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# Recent Progress in High-performance Anion-exchange Chromatography/Ionspray Mass Spectrometry for Molecular Mass Determination and Characterization of Carbohydrates Using Static and Scanning Array Detection

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**Position- and time-resolved ion counting (PATRIC) array detection with a magnetic sector mass spectrometer equipped with an ionspray interface in the on-line coupling of high-performance anion-exchange chromatography (HPAEC) with mass spectrometry (MS) is described. PATRIC array detection is used in both the static and scanning mode for molecular mass determination and structure characterization caused by nozzle/skimmer-induced fragmentation in the ionspray interface. The virtual mass range is extended to over 7000 Da by the presence of triply and quadruply sodiated molecules in an enzyme digest of an exocellular polysaccharide (EPS) of *Lactococcus lactis* and quadruply sodiated molecules of β-1,4-xylose oligomers. After optimization of the HPAEC/MS system, molecular mass information could be obtained down to ~700 pg per injection for each α-1,4-glucose oligomer up to a degree of polymerization of 7 using PATRIC array detection in the static mode. Structure information was obtained for α-1,4 glucose oligomers, galacturonic acid oligomers and an EPS digest using PATRIC array detection in the scanning mode. © 1998 John Wiley & Sons, Ltd.**

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## INTRODUCTION

Although the on-line coupling of high-performance anion–exchange chromatography (HPAEC) with mass spectrometry (MS) for the analysis of carbohydrates has been described[1–4] using both thermospray (TSP) and ionspray (IS), the method has some drawbacks. HPAEC using pulsed amperometric detection (PAD) measures underivatized carbohydrates at low picomole levels. With TSP the overall sensitivity of the cationization process of the carbohydrates is hampered by the high chemical background of sodium acetate cluster ions to masses above 1000 Da. This phenomenon limits the power of the on-line coupling to single ion monitoring (SIM) at nanomole levels for oligosaccharide mixtures from different sources, e.g. maltodextrins and digests of plant cell wall polysaccharides.[4] Using IS instead of TSP, first described for the analysis of oligosaccharides by Conboy and Henion,[5] the influence of the sodium acetate cluster ions is diminished and the optional nozzle/skimmer-induced dissociation could be used to obtain structure information. Recently, the on-line coupling of HPAEC/MS with IS as an ionization technique without using a booster pump, and a newly developed cation-exchange system to replace sodium for hydronium ions in the eluent stream, was described for measurements at medium picomole levels.[6] Owing to the absence of the sodium acetate cluster ions, and by using only the electrolysis mode of the cation-exchange system by leaving out the sulphuric acid as a regenerant, the method becomes more sensitive and, as a consequence, becomes more useful for the characterization of complex mixtures of homo- and heterogeneous oligosaccharides using full-scan analysis.

Further sensitivity improvements can be obtained by using an array detector, which provides 1–3 orders of magnitude higher sensitivity over conventional detectors, such as secondary electron multipliers (SEM),[7,8] depending on the scanning mode. Reinhoud *et al.*[9] demonstrated the power of a position- and time-resolved ion counting (PATRIC) detector in the coupling of capillary electrophoresis with MS.

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In the static detection mode, the information is obtained over a mass range of, e.g., 4% around a central mass and the sensitivities are comparable to SIM, but more mass spectrometric information, such as the charge state of an ion, is obtained. In the scanning mode the improvement in sensitivity is 1–2 orders of magnitude.

Electrospray (ESI) and IS are powerful ionization techniques, partly due to the generation of multiply charged ions of macromolecules such as proteins[10] and oligonucleotides,[11] extending the virtual mass range of the mass spectrometer. The mechanism for the ion formation and the charge distribution has been discussed by many authors.[12–15] Using proteins as model compounds, the maximum charge state has been described in relation to basic amino acid residues,[13] aqueous solution-phase equilibria models[14] and gas-phase basicity,[15] whereas for polyethylene glycols a simple model was suggested for the maximum possible number of charges[16] depending on different parameters among these, the possible bonding sites and configuration. The maximum charge capacity of a molecule is limited by Coulomb repulsion. Depending on the configuration of the molecule, and more or less as a rule of thumb, one charge per 1000 Da can be expected. However, for sugar oligomers only singly and doubly sodiated molecules could be observed so far with both TSP and ESI.[17]

In this paper, the presence of multiply charged molecules of sugar oligomers is described. A mixture of $\beta$-1,4-xylose oligomers shows quadruply sodiated molecules starting at a degree of polymerization (DP) of 19 up to DP55 having molecular masses of 2550 and 7303 Da, respectively. An exocellular polysaccharide (EPS) digest shows triply and quadruply sodiated molecules up to DP47 with a mass of 6849 Da. The characterization of the EPS digest and a series of saturated galacturonic acid oligomers by nozzle/skimmer induced fragmentation is described. Improvements in HPAEC/MS resulting in detection limits down to ~700 pg per injection for each $\alpha$-1,4-glucose oligomer up to DP7 with PATRIC array detection in the static mode are demonstrated.

## EXPERIMENTAL

The HPAEC/MS system consisted of a Dionex (Sunnyvale, CA, USA) DX-300 chromatography system coupled to a Finnigan (San Jose, CA, USA) MAT 900 double-focusing mass spectrometer equipped with a Finnigan MAT API ion source, which was operated in the IS mode.

The Dionex DX-300 chromatography system consisted of an EDM-2 solvent degas unit, an AGP-1 pump module, an LCM-3 chromatography module, containing a Rheodyne (Cotati, CA, USA) Model 9126 all-PEEK injector with a 6.3 μl loop, a pulsed amperometric detector (PAD) with a gold electrode and a membrane desalting device (MDD), which is in fact a cation-exchange system to remove the sodium ions from the effluent stream prior to introduction into the MS. The regenerating solvent for the MDD was water and the exchange of $Na^+$ for $H_3O^+$ was accomplished by electrolysis of water by means of a Dionex SRS controller operating at 500 mA. The water reservoir was pressurized by 50 kPa of helium resulting in a water flow rate of 2 ml min$^{-1}$, when the current of the Dionex SRS controller was on.

