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Recent progress in high-performance anion-exchange chromatography–thermospray mass spectrometry of oligosaccharides

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ABSTRACT

The on-line combination of high-performance anion-exchange chromatography and mass spectrometry via a thermospray interface has proved to be a powerful tool in the characterization of sugar oligomers obtained by enzymatic digestion of plant cell wall polysaccharides. The potential of the method can be improved by the use of a new column material, CarboPac PA100, which requires lower sodium acetate concentrations for the elution of large sugar oligomers. Further, the application of multiple-ion detection optimizes the information obtained from the analysis by improving both the signal-to-noise ratio and the conservation of the chromatographic resolution. Negative-ion instead of positive-ion detection results in significantly better signals, especially for the larger sugar oligomers.

INTRODUCTION

One of the major themes in the developments in on-line liquid chromatography–mass spectrometry (LC–MS) in the past few years has been its application in biochemistry. LC–MS has found wide application in the analysis of bio(macro)molecules, such as nucleosides and nucleotides [1] and peptides and proteins [2–5]. However, relatively little attention has been paid to the LC–MS analysis of sugar oligomers. Small oligosaccharides have frequently been used to establish the absence of thermal degradation in LC–MS interfaces. In LC–MS studies of starch hydrolysates using a thermospray interface, thermally induced hydrolysis of oligosaccharides to monomeric units was observed [6–9]. Intact oligosaccharides have been studied by LC–MS using a moving-belt interface with fast atom bombardment ionization [10].

Recently, considerable progress has been made in the LC–MS analysis of oligosaccharides. Simpson *et al.* [11] demonstrated the possibility of direct coupling of high-performance anion-exchange chromatography (HPAEC) to MS by means of an anion micromembrane suppressor (AMMS). HPAEC can be considered to be one of the most powerful LC methods available for the separation of oligosaccharides [12]. Simpson

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et al. [11] demonstrated the analysis of monomeric and dimeric amino sugars using a thermospray interface. However, the protonated and ammoniated sugar dimer molecules analysed showed poor abundances, indicating thermal degradation in the thermospray interface.

One of the ways in which the thermal degradation of the sugar oligomers can be avoided is the application of the ionspray interface [13]. In contrast to the thermospray interface, no heat is used for the nebulization of the column effluent in the ionspray interface. On-line coupling of HPAEC and ionspray MS via an AMMS has been demonstrated by Conboy and co-workers [14,15]. The method was applied to the determination of high-mannose oligosaccharides that were obtained by the treatment of RNase B with endoglycosidase. Sugar oligomers of the type GlcNAc-(Man)$_n$ with $n = 5$–9 were observed in these experiments [15].

Recently, we demonstrated that the thermal degradation of sugar oligomers in the thermospray interface is due to the presence of ammonium acetate in the solvent used and can be attributed to thermally induced ammoniolysis of the oligosaccharides to their monomeric units. However, with a mobile phase containing low concentrations of sodium acetate, intact sodiated molecules can be observed in the positive-ion mode for maltodextrins up to a degree of polymerization (DP) of 10, e.g., after reversed-phase LC–MS [8,9]. This solvent system is also readily compatible with HPAEC in combination with an AMMS [16,17]. The HPAEC–MS system with the thermospray interface was applied to the analysis of oligosaccharides obtained by the enzymic degradation of plant cell wall polysaccharides. Sugar oligomers up to $DP = 10$ could be detected in these experiments [16,17].

The upper DP value achievable in HPAEC–MS is limited by two effects. First, larger sugar oligomers generally require higher sodium acetate concentrations for their elution. In our system, two AMMS in series are used to reduce the sodium concentration in the mobile phase to below 10^{-3} mol/l. This approach is successful as long as the sodium concentration in the mobile phase is kept below 0.4 mol/l. Sugar oligomers eluting at higher sodium concentrations are lost,

because the HPAEC system has to be disconnected from the LC–MS interface in order to avoid source contamination. Intense sodium acetate cluster ions are detected at higher sodium concentrations, resulting in signal instabilities and baseline noise [16,17]. Second, the response at higher DP values generally is significantly lower in thermospray MS under these conditions [17].

