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Characterization of sugar oligomers by on-line high-performance anion-exchange chromatography–thermospray mass spectrometry

R. A. M. van der Hoeven and W. M. A. Niessen

Division of Analytical Chemistry, Center for Bio-Pharmaceutical Sciences, University of Leiden, P.O. Box 9502, 2300 RA Leiden (Netherlands)

H. A. Schols

Department of Food Science, Wageningen Agricultural University, Bomenweg 2, 6703 HD Wageningen (Netherlands)

C. Bruggink

Dionex BV, P.O. Box 9338, 4801 LH Breda (Netherlands)

A. G. J. Voragen

Department of Food Science, Wageningen Agricultural University, Bomenweg 2, 6703 HD Wageningen (Netherlands)

J. van der Greef

Division of Analytical Chemistry, Center for Bio-Pharmaceutical Sciences, University of Leiden, P.O. Box 9502, 2300 RA Leiden (Netherlands)

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ABSTRACT

The on-line coupling of high-performance anion-exchange chromatography and thermospray mass spectrometry via an anion-micromembrane suppressor and a booster pump is described. The system was applied to the analysis of homologous series of oligosaccharides. Among others, the mass spectrometric detection of β-1,4-xylose oligomers up to degree of polymerization (DP) 25 is demonstrated. Further, the system was used in the analysis of more complex oligosaccharide samples, containing mixed oligomers of hexoses, pentoses and uronic acids. In such samples oligomers up to DP 10 can be detected. The potential use of this approach in the characterization of oligosaccharides obtained from (enzymic) degradation of plant cell wall oligosaccharides is discussed.

INTRODUCTION

High-performance anion-exchange chromatography (HPAEC) is a powerful method for the separation of sugars [1–5]. The technique is widely used in the analysis of food for sugars and in the characterization of sugar oligomers obtained by (enzymic) deg-

Correspondence to: W. M. A. Niessen, Division of Analytical Chemistry, Center for Bio-Pharmaceutical Sciences, University of Leiden, P.O. Box 9502, 2300 BA Leiden, Netherlands.

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radation of glycoproteins and plant cell wall polysaccharides. One of the problems with HPAEC, especially in the latter fields of application where in most instances no standards are available, is the difficulty of predicting the elution order of heterogeneous sugar oligomers. Further, a full characterization of sugar oligomers requires the determination of the sugar composition and the sugar linkage positions. In these respects, the abilities of mass spectrometry (MS), especially in an on-line combination with HPAEC, are expected to be extremely useful. However, the solvent conditions generally used in the HPAEC of sugar oligomers are not compatible with on-line MS detection. The problems with the high salt concentrations of the mobile phase, *e.g.*, 0.1 mol/l sodium hydroxide and a gradient of 0–0.5 mol/l sodium acetate, as used in the separation of sugar oligomers, have already been solved for conductivity detectors in ion chromatography by the introduction of a micromembrane suppressor [4–7], which efficiently replaces either cations or anions by protons or hydroxyl ions. An anion micromembrane suppressor (AMMS) can be used in combination with HPAEC in sugar analysis to remove the excess sodium ions. On-line desalting with an AMMS prior to fraction collection for off-line fast atom bombardment MS or ^{1}H NMR analysis has been described by Basa and Spellman [4]. Simpson *et al.* [7] demonstrated that with an AMMS the sodium concentration can be decreased from 0.1 mol/l to a level compatible with on-line thermospray mass spectrometry. The system was applied to analyse for mono- and diamino sugars [7]. Next, coupling of ion-exchange chromatography to MS via a micromembrane suppressor was described for ionspray [8] and particle-beam [9] interfaces.

In this paper, the coupling of HPAEC to thermospray MS via an AMMS is described for the characterization of sugar oligomers. The technology as already described by Simpson *et al.* [7] was applied without many changes. However, the application range has been significantly widened, especially in terms of the maximum degree of polymerization of the oligosaccharides analysed [degree of polymerization (DP) = 2 by Simpson *et al.* [7] and DP > 10 in this work] and the maximum allowable sodium concentrations in the mobile phase (0.1 mol/l by Simpson *et al.* [7] and up to 0.4 mol/l in this work). Attention is mainly focused on the characterization of the HPAEC–MS system by means of analyses for homologous non-branched sugar oligomers. An example of the HPAEC–MS analysis of a more complex sample obtained by enzymic degradation of plant cell wall polysacchsrides is also described. The applicability of HPAEC–MS in the latter field is discussed in more detail elsewhere [10].

EXPERIMENTAL

Apparatus

The general experimental set-up for HPAEC–MS with a thermospray interface is similar to that used by Simpson *et al.* [7]. The total system consisted of a Dionex (Sunnyvale, CA, USA) DX-300 chromatography system, a Kratos (Manchester, UK) Spectroflow 400 LC pump, acting as booster pump, and a Finnigan MAT (San José, CA, USA) TSQ-70 mass spectrometer equipped with a Finnigan MAT thermospray interface. A schematic diagram of the set-up is given in Fig. 1.