A 250 mm × 2 mm i.d. Dionex CarboPac PA1 column was used. Gradient elution was performed using water (A), 0.5 M sodium hydroxide (B) and 0.625 M sodium acetate (C). The flow rate through the column was 0.2 ml min$^{-1}$. The linear gradients for the different samples were as follows: for $\alpha$-1,4-glucose oligomers, starting at 0.1 M sodium hydroxide (A : B : C = 80 : 20 : 0), increasing to 50% C in 25 min and kept constant for 10 min (A : B : C = 30 : 20 : 50); for $\beta$-1,4-xylose oligomers, starting at 0.1 M sodium hydroxide, increasing to 30% C in 30 min, to 80% C in 45 min; for the EPS digest, starting at 0.1 M sodium hydroxide, increasing to 64% C in 40 min; for galacturonic acid oligomers, starting at 0.1 M sodium hydroxide, increasing to 80% C in 45 min, and kept constant for 30 min.

Owing to the absence of back-pressure in the API interface, the outlet of the MDD could be coupled directly to the MS mass spectrometer. However, optimum mass spectrometric sensitivity was obtained by splitting the flow rate to about 40 μl min$^{-1}$ directed to the mass spectrometer by means of a splitting device to regulate the flow rate. The experimental set-up, but without the splitting device, has been published elsewhere.[6] For optimum performance and electrical contact, a liquid sheath flow rate of 10 μl min$^{-1}$ consisting of a mixture of propan-2-ol and water containing $10^{-4}$ M sodium acetate (80 : 20, v/v), together with nitrogen as a sheath gas at a flow rate of 6 l min$^{-1}$ was used. To prevent electrical breakdown due to condensation of the solvents used, nitrogen was used as auxiliary gas at a flow rate of 2 l min$^{-1}$. The mass spectrometer was operated at an accelerating voltage at 5 kV and was optimized daily with respect to voltages on sampling capillary, nozzle, skimmer and octapole to obtain the optimum response for a combination of molecular information and nozzle/skimmer-induced fragmentation. The sampling capillary was kept at 200 °C during all the experiments. All spectra were obtained in the positive ionization mode using the PATRIC array detector, either in the scanning mode from $m/z$ 200 to 2000 in 3 s or in the static mode at certain predefined masses, with a mass window of 4%.

### Chemicals

Propan-2-ol and sodium hydroxide were purchased from Baker (Deventer, The Netherlands). Water was purified with a Milli-Q apparatus (Millipore, Bedford, MA, USA). Sodium acetate was obtained from Merck (Darmstadt, Germany). The pure $\alpha$-1,4-glucose oligomers were obtained from Boehringer Mannheim (Mannheim, Germany). Maltodextrin MD-20 was supplied by Roquette (Lille, France). The mixture of oligomeric galacturonic acids was obtained by degrading polygalacturonic acid with polygalacturonase.[18] The $\beta$-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). The EPS digest was obtained as follows: EPS produced by *Lactococcus lactis* subsp. *cremoris B4o* (NIZO)[19] was purified and subsequently treated with 48% HF (48 h, 0 °C). The resulting, partially debranched, polymer was incubated (24 h, 30 °C) with the crude enzyme preparation Maxazyme CL 2000[20] (Gist Brocades, Delft, The Netherlands). This resulted in a mixture of oligosaccharides containing glucose, galactose and rhamnose.

## RESULTS AND DISCUSSION

The combination of HPAEC/MS with IS as ionization technique and a recently developed anion membrane suppressor, a membrane desalting device (MDD)[21] and Patric array detection[7–9] results in a significant improvement, with respect to sensitivity, for the mass spectrometric analysis of oligosaccharides. Recently, the influence of changing from TSP to IS as ionization technique and the performance of the MDD were evaluated.[6] Using IS no sodium acetate cluster ions could be observed in the mass spectra, making the method suitable for full-scan measurements. The performance of the MDD, working in the electrolysis mode only, in combination with a 2 mm CarboPac column, could be used with sodium acetate gradients up to a total sodium load of 0.6 M without loss of sensitivity. Avoiding sulphuric acid as an additional regenerant prevents the breakthrough of sulphuric acid through the membrane, which influences the sensitivity unfavourably.

The coupling of the HPAEC system to the API source of the MAT900 in the same way as was performed with a TSQ-700 gave rise to problems with respect to spray stability and sensitivity at a flow rate of 0.2 ml min$^{-1}$ directed to the mass spectrometer. Therefore, it was decided to apply a splitting device consisting of a zero low-dead volume T-piece and a metering valve to regulate the flow rate to waste. Optimum mass spectrometric sensitivity was obtained by splitting the flow rate to the extent that ~40 μl min$^{-1}$ was directed to the mass spectrometer. Only 0.2 m of 100 μm i.d. ($V = 1.6$ μl) fused-silica tubing was used between the splitting device and the needle assembly and no extra peak broadening could be observed.

Instead of more conventional detection systems, by using PATRIC array detection the sensitivity could be improved drastically.[9] In all the described examples array detection was used, either in the static or in the scanning mode. The static mode was used for optimum sensitivity and for the confirmation of the charge mode of the ions formed, whereas in the scanning mode most attention was paid to the observation of multiply sodiated molecules, minor components in enzymatic digests and the fragmentation generated by nozzle/skimmer induction.