The studies described in this paper were aimed at the partial removal of these two limitations in order to extend the applicability of the approach. The discussion can be subdivided into two parts. The first part primarily describes improvements to the previously reported HPAEC–MS approach [16,17]. For instance, the use of a new commercially available column material, CarboPac PA100, which permits the elution of sugar oligomers at relatively lower sodium-acetate concentrations, has been tested in HPAEC–MS and the effects on the response of sugar oligomers are reported here. Further, a comparison is made between positive- and negative-ion detection of oligosaccharides under these conditions. Previously only positive-ion detection has been reported [16,17]. Negative-ion detection was investigated in order to improve the response, especially for the larger oligomers. In most experiments α-1,4-glucose oligomers (maltodextrins) were used as model compounds, but some results with other sugar oligomers are also described.

EXPERIMENTAL

The general experimental set-up was similar to that reported previously [16,17]. A Dionex (Sunnyvale, CA, USA) DX-300 modular ion chromatographic system, consisting of an EDM-2 solvent degassing unit, an AGP pump module, an LCM-3 chromatography module containing a Rheodyne (Cotati, CA, USA) Model 9126 all-PEEK injector with a 25-μl loop, a pulsed electrochemical detector (PED) with a gold electrode and two AMMS-II in series, was coupled via a Kratos (Manchester, UK) Spectroflow 400 LC pump, which acted as a booster pump, to a Finnigan MAT (San Jose, CA, USA) thermospray interface fitted on to a Finnigan MAT

TSQ-70 tandem mass spectrometer equipped with a 20-kV conversion dynode detection system. Regeneration of the AMMS systems was performed by a continuous flow of 0.1 mol/l sulphuric acid at a flow-rate of ca. 15 ml/min through the system.

Two different columns (250 mm × 4 mm I.D.) were used in these experiments: a CarboPac PA1 column which had also been used in the earlier experiments [16,17] and the new CarboPac PA100 column, which is expected to give oligosaccharide elution at lower sodium acetate concentrations. Gradient elution was applied using two solvents: (A) 0.1 mol/l aqueous sodium hydroxide and (B) 1 mol/l sodium acetate in 0.1 mol/l aqueous sodium hydroxide. Each chromatographic run was followed by a washing and re-equilibration step (10 min at 100% solvent B and 10 min at 100% solvent A). A flow-rate of 1.0 ml/min was used. For maltodextrin analysis, a linear gradient from 0 to 30% solvent B in solvent A in either 20 or 30 min was used. At a T-piece between the AMMS and the booster pump 10^{-4} mol/l aqueous sodium acetate was added at 0.5 ml/min.

Typical operating conditions of the thermospray interface were block temperature 350°C, vaporizer temperature 80°C and repeller potential 50 V in the positive-ion mode and −50 V in the negative-ion mode. Mass spectra were acquired from m/z 150 to 1500 at 3–5 s per scan. In multiple ion detection (MID) 5–10 ions were selected per segment, e.g., ions for $DP = 1$–5, ions for $DP = 6$–10. Switching from one segment to another was done at an appropriate moment during the run. The segments were acquired in 3–5 s.

Maltodextrin MD-25, a mixture of α-1,4-glucose oligomers, was obtained from Roquette (Lille, France).