The Dionex DX-300 chromatography system consisted of an EDM-2 solvent degas unit, an AGP pump module and an LCM-3 chromatography module, containing a Rheodyne (Cotati, CA, USA) Model 9126 injector (25-μl loop), a pulsed electrochemical detector with gold electrode and two AMMS-II micromembrane suppressors in series, regenerated with 10–15 ml/min of 0.15 mol/l sul-

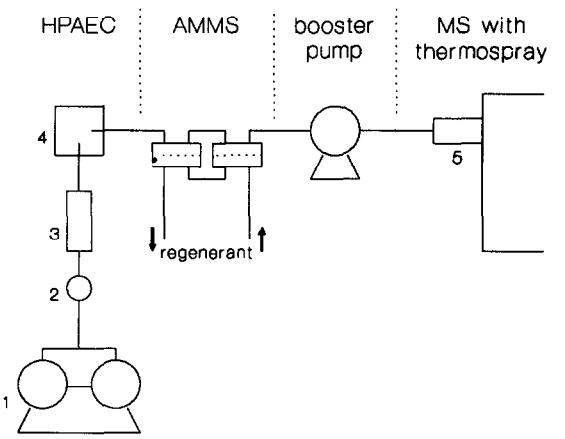

Fig. 1. Schematic diagram of the experimental set-up. 1 = AGP pump module; 2 = injection valve with 25-μl loop; 3 = 250 mm × 4 mm I.D. CarboPac PA1 column; 4 = electrochemical detector; 5 = thermospray interface.

phuric acid delivered by a Chem/Tech Iwaki (Lowell, MA, USA) Model EP B15 pump. The effluent from the AMMS was mixed with 1 ml/min of 0.1 mmol/l aqueous sodium acetate and delivered to the thermospray interface by means of the booster pump. The thermospray interface was operated in the positive-ion mode [scanning over the range m/z = 150–1500 or with multiple ion detection (MID) at 3–5 s per scan] with a block temperature of 350°C, a vaporizer temperature of 100°C and a repeller potential of 50 V.

Ion-moderated partition chromatography was performed using a Spectra-Physics (San José, CA, USA) Model SP8800 pump in combination with a Model SP8800 autosampler, a column oven and a Winner Integration Software package. The effluent was monitored using a Shodex Model SE 61 refractive index detector (Showa Denka, Tokyo, Japan).

Chromatography

In HPAEC, a Dionex CarboPac PA1 column (250 mm × 4 mm I.D.) was used. Gradient elution was performed using mixtures of 0.1 mol/l sodium hydroxide (solvent A) and 1 mol/l sodium acetate in 0.1 mol/l sodium hydroxide (solvent B). The flow-rate was 1 ml/min. The gradient programmes used are given in Table I.

In ion-moderated partition chromatography, a Aminex (Bio-Rad Labs., Richmond, CA, USA) HPX-22H column (300 mm × 7.8 mm I.D.) was used with 5 mmol/l sulphuric acid as the mobile phase at a flow-rate of 0.2 ml/min at 85°C. Glucose and xylose were used as standards for the determination of the mass percentages of α-,4-glucose and β-1,4-xylose oligomers, respectively.

TABLE I

GRADIENT PROGRAMME FOR THE SAMPLES ANALYSED

CarboPac PA1 column. Solvent A: 0.1 mol/l sodium hydroxide. Solvent B: 1 mol/l sodium acetate in 0.1 mol/l sodium hydroxide. Flow-rate: 1 ml/min. Each programme ends with a washing step consisting of a linear gradient up to 100% B in 5 min and a re-equilibration step of 15 min at 0% B.

Sample	Gradient programme (%B in A)
α-1,4-Glucose	0–30% B in 40 min
α-1,5-Arabinose	0% B for 5 min, 0–40% B in 28 min
β-1,4-Xylose	0–5% B in 5 min, 5–30% B in 40 min
Pectin digest	0% B for 5 min, 0–30% B in 21 min
Arabinoxylan digest	0–30% B in 30 min

Samples

All monomeric sugars were obtained from commercial sources. Maltodextrins (α-1,4-glucose oligomers, MD-25) were obtained from Roquette (Lille, France). The α-1,5-arabinose oligomers were prepared by degrading a linear arabinan with pure enzymes as described by Voragen *et al.* [11]. Acetylated β-1,4-xylose oligomers were isolated from birchwood by steam extraction and were kindly provided by Dr. J. Puls (Institute of Wood Chemistry and Chemical Technology of Wood, BFH, Hamburg, Germany). A pectin digest was obtained by incubation of a pectic polysaccharide fraction with Pectinex Ultra SP (Novo Ferment, Basle, Switzerland). Arabinoxylan oligomers were isolated by chromatography over Bio-Gel P2 after incubation of wheat arabinoxylan with endoxylanase [12].