### α-1,4-Glucose oligomers

A series of α-1,4-glucose oligomers were used for sensitivity measurements using static array detection. In Fig. 1 the sum of the sodiated molecules and relevant

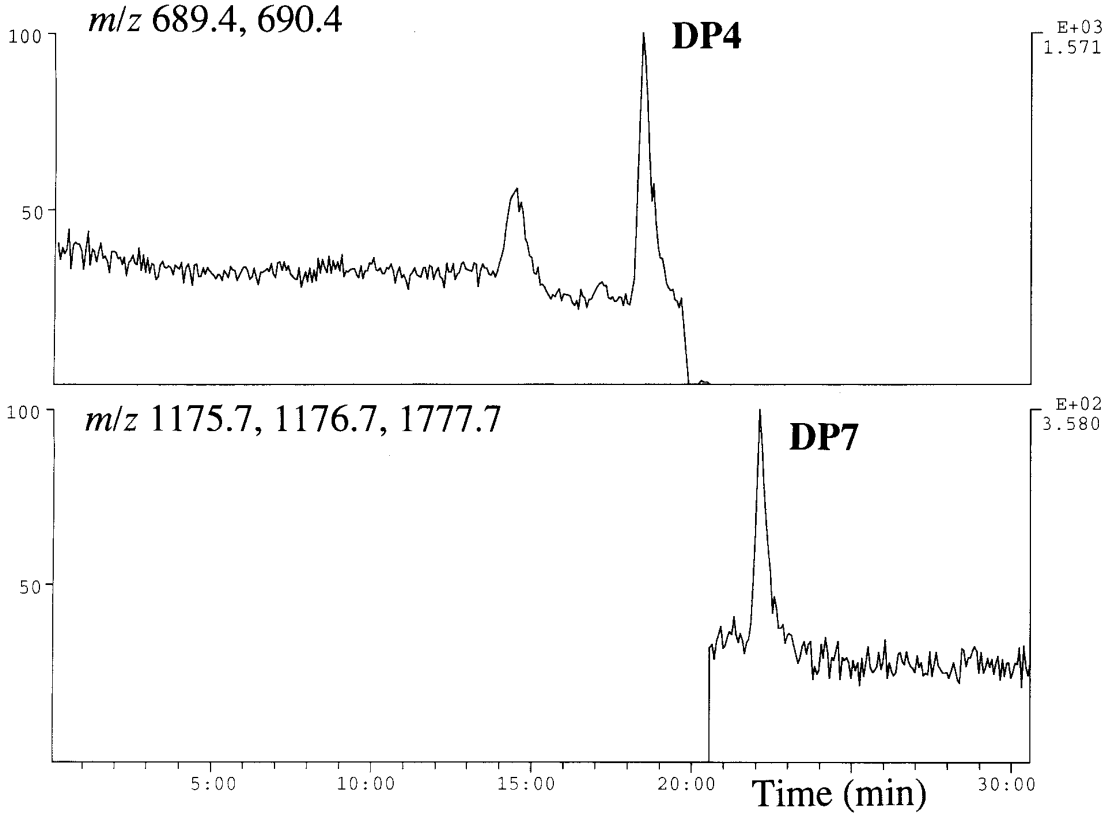

**Figure 1.** HPAEC/MS of maltotetraose (DP4) and maltoheptaose (DP7) at a concentration of 300 ng ml$^{-1}$ (700 pg per injection of each oligomer). Until 20 min static array detection with *m*/*z* 689 as centre mass, and subsequently with *m*/*z* 1175 as centre mass. For conditions, see Experimental.

isotope peaks of DP4 and DP7 (maltotetraose and maltoheptaose) at 700 pg per injection for each compound are shown. Two procedures were started after each other in this experiment. Starting with static array detection with *m/z* 689 as the centre mass and with a 4% mass window, after ~20 min static array detection with *m/z* 1175 as the centre mass was used. Regarding the signal-to-noise ratio the concentration detection limit is ~100 ng ml$^{-1}$ for DP7, and even lower for DP4. The trace of DP4 (*m/z* 689) shows two peaks at retention times of 14 min 29 s and 18 min 25 s, respectively. The first peak, eluting exactly at the retention time of DP2, is believed to be a gas-phase formed dimer of DP2 minus the loss of water, although this was not investigated in more detail.

Recent work evaluating the coupling of HPAEC/MS with IS on a quadrupole mass spectrometer[6] showed the capability of detecting maltooligosaccharides up to DP20, although the signal-to-noise ratio above DP15 was poor. However, using static array detection with *m/z* 1530 and 1700 as the centre mass the signal-to-noise ratio was improved impressively, as shown in Fig. 2 for DP18–21. The ions used are the doubly sodiated molecules of the different DP values, corresponding to masses of 2958, 3120, 3282 and 3444 Da, respectively. Another phenomenon which could be observed is the appearance of two peaks at every *m/z* ratio, none of them with overlap of retention time. The appearance of two peaks starting at about DP15 with a ratio at DP21 of ~1 : 1 cannot be understood readily. Some kind of an isomaltose series could be present next to the linear α-1,4-glucose oligomers. The reason why this phenomenon is not observed during PAD experiments is that these higher oligomers are only minor components in the maltodextrin sample and PAD is not a specific detection method. Until now, maltohexoses have been characterized as singly and/or doubly sodiated molecules with TSP, and also with IS, up to DP21. Although the double sodiation starts at DP5 with TSP and at DP8 with IS, no multiply sodiated molecules could be observed with either ionization technique so far.