RESULTS AND DISCUSSION

Improved response in HPAEC–MS over reversed-phase LC–MS

HPAEC is a very powerful tool in the analysis of oligosaccharides. The on-line coupling to thermospray mass spectrometry allows rapid molecular mass determination of the various constituents of (complex) oligosaccharide samples. In Fig. 1a, the peak areas measured with reversed-phase LC–MS (data from ref. 9) and HPAEC–MS for various α-1,4-glucose oligomers are plotted as a function of the DP value. The peak areas were measured under comparable conditions in multiple-ion detection and were corrected for the difference in the amount injected. Depending on the DP, a 2–8-fold improvement in the response is achieved in HPAEC–MS. Therefore, despite the unfavourable mobile phase composition, the HPAEC–MS combination with the AMMS for suppression of the sodium ions results in considerably better ionization characteristics than reversed-phase LC–MS
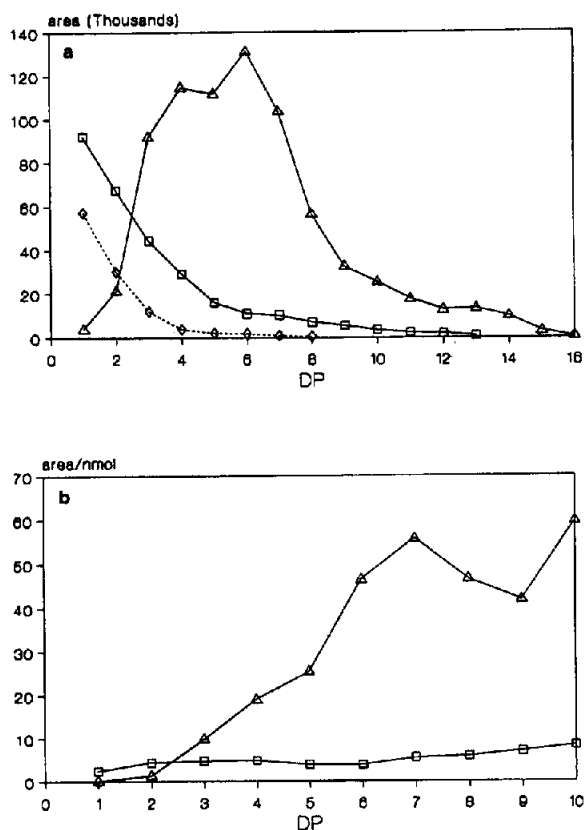

Fig. 1. Comparison of peak areas obtained by (◇) reversed-phase LC–MS, (□) positive-ion HPAEC–MS and (△) negative-ion HPAEC–MS as a function of the degree of polymerization (DP) for α-1,4-glucose oligomers. (a) Absolute peak areas corrected for the difference in injected amount; (b) peak area per nmole of oligomers actually present in the injected amount. HPAEC–MS with a PA100 column. Peak areas in arbitrary units.

with 10^{-4} mol/l aqueous sodium acetate as the mobile phase.

Comparison of PA1 and PA100 columns

The high sodium acetate concentrations needed in HPAEC, especially in the analysis of larger sugar oligomers, present difficulties in the coupling to thermospray mass spectrometry. Therefore, the introduction of the CarboPac PA100 column material is expected to be advantageous for HPAEC–MS. The most important practical difference between the older CarboPac PA1 and the new CarboPac PA100 packing materials is that the latter allows the elution of oligosaccharides at lower sodium acetate concentrations. This can be explained by the smaller particle diameter, i.e., 8.5 μm for PA100 compared with 10 μm for PA1, which enhances the ion exchange, and the lower capacity, i.e., 90 μequiv. for the PA100 compared with 100 μequiv. for the PA1 column. In Fig. 2, the sodium acetate concentration required for the elution of a series of α-1,4-glucose oligomers is plotted as a function of DP. Significant lower sodium concentrations are required to elute most of the oligomers. As the sodium acetate concentration is a limiting factor in HPAEC–MS, sugar oligomers with higher DP values can be analysed. Whereas α-1,4-glucose oligomers up to $DP = 6$ could be detected with the PA1 column [17], oligomers up to $DP = 12$ were detected in
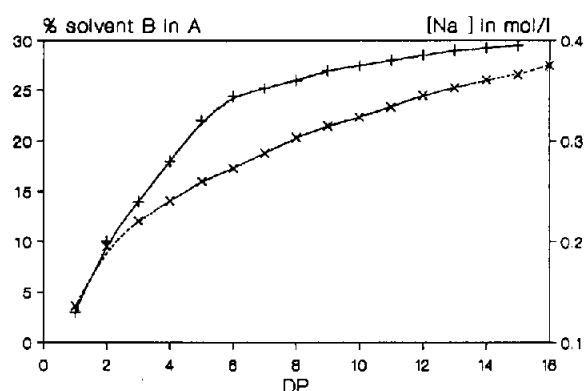