RESULTS AND DISCUSSION

HPAEC–MS coupling

The most important aspect of coupling HPAEC and MS via a thermospray interface is the proper functioning of the AMMS. With the present set-up, where two AMMS systems are used in series, the sodium ions could be removed sufficiently to allow thermospray MS detection as long as the sodium concentration in the mobile phase did not exceed 0.4 mol/l. Otherwise, significant salt deposits will occur in the ion source, which hampers further analysis. When higher sodium concentrations are required in the gradient programme, such as in the washing procedure performed after each run, the HPAEC system was generally disconnected from the mass spectrometer as soon as the 0.4 mol/l sodium limit was exceeded (corresponding to 30% solvent B in solvent A). As a result, the maximum degree of polymerization that can be determined by HPAEC–MS is limited, the extent depending on the type of sugar oligomers. Given the gradient programmes in Table I, the percentage of solvent B and the sodium concentration in the mobile phase at which a particular oligomer elutes are plotted in Fig. 2 as a function of DP for homologous oligomers of α-1,4-glucose, α-1,5-arabinose and β-1,4-xylose.

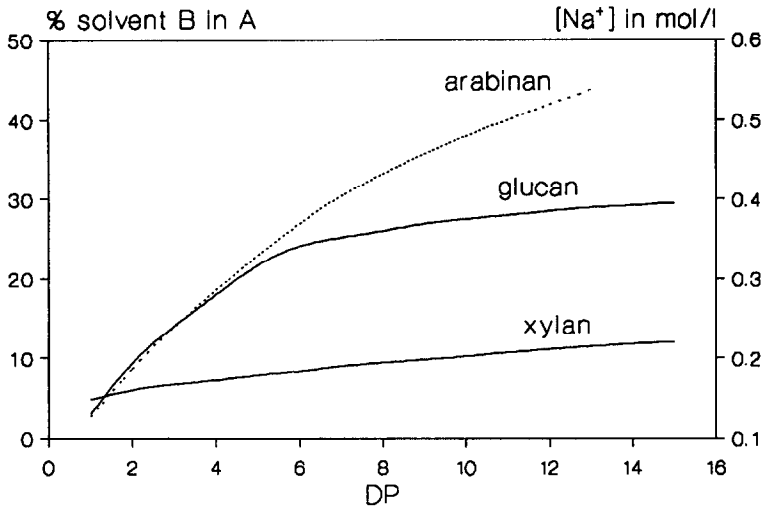

Fig. 2. Plot of the percentage of solvent B and the sodium acetate concentration at which a particular oligomer elutes as a function of the degree of polymerization for oligomers of α-1,4-glucose, α-1,5-arabinose and β-1,4-xylose. For conditions, see Table I.

The data handling, especially after the analysis of unknown samples, is elaborate. As a result of the significant background signal from sodium acetate cluster ions, especially with sodium concentrations between 0.2 and 0.4 mol/l, no peaks are generally detected in the total ion current chromatograms. Therefore, mass chromatograms must be constructed for the known m/z values of series of oligomers and searched for the presence of peaks. In practice, with the type of samples analysed, the searching can be performed using a limited number of m/z values. It must be emphasized that poor total ion current chromatograms are frequently found in LC–thermospray MS.

Mass spectra of oligosaccharides

Intact oligosaccharides can be studied by thermospray MS via ionization with akali metal ions, *e.g.*, sodium or lithium, but not in the presence of the commonly used ammonium acetate [13,14]. This is also possible using the solvent system obtained after desalting by AMMS in HPAEC–MS. The mass spectra of the sugar oligomers under these conditions are dominated by the presence of strong sodiated molecules at $m/z = M_r + 23$, while with higher DP values doubly charged disodiated molecules are observed at $m/z = (M_r + 46)/2$. No fragmentation is observed.

The peak positions in the mass spectra of homologous sugar oligomers are readily predictable, as subsequent members in a series differ by either 132 (pentose units) or 162 (hexose units) for singly charged ions, and 66 or 81, respectively, for doubly charged ions. From the mass spectrum obtained under these conditions no discrimination is possible between various isomeric sugars, *e.g.*, the pentoses xylose and arabinose or the hexoses glucose and galactose.

α-1,4-Glucose oligomers

Previously, reversed-phase LC–MS data for α-1,4-glucose oligomers up to DP = 10 have been reported [14]. In that work, it was primarily the sensitivity, badly influenced by the peak broadening owing to the separation of the sugar anomers, which prevented the observation of peaks at higher DP values. With HPAEC–MS the same sample of α-1,4-glucose oligomers (MD-25, 25 μg injected) was analysed. Glucose oligomers could be detected up to DP = 6. Oligomers with higher DP values were not detected owing to a lack of sensitivity, the low concentration of the higher oligomers and the high sodium concentration necessary for the elution of these oligomers (*cf.*, Fig. 2).

The response of the first oligomer in the series (glucose) is significantly lower than that of the dimer and trimer. Whereas in the reversed-phase LC–MS experiments the observed peak areas of the first

three oligomers are in the proportions *ca.* 10:5:2, in HPAEC–MS it is 5:10:8 for the monomer, dimer and trimer, respectively. From ion-moderated partition chromatography with refractive index detection it was calculated that the MD-25 sample contains *ca.* 14% monomer, 10% dimer and 9% trimer. The decrease in response in the HPAEC–MS experiments can be attributed to losses of the small monomers in the AMMS system, although experiments with a single AMMS performed by Dionex indicated losses of only *ca.* 20% for the monomers [15]. However, an unexpected low response for the monomeric sugar was observed with the other samples analysed also (see, for instance, the data on xylose oligomers discussed below).