### β-1,4-Xylose oligomers

Although it was not possible to observe multiply sodiated molecules with aldohexoses, quadruply sodiated oligosaccharides could be observed for a series of aldopentoses. A β-1,4-xylose oligomer mixture was analysed with PATRIC array detection in the scanning mode. Singly sodiated molecules could be observed up to DP14, whereas the doubly sodiated molecules could be detected from DP8 to 29. The observation of singly and double sodiated molecules was limited by scanning

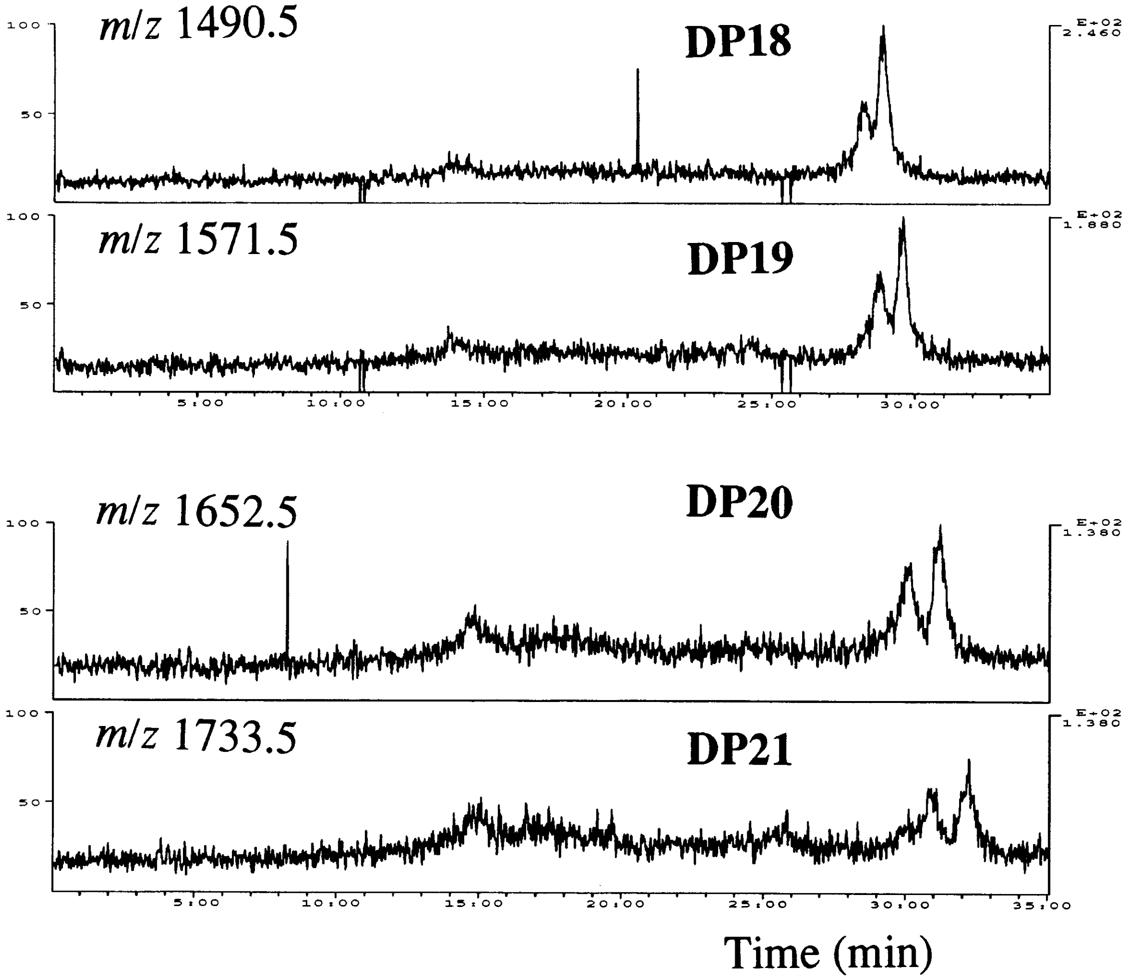

**Figure 2.** HPAEC/MS of α-1,4-glucose DP18–21 oligomers. Alternating static array detection with *m/z* 1530 and 1700 as centre masses. For conditions, see Experimental.