Fig. 2. Percentage of solvent B and corresponding sodium concentration in the mobile phase required for the elution of α-1,4-glucose oligomers as a function of the degree of polymerization (DP) for (+) CarboPac PA1 and ($\times$) CarboPac PA100 columns.

experiments with the PA100 column (see also Fig. 1). With this particular sample, the detection of higher DP values is limited by the low concentration of these oligomers in the sample analysed [less than 1% (w/w) for $DP > 10$] and by the decrease in the signal at higher DP values. The response for α-1,4-glucose oligomers, expressed in peak area per nmole, is plotted as a function of the DP value in Fig. 1b. In practice, less than 0.2 nmol of $DP = 10$ is injected.

Improved resolution in HPAEC–MS by multiple-ion detection

In a previous report [16], it was claimed that the poor chromatographic peak shapes often observed at low sample concentrations in HPAEC–MS when operated in the full-scan mode would be significantly improved by the use of MID. In Fig. 3a, mass chromatograms from the HPAEC–MS analysis of an arabinogalactan digest are shown for Gal_2Ara obtained with both full-scan (upper trace) and MID acquisition (lower trace). Whereas from the full-scan mass chromatogram no conclusions can be drawn on the number of Gal_2Ara isomers present, three isomers are readily observed in the MID chromatogram.

With the same sample, it was observed that the separation in some instances is influenced by the amount of compound injected. In Fig. 3b, mass chromatograms are shown for Gal_2. A broad peak is observed in the upper trace, leading to the assumption that two components are present. When the sample is diluted tenfold and re-injected, these two components are well separated and apparently present in a ca. 1:15 ratio. With full-scan acquisition, the existence of two oligomers was not observed. The apparent column overloading with respect to the galactose dimers is used to permit the detection of minor components with higher DP values in the mixtures.

The presence of these galactose dimers is determined by the structure of the arabinogalactan and reveals the complex nature of this type of polysaccharide. Although one of the galactose dimers found originates from the linear β-1,4-linked galactan backbone, the second galactose
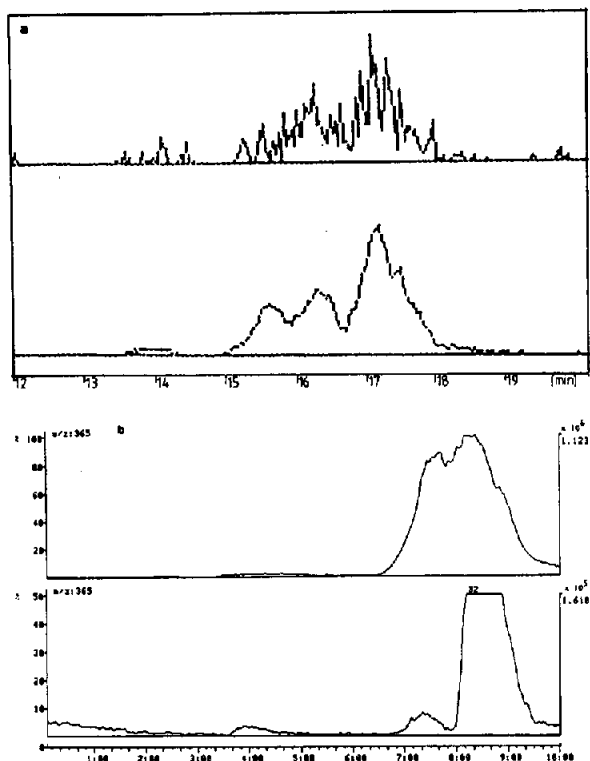
Fig. 3. Selected mass chromatograms from the HPAEC–MS analysis of arabinogalactan digests. (a) Comparison of full-scan (upper trace) and MID (lower trace) acquisition for Gal$_2$Ara. Estimated amount injected: ca. 100–200 ng per component. (b) Comparison of two sample concentrations, differing by a factor of 10, for Gal$_2$. CarboPac PA100 column. Estimated amount injected: ca. 150 ng for the major component.