Significant additional peak broadening in HPAEC–MS is expected owing to the large dead volume between the electrochemical detector and mass spectrometer, *i.e.*, two AMMS-II systems, each with 40 μl dead volume, the pump head of the booster pump and the necessary tubings and connections. The peak broadening in terms of peak standard deviation (measured at 0.6 of the peak height) was evaluated for the glucose dimer and trimer from the α-1,4-glucose sample. Peak standard deviations of *ca.* 6 and 7.7 s were observed in the chromatogram with the electrochemical detector and in the mass chromatograms, respectively, yielding an effective peak standard deviation of the dead volume of *ca.* 4.8 s (assuming independence of peak standard deviations). In general, no significant decrease in resolution due to the additional peak broadening was experienced.

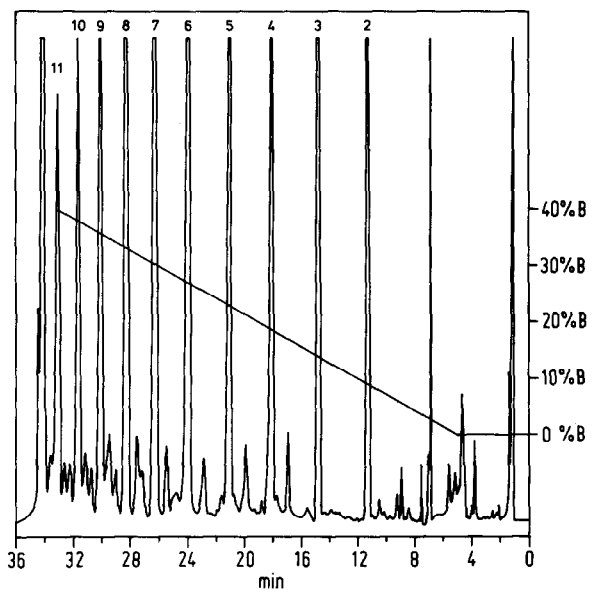

Fig. 3. HPAEC of α-1,5-arabinose oligomers monitored with electrochemical detection. The gradient programme is indicated (*cf.*, Table I); for other experimental conditions, see text.

α-1,5-Arabinose oligomers

The chromatogram of α-1,5-arabinose oligomers, as obtained with electrochemical detection, is given in Fig. 3; the gradient programme is also indicated. In this particular chromatogram, arabinose oligomers were detected up to DP = 11. By extending the gradient programme to 50% B, oligomers of higher DP values can also be detected. In the HPAEC–MS of this α-1,5-arabinose sample, oligomers could be detected up to DP = 9 (using MID). At low DP values, only singly charged ions are detected, whereas at DP values exceeding 5 an increasing intensity of doubly charged ions is observed. The relative intensities of singly and doubly charged ions as a function of the DP value for the α-1,5-arabinose oligomers are shown in Fig. 4. The response for the arabinose oligomers with DP > 5, based on summed peak areas of singly and doubly charged ions, is fairly constant. From the gradient programme indicated in Fig. 3, it can be concluded that DP = 7 is eluting at 30% B (0.4 mol/l sodium in the mobile phase), whereas DP = 8 and 9 are eluting at even higher sodium concentrations.

β-1,4-Xylose oligomers

While the study of oligomers with high DP values is hampered by the required sodium acetate concentration in the mobile phase with α-1,4-glucose and α-1,5-arabinose oligomers, this is not the case with β-1,4-xylose oligomers. The latter elute at significantly lower sodium acetate concentrations, as demonstrated in Fig. 2. Therefore, it must be possible to detect oligomers with higher DP values and thus with higher *m/z* ratios.

A series of mass chromatograms reconstructed from the full-scan data for the *m/z* values of the doubly charged ions of DP = 12–17 are given in Fig. 5. In full-scan acquisition (*m/z* = 150–1500 with 5 s per scan), xylose oligomers could be detected up to DP = 16 as doubly charged ions at *m/z* = 1088.

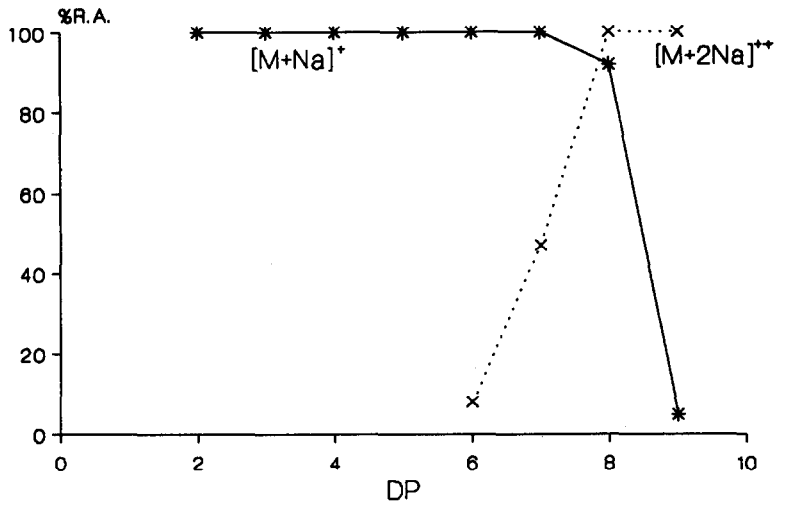

Fig. 4. Relative intensities of the $[M + Na]^+$ and $[M + 2Na]^{2+}$ ions of α-1,5-arabinose oligomers as a function of the degree of polymerization. R.A. = Relative abundance.