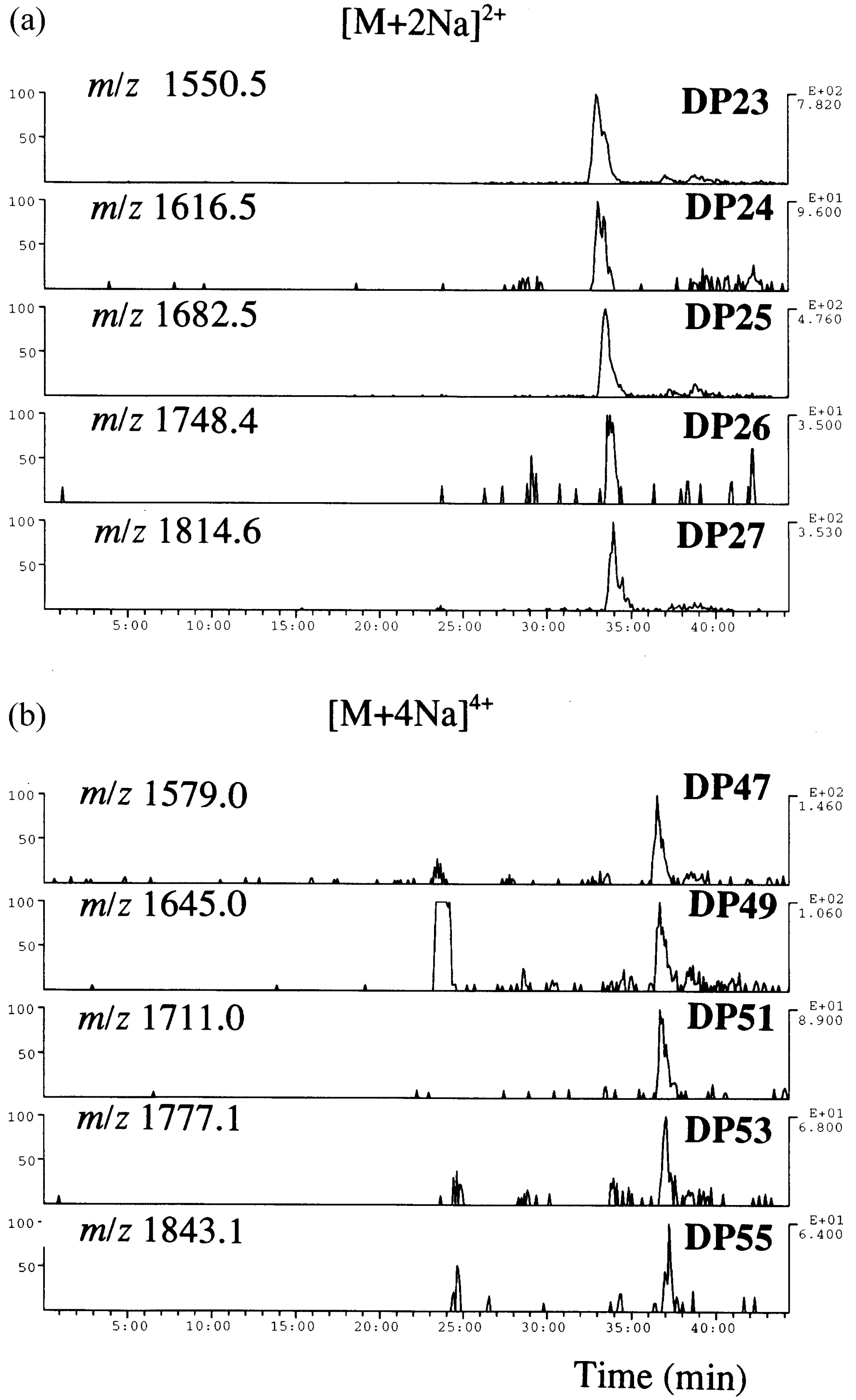

**Figure 3.** (a) HPAEC/MS of doubly sodiated molecules of β-1,4-xylose DP23–27 oligomers, using scanning array detection; (b) HPAEC/MS of quadruply sodiated molecules of β-1,4-xylose DP47–55 oligomers, using scanning array detection.

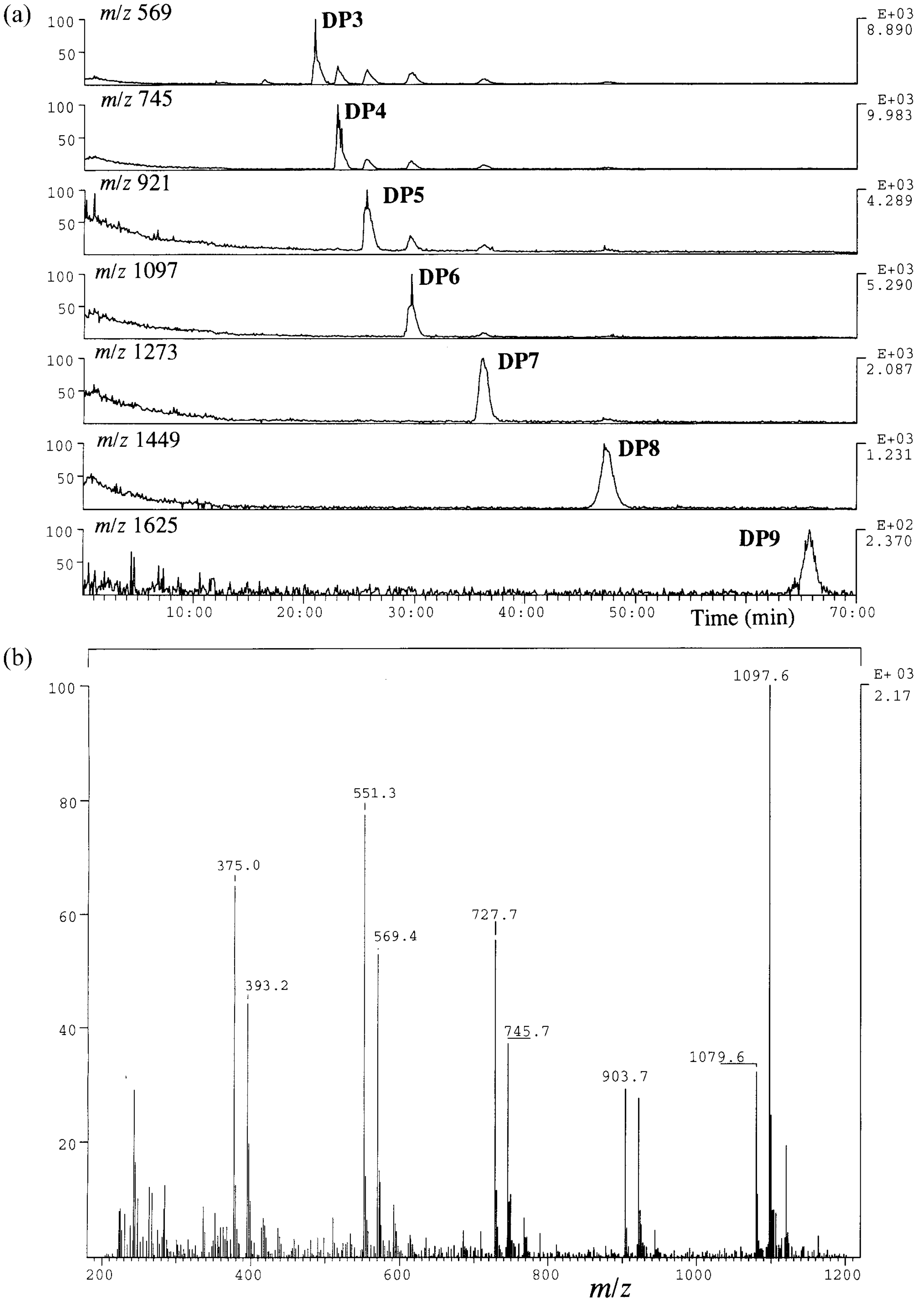

**Figure 4.** (a) HPAEC/MS of enzyme digest of polygalacturonic acid, showing oligomers up to DP9; (b) Typical mass spectrum of a galacturonic acid oligomer, DP6, with nozzle/skimmer-induced fragmentation.