dimer (present in minor amounts) suggests the presence of single-unit side-chains of galactose. This observation is confirmed by methylation analysis. Further results with the HPAEC–MS analysis of arabinogalactan digests are discussed in detail elsewhere [18].

As the application of MID leads to improvements in both signal-to-noise ratio and peak shape, our strategy in handling unknown samples has been changed. Initially, unknowns were only analysed with full-scan acquisition. Now, each sample is analysed twice. First, the sample is analysed with full-scan acquisition to detect major peaks and obtain a general view on the sample composition. Next, it is analysed using various appropriate segments of MID. The instrument control language (ICL) of our instru-

ment allows an automatic time-based or event-based switching between the various MID segments. In composing the MID segments, additional m/z values are taken into account for sugar oligomers differing by one or two units from the oligosaccharides already detected with full-scan acquisition. This strategy led to the acquisition of various pieces of new information, e.g., in the analysis of arabinogalactan oligomers [18]. Switching between the various MID segments must be done with care, considering the unpredictable elution order often observed in HPAEC–MS. The retention times of some peaks observed in a glucuronoarabinoxylan digest may serve as an example. The oligosaccharides in this sample consist of a linear β-1,4-xylose oligomer, branched with α-1,2- and α-1,3-arabinose units and a glucuronic or 4-O-methylglucuronic acid unit. Components with identical sugar composition but differing in the position of the arabinose units on the xylose backbone are found to give widely differing retention times; for instance, two GlcA-Pentose$_5$ isomers are detected at 21:23 and 27:16 min, whereas the latter peak almost co-elutes with a GlcA-Pentose$_6$ isomer eluting at 27:29 min. Sometimes a large time window is required in the MID segments. This example also indicates a current limitation of the HPAEC–MS approach: no structural information has yet been obtained. Therefore, the identity of the arabinoxylan isomers cannot be determined as arabinose and xylose are isomers. Obviously, on-line coupling of HPAEC and tandem mass spectrometry, permitting the determination of the sequence of monomeric units in the oligosaccharide, would be of great help, but at present little structural information has been obtained in attempts to fragment the sodiated sugar oligomers by collision-induced dissociation.

Negative-ion detection

In previous experiments only positive-ion detection was performed [16,17]. In order to investigate whether better signal-to-noise ratios can be obtained, some experiments were performed in the negative-ion mode. In principle, one would expect that optimization of the experimental conditions, especially the solvent

composition, would be required but these experiments were not performed. The solvent composition used in the negative-ion mode was identical with that used in the positive-ion mode. Possible improvement by optimization of the conditions is currently under investigation. Obviously, the present conditions ideally match the operation of HPAEC–MS.

The negative-ion mode was tested for α-1,4-glucose and arabinogalactose oligomers. The mass spectra obtained under these conditions are surprisingly complex. Typical negative-ion mass spectra for Glc_4 and Glc_8 are shown in Fig. 4.