For xylose oligomers and for other pentose oligomers the singly charged ions have odd m/z ratios and the doubly charged ions even m/z ratios.

In MID, using various groups of 5–10 ions and stepping from one appropriate group to another during elution, the peak shapes and signal-to-noise ratios significantly improve. As a result, detection of xylose oligomers up to DP = 25 (molecular mass 3318) is now achievable. The peak of the doubly charged ion of DP = 25, still having an acceptable signal-to-noise ratio, is found at $m/z = 1682$. A series of mass chromatograms demonstrating the detection of these large oligomers is shown in Fig. 6. At high DP values the separation of the various

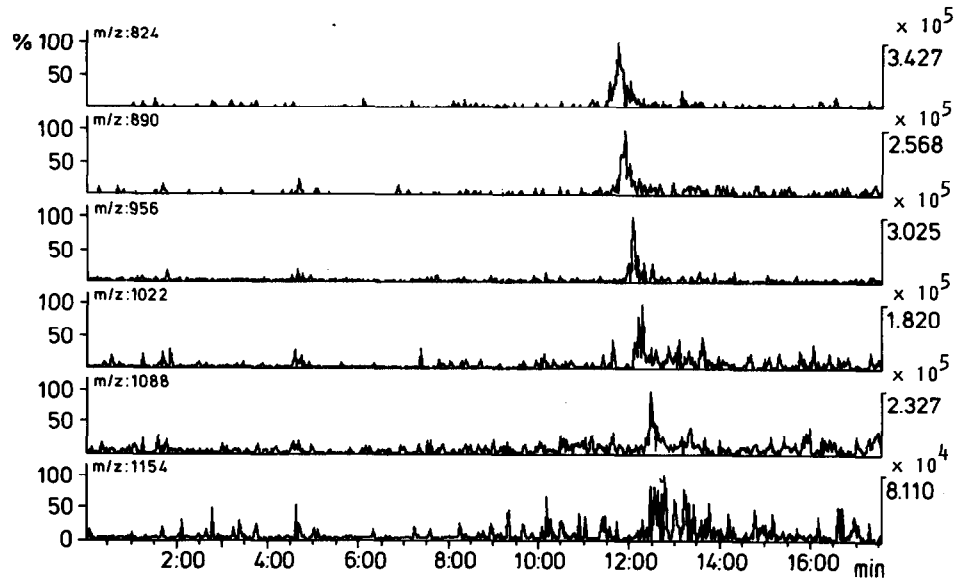

Fig. 5. HPAEC–MS of β-1,4-xylose oligomers. Mass chromatograms of doubly charged ions for DP = 12–17 reconstructed from full-scan acquisition (m/z = 150–1500 with 5 s per scan). Right-hand abscissa is ion intensity in arbitrary units. For conditions, see text.