**Table 1.** ***m/z* ratios of expected homologous series**[a]

| | | 3 Hexose + [3 hexose + deoxyhexose]$_n$ | | |
|---|---|---|---|---|
| *n* | [M + Na]$^+$ | [M + 2Na]$^{2+}$ | [M + 3Na]$^{3+}$ | [M + 4Na]$^{4+}$ |
| 3 | *527.2* | 275.1 | 191.1 | 149.0 |
| 7 | *1159.4* | 591.2 | 401.8 | 307.1 |
| 11 | *1791.6* | *907.3* | 612.5 | 465.2 |
| 15 | 2423.8 | _1223.4*_ | 823.3 | 623.2 |
| 19 | 3056.0 | *1539.5* | *1034.0* | 781.3 |
| 23 | 3688.3 | *1855.6* | _1244.8*_ | 939.3 |
| 27 | 4320.5 | 2171.7 | *1455.5* | 1097.4 |
| 31 | 4952.7 | 2487.9 | *1666.2* | *1255.4* |
| 35 | 5584.9 | 2804.0 | 1877.0 | _1413.5*_ |
| 39 | 6217.1 | 3120.1 | 2087.7 | _1571.5*_ |
| 43 | 6849.4 | 3436.2 | 2298.5 | *1729.6* |
| 47 | 7481.6 | 3752.3 | 2509.2 | 1887.6 |

[a] For explanation of values in italics and asterisks, see text.

only to *m/z* 2000. However, the virtual mass range could be extended to over 7000 Da, as shown by the presence of quadruply sodiated molecules starting at DP20–55. The charge state was confirmed with static array detection. This means that DP55, having a molecular mass of 7303 Da, was detected as $[M + 4Na]^{4+}$ at *m/z* $(7303 + 92)/4 = 1843$. In Fig. 3(a), the doubly sodiated molecules of DP23–27 and in Fig. 3(b) the quadruply sodiated molecules of the odd numbers between DP47 and 55 are presented. Despite the use of scanning array detection, triply sodiated molecules could not be observed. According to the theory that the charge capacity is dependent on the Coulomb repulsion by other charges, the selective discrimination probably is due to the configuration of the oligomers.[16] It is not clear whether such oligomers can form, for instance, a ring structure, or a twisted ring structure stabilized by hydrogen bonding if the oligomer is over DP20. Obviously, the triply sodiated molecules are not stable in the gas phase. It also appeared that only the odd DP values could be observed by their quaduply sodiated molecules, as shown in Fig. 3(b) for DP47–55. Since it is hard to believe that the even oligomers were absent in the digest, the quadruply sodiated molecules of those specific oligomers might be unstable in the gas phase. No further experiments were performed to clarify this phenomenon.

## Galacturonic acid oligomers

Although the robustness of the HPAEC/MS system has already been tested and discussed earlier,[6] only the results of galacturonic acid oligomers up to DP4 were presented. With an enzymatic degraded substrate of polygalacturonic acid the presence of a DP9 oligomer at *m/z* 1625 could be confirmed. In Fig. 4(a) the ion chromatogram of DP3–9 is presented. Owing to the choice of effluents introducing a maximum load of 0.6 M of sodium ions to the MDD, the method is limited for