For small α-1,4-glucose oligomers, i.e., up to $DP = 4$, primarily three ions are detected in the negative-ion mode, i.e., $[M - H]^-$ at $m/z = M_r - 1$, $[M + OAc]^-$ at $m/z = M_r + 59$ and $[M +$

$HSO_4]^-$ at $m/z = M_r + 97$. The peak areas in the mass chromatograms for these ions as a function of DP are plotted in Fig. 5a. At $DP < 4$ the acetate or hydrogensulphate adducts are most abundant, whereas for DP between 5 and 7 the deprotonated molecule is most abundant, and at even higher DP values the doubly charged ions (see below) are most abundant. Further, some peaks due to dimers are detected, e.g., $[2M - H]^-$ at m/z 359, 683, 1007 and 1331 and $[2M + HOAc + OAc]^-$ at m/z 479, 803, 1127 and 1451 for $DP = 1–4$, respectively. The relative abundance of these ions is generally below 25%. Frequently, additional adduct peaks are detected at $m/z + 120$, which are probably due to the
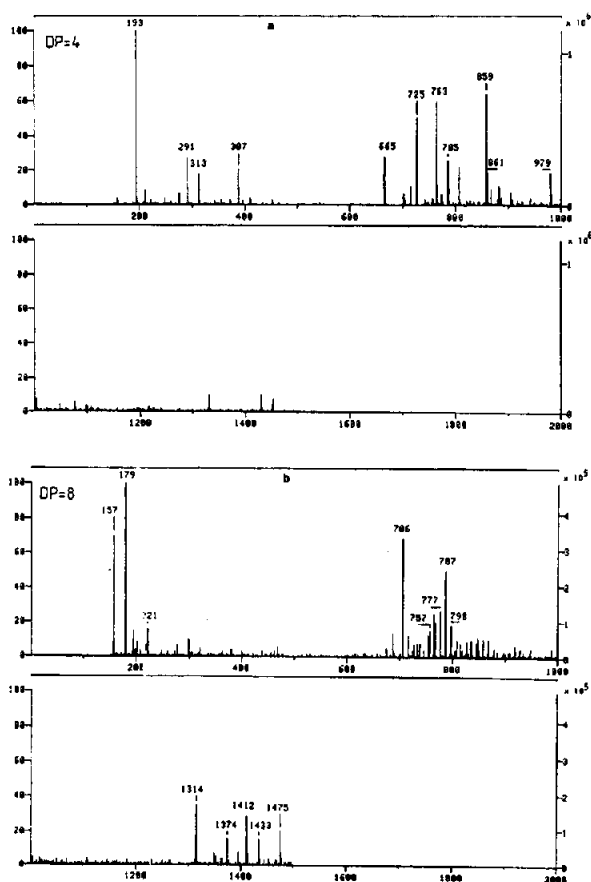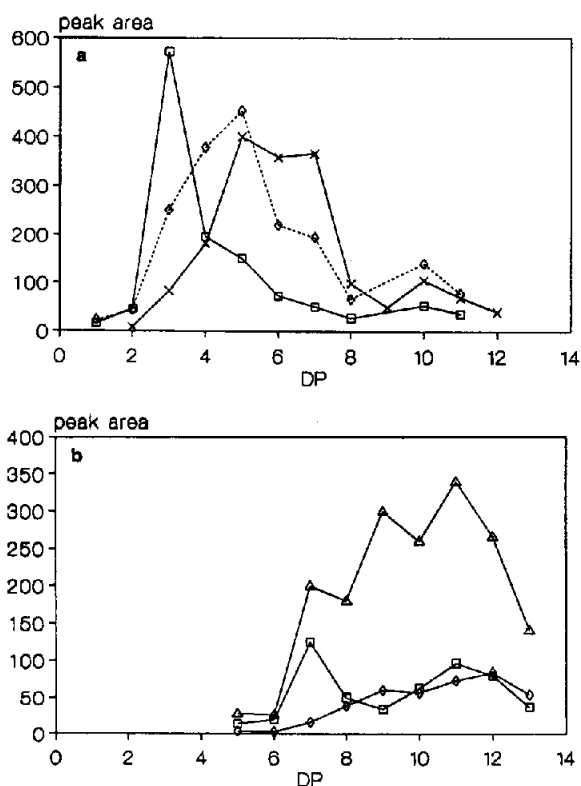

Fig. 4. Negative-ion thermospray mass spectra of α-1,4-glucose oligomers with (a) $DP = 4$ and (b) $DP = 8$ obtained by HPAEAC–MS (cf., Table I). For conditions, see text.

