIG. 8)

Hexose	Pentose	Deoxyhexose	Hexuronic acid	4-O-Methylhexuronic acid
8	2	2	0	0
7	3	1	1	0
6	4	0	2	0
6	0	0	5	0
7	4	0	0	1
7	0	0	3	1
8	0	0	1	2

with the interpretation of NMR data and in the characterization of enzyme activity on certain polysaccharides. Further work along these lines is in progress.

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