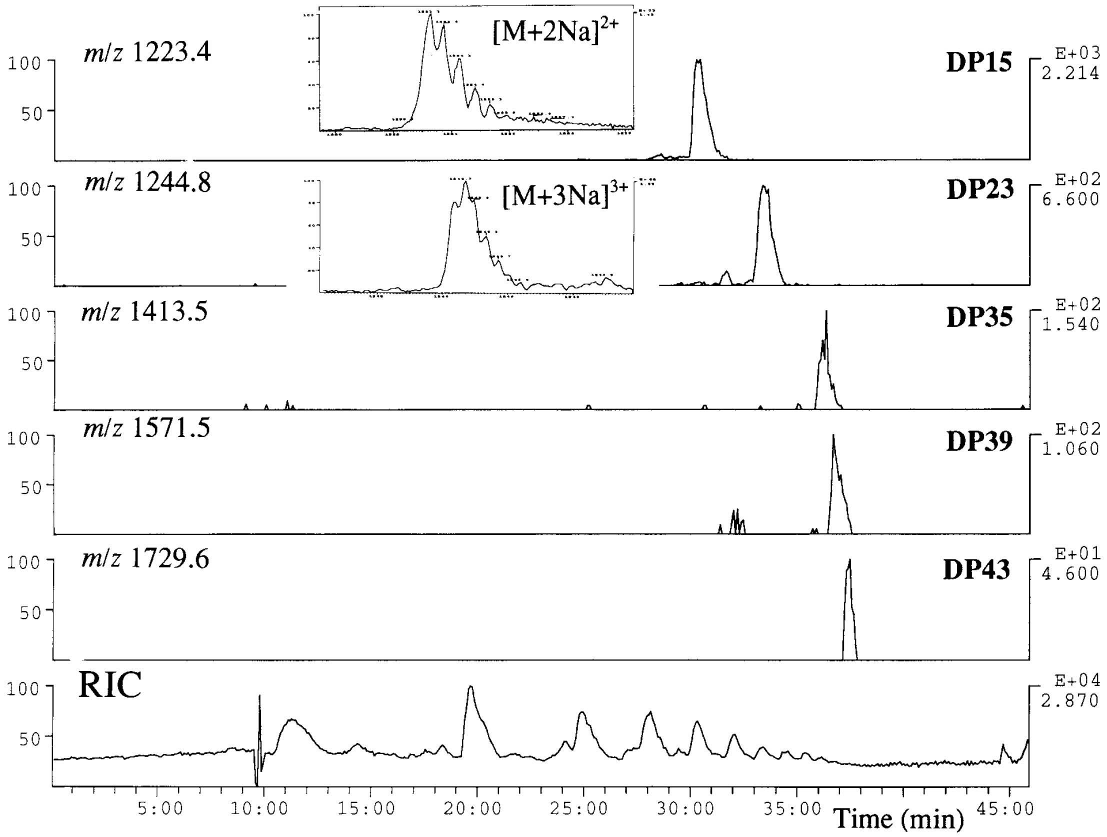

**Figure 5.** HPAEC/MS of an EPS digest. The ion traces are doubly, triply and quadruply sodiated molecules of a homologous series. Experiments were performed using scanning array detection. Charge states of *m/z* 1223 and 1245 were confirmed by static array detection.

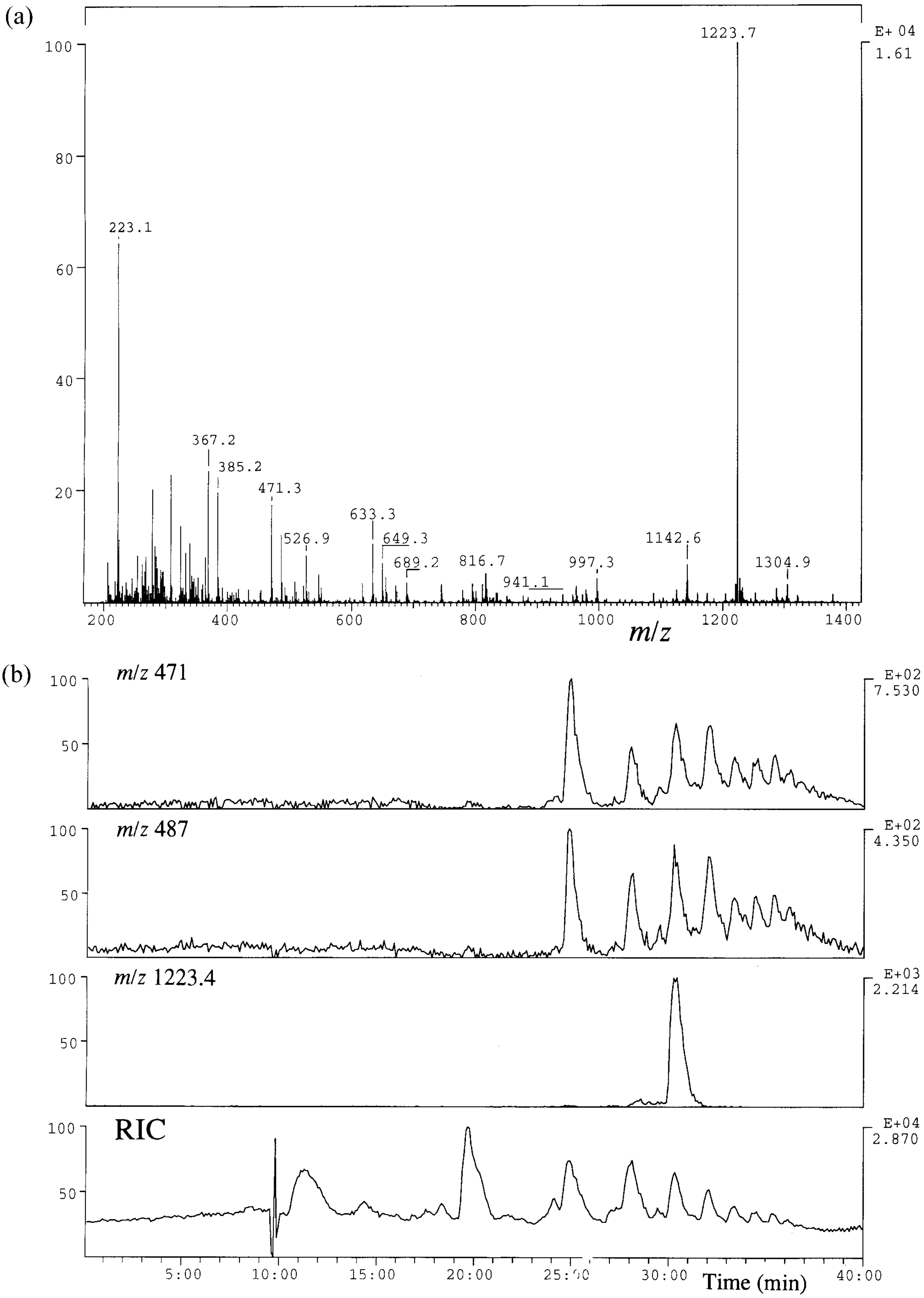

**Figure 6.** (a) Mass spectrum of DP15 from the homologous series of the EPS digest; (b) ion traces of typical fragmentation, *m/z* 471 and 487, and the doubly sodiated molecule of DP15 at *m/z* 1223, observed in this homologous series.

the determination of galacturonic acid oligomers on this type of column.