Fig. 5. Peak areas with negative-ion detection as a function of DP. (a) Peak areas for the singly charged ions, ($\times$) $[M - H]^-$ at $m/z = M_r - 1$, ($\square$) $[M + OAc]^-$ at $m/z = M_r + 59$ and ($\Diamond$) $[M + HSO_4]^-$ at $m/z = M_r + 97$. (b) Peak areas for the doubly charged ions, ($\triangle$) $[M + SO_4]^{2-}$ at $m/z = (M_r + 96)/2$, ($\square$) $[M - H + OAc]^{2-}$ or $[M + 2OAc]^{2-}$ at $m/z = (M_r + 58)/2$ and $(M_r + 118)/2$, respectively, and ($\Diamond$) $[M + 2HSO_4]^{2-}$ or $[M - 2H]^{2-}$ at $m/z = (M_r + 194)/2$ and $(M_r - 2)/2$, respectively. Peak areas in arbitrary units. For further explanation, see text.

addition of two acetic acid molecules. The latter is also observed in the positive-ion mode.

For larger sugar oligomers, typically $DP > 5$, various doubly charged peaks are detected. The most abundant doubly charged peak is due to $[M + SO_4]^{2-}$, and doubly deprotonated ions and acetate and hydrogensulphate adducts are also observed. A summary of the various peaks detected and typical m/z values for α-1,4-glucose oligomers with $DP = 4$ and 8 is given in Table I. The peak areas in the mass chromatograms for these doubly charged ions as a function of DP are plotted in Fig. 5b and the peak areas for the singly charged ions in Fig. 5a. For clarity, the peak areas for only three types of ions are plotted in Fig. 5b. The peak areas of $[M - 2H]^{2-}$ and $[M + 2OAc]^{2-}$ are almost identical with those of $[M + 2HSO_4]^{2-}$ and $[M - H + OAc]^{2-}$, respectively. As in these experiments the mass spectrometer was scanning up to m/z 2000, the singly charged ions are only detected up to $DP = 12$. The relative abundance of the singly charged ions of the oligomers with high DP values may be underestimated as a result of the discrimination effects common to a quadrupole mass analyser.

Apparently, some fragmentation is also observed, especially to monomeric units, resulting in a peak at m/z 179 in the mass spectra for some of the DP values. In other mass spectra, other peaks are detected that are not readily explained, e.g., m/z 193 in the spectrum for $DP = 4$ (see Fig. 4a). The presence of glucuronic acid in this commercial sample is not expected. In general, little attention was paid to these not very reproducible peaks.

In general, the formation of (many) adduct ions is unfavourable to the sensitivity, because the ion intensity is spread over a number of peaks instead of being concentrated in only one peak. For quantification but also for molecular mass determination, as provided in this study, a single peak in the mass spectrum is preferred. However, in the present instance the observation of adduct ions is hardly a problem in that respect. The summed peak areas for $[M - H]^-$, $[M + OAc]^-$, $[M + HSO_4]^-$ and $[M + SO_4]^{2-}$ are plotted as a function of the DP value and compared with peak areas observed with positive-ion detection (see Fig. 1). It appears that a much better response is achieved in the negative- than in the positive-ion mode for most