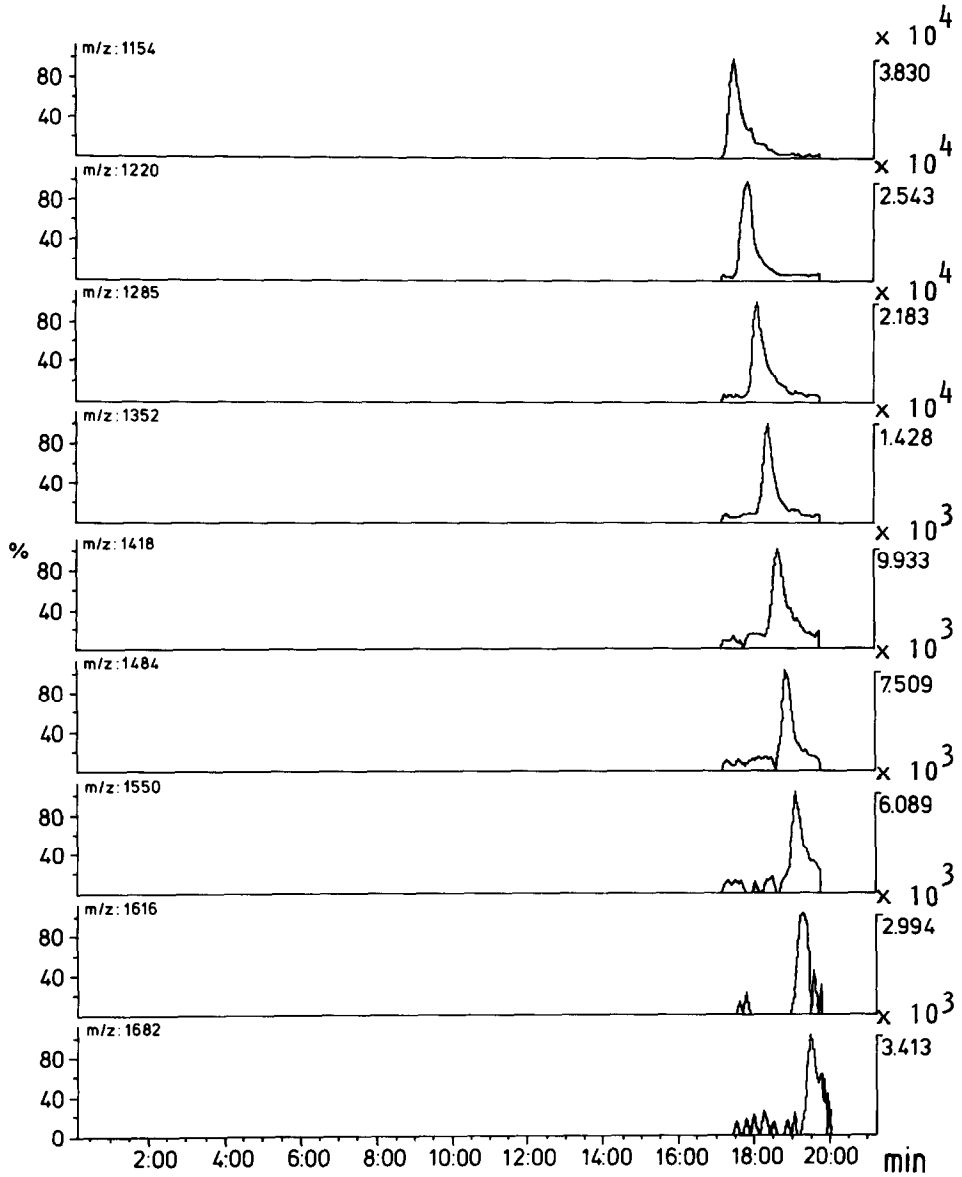

Fig. 6. HPAEC–MS of β-1,4-xylose oligomers. Mass chromatograms of doubly charged ions for DP = 17–25 obtained by multiple ion detection. Right-hand abscissa is ion intensity in arbitrary units.

peaks under the present chromatographic conditions is rather poor, but the peaks are readily distinguishable as a result of the selectivity of MS detection. A good chromatographic separation of the peaks of the xylose oligomers under the conditions used was observed with electrochemical detection only up to DP = 18.

From these data, it may be concluded that the response in thermospray MS appears to be sufficient also for the larger sugar oligomers (DP > 10). However, in order to allow HPAEC–MS detection of higher oligomers other than xylans, more attention must be paid to the efficient removal of the sodium ions from the column effluent prior to

thermospray MS. In that way the range of applicability of the HPAEC–MS approach can be expanded.

In the experiments with β-1,4-xylose oligomers, it was found that singly charged ions could be detected up to DP = 11, whereas doubly charged ions could be detected from DP = 5 upwards. The peak areas observed for singly and doubly charged ions are plotted as a function of the DP value (from 5 to 25) in Fig. 7a. This plot provides information on the relative importance of singly and doubly charged ions to the total peak area observed. Fig. 7a not only demonstrates the growing importance of the doubly charged ions with increasing DP value, but also provides information of the decrease in response as a function of DP. However, the decrease in response as a function of DP, which earlier was found to be large for α-1,4-glucose oligomers [14], is more readily evaluated by plotting the peak area per mass percentage or the peak area per mole of each individual xylose oligomer injected (Fig. 7b). These plots were constructed by using data on the actual composition of the β-1,4-xylose sample as obtained from an ion-moderated partition separation with refractive index detection, using xylose as a stan-