Nozzle/skimmer fragmentation is easily induced, as can be seen from, for instance, the ion traces of *m/z* 569, 745 and 921 in Fig. 4(a), which can be explained by the subsequent cleavage of galacturonic acid units. In Fig. 4(b) the mass spectrum of the DP6 peak is presented, showing in addition to the $[\mathrm{M} + \mathrm{Na}]^+$ ion at *m/z* 1097, the $[\mathrm{M} - \mathrm{H} + 2\mathrm{Na}]^+$ ion at *m/z* 1119 and the $[\mathrm{M} + \mathrm{Na} - \mathrm{H_2O}]^+$ ion at *m/z* 1079. The observed fragmentation can be explained by the same type of ions, each time minus a galacturonic acid unit, e.g. $[\mathrm{M} - (\mathrm{GalA})_n + \mathrm{Na}]^+$. The series of galacturonic acid oligomers could only be characterized by singly sodiated molecules and even at DP9 no doubly sodiated molecules could be observed.

### EPS-derived oligosaccharides

Since the HF-treated EPS consists of a repeating unit with glucose, galactose and rhamnose: →)-β-Glc-(1 → 4)-[α-Rha-(1 → 2)]-β-Gal-(1 → 4)-β-Glc(1 →, where the rhamnose branching might be absent in some repeating units owing to the treatment. The enzyme digest is believed to contain a homologous series, starting at a trimer, glucose–galactose–glucose (Glc–Gal–Glc), with an increment of 632 Da (Glc–[Rha]Gal–Glc). PATRIC array detection in the scanning mode is used from *m/z* 200 to 2000 in 3 s. This means that oligosaccharides above DP11 have to be characterized by multiply charged molecules. With this series of oligosaccharides triply and even quadruply sodiated molecules could be observed. The branching of these oligosaccharides probably disables the formation of ring structures, so the formation of multiply sodiated molecules can be explained by charge density. In Table 1 all the possible *m/z* ratios of the expected homologous series are summarized, and the observed *m/z* ratios are shown in italics. In Fig. 5 some of these ratios are shown as ion chromatogram, marked in Table 1 with asterisks, including a doubly sodiated molecule at *m/z* 1223.4, a triply sodiated molecule at *m/z* 1244.8 and quadruply sodiated molecules at *m/z* 1413.5, 1571.5 and 1729.6. The presence of doubly and triply sodiated molecules is confirmed with PATRIC array detection in the static mode for DP15 and 23. The isotope pattern of doubly and triply sodiated molecules must be divided by *m/z* 0.5 and 0.33 Da, respectively. The isotope pattern for these two oligosaccharides is inserted in the relevant traces of the ion chromatogram in Fig. 5.

To differentiate between linkage types in disaccharides, Garozzo *et al.*[22] used negative fast atom bombardment (FAB), because negative ionization is believed to induce selective deprotonation of the anomeric hydroxyl group. Although, the fragmentation pattern of oligosaccharides in both the positive and negative ionization modes is found to be more or less similar, mechanistic details are outside the scope of this discussion. Although the relative abundances of the fragments formed in the positive ionization mode are linkage dependent, the ions formed are mainly due to losses of 18, 30, 60 and 90 Da and the loss of sugar units from the sodiated molecules forming sodiated fragments. Recently, Asam and Glish[23] discussed the fragmentation of alkali metal-cationized polysaccharides in a quadrupole ion trap to distinguish between different linkage types.

Only DP3 with *m/z* 527 (Glc–Gal–Glc), eluting at 19 min 38 s, shows the above-mentioned fragmentation pattern. All the other oligosaccharides of the series, beginning at *m/z* 1159 and with a mass increment of 632 Da, behave differently. Obviously, there is a loss of one Glc or Rha, but the most important fragmentation is caused by the elimination of a sodiated Glc (Glc − H + Na), probably at the reducing end, forming a protonated fragment, followed by successive losses of sugar units. As an example, the mass spectrum of DP15 is shown in Fig. 6(a). This fragmentation, resulting in fragments at, for instance, *m/z* 471, 487 and 633, 649, etc., is specific for this series of oligosaccharides, as is shown in Fig. 5(b). The only DP value which does not fit is DP3 at 19 min 38 s, as mentioned earlier. The repetitive mass difference of 16 Da can be explained by the presence of two fragmentation series, one series with first the cleavage of Rha, a deoxyaldohexose and then successive losses of aldohexoses, and the other series with only losses of aldohexoses.

Any change in structure, such as one aldohexose less, changes the fragmentation pattern. For instance, at 24 min a DP6 with *m/z* 997 is eluting just before *m/z* 1159, which does not show the specific fragments at *m/z* 471 and 487 as shown in Fig. 6(b). Probably the aldohexose is the Glc at the reducing end, which makes it impossible to cleave off a sodiated Glc, thus hampering the described fragmentation.

In addition to the homologous series mentioned above, information on at least three other series, which were minor components, could be recognized using the scanning array detection.

## CONCLUSIONS

PATRIC array detection broadens the application of HPAEC/MS with respect to overall sensitivity. Static array detection shows concentration detection limits down to 700 pg per injection for each member of an α-1,4-glucose oligomer series and can be used for the confirmation of the charge state. The virtual mass range for the analysis of oligosaccharides is extended to over mass 7000 by the observation of triply and quadruply sodiated molecules. Those multiply sodiated species were observed in both a series of β-1,4-xylose oligomers and in an EPS digest, consisting of Gal, Glc and Rha above a certain DP value. The absence of triply sodiated molecules in the β-1,4-xylose series cannot be understood readily, but probably the configuration of the oligomer hampers the formation of triply sodiated molecules.

Array detection in the scanning mode has been shown to be powerful in full-scan measurements for the structure characterization of, for instance, galacturonic acid oligomers, and also for the characterization of minor components in enzymatic digests.

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