TABLE I

TYPICAL IONS DETECTED FOR α-1,4-GLUCOSE OLIGOMERS IN THE NEGATIVE-ION MODE WITH HPAEC–MS USING A THERMOSPRAY INTERFACE

Charge	Ion identity	m/z value relative to M_r	m/z value for Glc$_4$	m/z value for Glc$_8$
−1	$[M - H]^-$	$M_r - 1$	665	1313
−1	$[M + OAc]^-$	$M_r + 59$	725	1373
−1	$[M + HSO_4]^-$	$M_r + 97$	763	1411
−1	$[M + NaSO_4]^-$	$M_r + 119$	785	1433
−1	$[M + HOAc + OAc]^-$	$M_r + 119$	785	1433
−1	$[2M - H]^-$	$2M_r - 1$	1331	n.d.[a]
−1	$[2M + HSO_4]^-$	$2M_r + 97$	1429	n.d.
−1	$[2M + HOAc + OAc]^-$	$2M_r + 119$	1451	n.d.
−1	$[2M + NaSO_4]^-$	$2M_r + 119$	1451	n.d.
−2	$[M - 2H]^{2-}$	$(M_r - 2)/2$	n.d.	656
−2	$[M - H + OAc]^{2-}$	$(M_r + 58)/2$	n.d.	686
−2	$[M + SO_4]^{2-}$	$(M_r + 96)/2$	n.d.	705
−2	$[M + 2OAc]^{2-}$	$(M_r + 118)/2$	n.d.	716
−2	$[M + 2HSO_4]^{2-}$	$(M_r + 194)/2$	n.d.	754

[a] Not detected.

DP values, especially the higher values. This would also be the case when the peak areas for only one specific m/z value, e.g. only for the $[M + SO_4]^{2-}$ ion, per DP value was used in the comparison (data not shown). In order to appreciate fully the comparison in Fig. 1, it must be pointed out that the peak areas in the negative-ion mode were obtained from data acquisition in the scanning mode (scan range 150–2000), whereas in the positive-ion mode multiple-ion detection on singly and doubly charged sodiated molecules was performed. In general, multiple-ion detection results in at least a tenfold better response than that obtained in the scanning mode. Hence the negative-ion mode appears to be preferred over the positive-ion mode for the characterization of oligosaccharides. Further study, especially directed at the optimization of the experimental conditions and application to a wider variety of samples, e.g., acidic oligosaccharides, is required. Negative-ion detection may be especially helpful in more advanced studies directed at structure elucidation, i.e., determination of sugar type, linkage type and position, as from the literature it appears that the deprotonated molecules are more readily fragmented by collision activation in a triple quadrupole instrument than the sodiated molecules observed in the positive-ion mode. Further research along these lines is in progress.

An interesting topic is the origin of the various ions detected. The observation of deprotonated ions and acetate adduct ions is not very surprising. As a result of the removal of sodium ions by the AMMS, the solvent entering the mass spectrometer is acetic acid with concentrations following the sodium acetate gradient. Sodium acetate is converted into acetic acid in the AMMS. The observation of hydrogensulphate and sulphate adduct ions indicates leakage through the membrane of the sulphuric acid that is used for regeneration of the AMMS. As the membrane system was functioning properly and was not ruptured, which may be concluded from the fact that the introduction of sodium acetate in the range 0.1–0.3 mol/l within a few minutes results in complete contamination of the thermospray ion source and complete loss of signal, it must be concluded that some sulphuric acid is

diffusing through the membrane. This was already known from previous studies, as protonated sulphuric acid and water adducts thereof have previously been detected at too high sulphuric acid concentrations of the regenerant solvent [16]. Obviously, limited diffusion of sulphuric acid through the membrane significantly influences the ionization conditions in the thermospray source. To our knowledge, the addition of sulphuric acid to the solvent for thermospray has not been reported previously. It might also prove useful in the negative-ion detection of other compounds.

CONCLUSIONS

Significant progress in the method development for oligosaccharide characterization by HPAEC–MS has been made. Higher oligomers can be detected as a result of the lower sodium acetate concentration needed for oligosaccharide elution on the new CarboPac PA100 column. The use of multiple-ion detection in addition to the scanning mode results in improved chromatographic peak shapes in MS detection. As a result, more information on the samples analysed is obtained. The use of negative-ion detection in HPAEC–MS with a thermospray interface looks promising, especially because of the improved response for oligosaccharides with larger DP values.

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