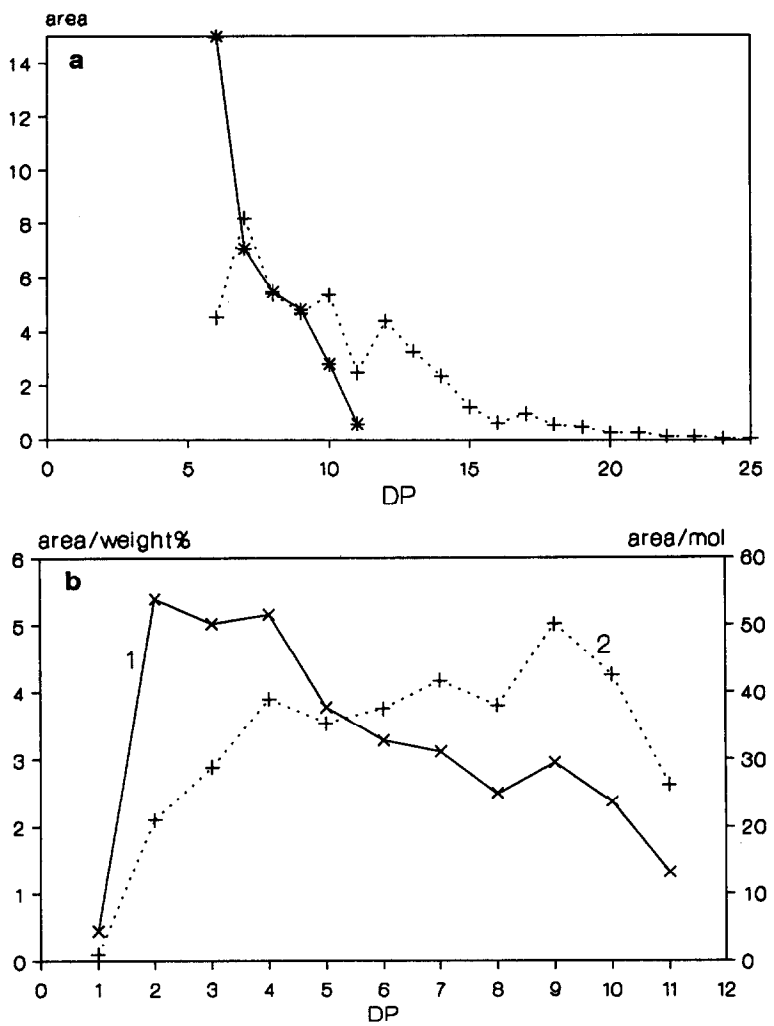

Fig. 7. (a) Peak areas (in arbitrary units) of singly charged (✳ = $[M + Na]^+$) and doubly charged ions (+ = $[M + 2Na]^{2+}$) (arbitrary units) in the MID run with β-1,4-xylose oligomers as a function of the DP value. (b) Peak area (in arbitrary units) per mass percentage (1) and peak area per mole (2) (both in arbitrary units) for DP = 1–11 oligomers of β-1,4-xylose in HPAEC–MS.

dard. It can be concluded that (i) the monomer is very efficiently lost in the AMMS, as it is present in the sample in a threefold excess over the dimer, (ii) the decrease in response in the peak area per mass percentage plot is approximately fivefold between DP 2 and 10, which is less than observed with α-1,4-glucose oligomers (fifteenfold between DP 2 and 10 [14]), and (iii) the response in peak area per mole is fairly constant between DP 4 and 10.

Heterogeneous oligosaccharides

In practice, the most interesting applications of the HPAEC–MS combination are in the characterization of complex mixtures of heterogeneous oligo-

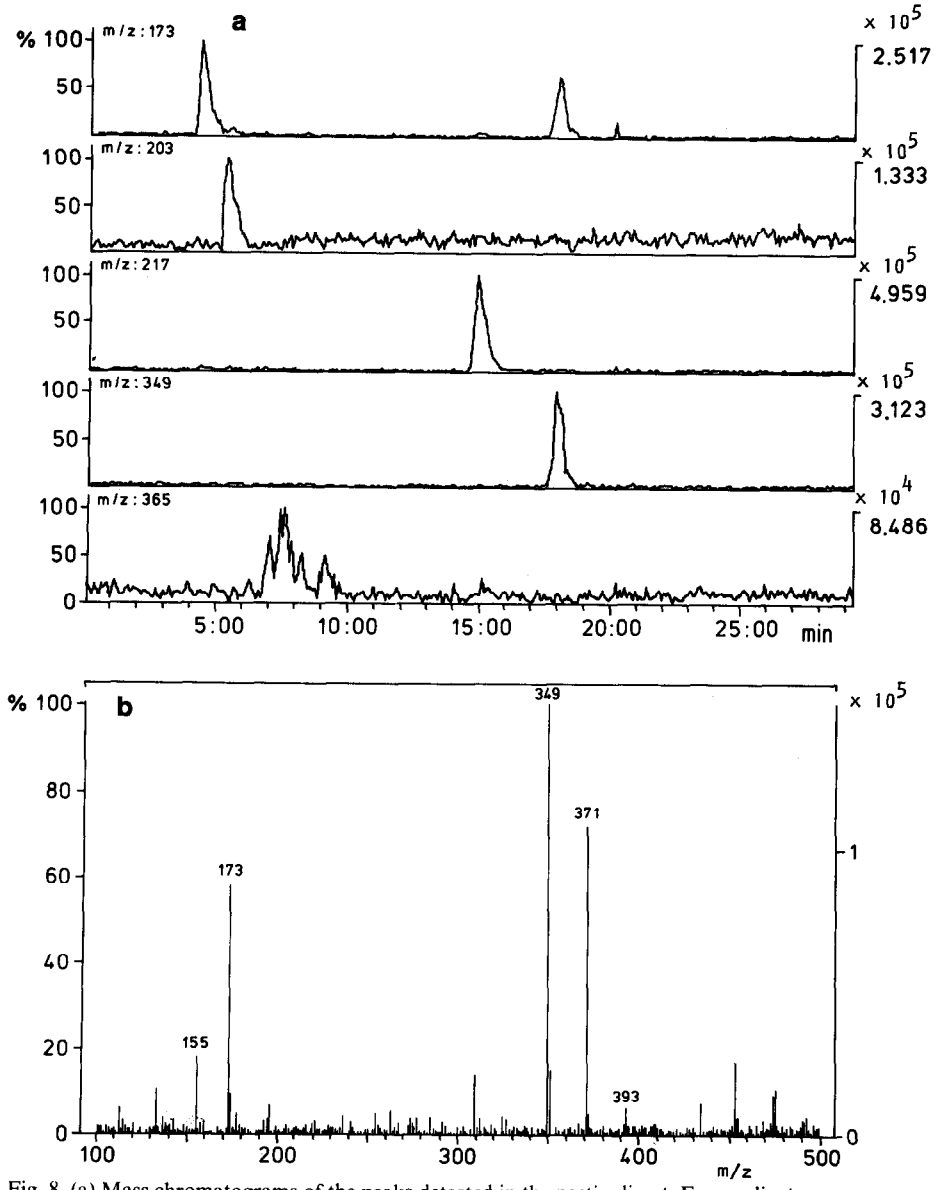

Fig. 8. (a) Mass chromatograms of the peaks detected in the pectin digest. For gradient programme, see Table I. (b) Mass spectrum of GalA–Xyl. Right-hand abscissa is ion intensity in arbitrary units.

saccharides. The ability to give rapid relative molecular mass information on-line with the separation of the various sample constituents is extremely helpful in the evaluation of enzymic behaviour and in the determination of the sugar composition of mixtures from the enzymic degradation of plant cell wall oligosaccharides. The HPAEC–MS method was developed in our laboratory for this field of application.

A digest of pectin was known to contain various monomeric sugars, *i.e.*, rhamnose, arabinose, galactose and galacturonic acid, in addition to an unknown dimer, most likely GalA–Xyl. The sample was analysed by HPAEC–MS. The mass chromatograms are shown in Fig. 8a. Peaks were detected with the m/z values expected for the monomeric sugars present, *i.e.*, (in order of elution) $m/z = 173$ for arabinose, $m/z = 203$ for galactose and $m/z = 217$ for galacturonic acid, while rhamnose was not detected as it was present in a low concentration and probably lost in the AMMS. Further, two dimeric species were found, *i.e.*, a hexose dimer at $m/z = 365$, which is most likely Gal_2 as deduced from its chromatographic behaviour, and at $m/z = 349$ for a uronic acid–pentose dimer, probably GalA–Xyl. The mass spectrum of GalA–Xyl is shown in Fig. 8b. Exceptionally, fragmentation owing to the loss of GalA with charge retention of the Xyl was observed in this spectrum. Further, some features of the mass spectra of uronic acids can be deduced. Uronic acids generally show two strong peaks, *i.e.*, a base peak due to the sodiated acid at $m/z = M_r + 23$ and a peak due to the sodiated sodium salt at $m/z = M_r + 45$. In polyuronic acids a series of sodiated sodium salt peaks can be observed. In some instances, a peak due to the loss of water from the sodiated acid is also observed (not in this instance).

An arabinoxylan sample was treated with endoxylanase and subsequently fractionated using Bio-Gel P2. One of the fractions, expected to contain hexamers of arabinose and xylose, was analysed by HPAEC–MS. In the sample two monomer peaks were found at $m/z = 173$, most likely due to xylose and arabinose; and two pentamer and two hexamer peaks at $m/z = 701$ and $m/z = 428$ and 833, respectively. Different pentamers and hexamers have been isolated and fully characterized by NMR [12]. Interestingly, the elution order of the latter four peaks is pentamer, hexamer, pentamer and hexamer. This example indicates the power of the on-line HPAEC–MS approach in elucidating the elution order of oligosaccharides in anion-exchange chromatography, where often unexpected orders are observed. Obviously, a next and highly desirable step is the elucidation of structural differences between the two pentamers and the two hexamers. The use of tandem mass spectrometry in this respect is currently under investigation.

As indicated before, the peaks of the oligomers present must be searched for by mass chromatography in the non-informative total ion current chromatograms. This can be considered a drawback, although the number of m/z values to be reconstructed is limited in the type of samples analysed in the present field of application. More extensive applications of HPAEC–MS to oligosaccharide mixtures obtained by enzymic degradation of plant cell wall polysaccharides are described elsewhere [10].

CONCLUSIONS

HPAEC–MS is a powerful technique in the characterization of oligosaccharides in mixtures. The method is fast and straightforward. Although at present only molecular mass information is obtained, this already is often very useful in combination with the available knowledge of the cell wall polysaccharides and in the evaluation of the action of enzymes and enzyme mixtures on plant cell wall material. The range of oligomers (DP values) that can be analysed depends greatly on the sugar composition. With β-1,4-xylose, oligomers up to DP = 25 could be observed. With heterogeneous sugar oligomers, compounds with DP values up to 10 are generally amenable to HPAEC–MS. In practice, the sample amounts used are 1–100 μg in a 25-μl injection volume (exact amounts in enzymic digests are unknown).

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