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Sensitivity Improvement in the Analysis of Oligosaccharides by On-line High-performance Anion-exchange Chromatography/Ion Spray Mass Spectrometry

R. A. M. van der Hoeven,[1] A. J. P. Hofte,[1] U. R. Tjaden,[1]* J. van der Greef,[1] Nelson Torto,[2]† L. Gorton,[2] G. Marko-Varga[3] and C. Bruggink[4]

[1]Division of Analytical Chemistry, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands
[2]Department of Analytical Chemistry, University of Lund, P.O. Box 124, S-221-00 Lund, Sweden
[3]Astra Draco AB, preclinical Research and Development, P.O. Box 34, S-221-00, Lund, Sweden
[4]Dionex B.V., P.O. Box 9338, 4801 LH Breda, The Netherlands

The on-line coupling of high-performance anion-exchange chromatography (HPAEC) with ion spray mass spectrometry, for the analysis of neutral and acidic oligosaccharides, is described. On-line desalting is performed by a cation-exchange membrane system which replaces sodium for hydronium ions from the effluent stream by means of either electrolysis of water or a combination of electrolysis and pneumatically supplied sulphuric acid. In contrast to formerly described systems no booster pump between the cation-exchange system and the ion spray interface is needed, due to the almost complete absence of back-pressure. The on-line removal of sodium ions prior to the interface by means of electrolysis, and use of ion spray as an ionization technique, enables the routine use of gradients of sodium acetate up to a total sodium concentration of 0.6 M. After optimization of the HPAEC/MS system molecular mass determination could be obtained at concentrations down to 3 μg/mL (20 ng of each compound) for α-1, 4-glucose oligomers up to a degree of polymerization of 7 (DP7). For maltodextrines the applicable mass range could be extended to over 3000 Da by the detection of at least DP20, as the doubly charged disodiated molecule. A series of galacturonic acid oligomers could be detected up to DP4 at a concentration of 10 μg/mL (67 ng per compound), using a sodium acetate gradient increasing to a total sodium concentration of 0.6 M. © **1998 John Wiley & Sons, Ltd.**

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Simpson *et al.*[1] used an anion micromembrane suppressor (AMMS) to exchange sodium ions hydronium ions by means of dialysis, prior to introducing the effluent into the MS. High-performance anion-exchange chromatography (HPAEC/MS) was first described by Conboy *et al.*,[2] who used an ion spray interface for the determination of quaternary ammonium compounds. HPAEC is a very powerful technique for the analysis of underivatized oligosaccharides using pulsed amperometric detection (PAD),[2] down to 10 pmol as minimum detectable amounts. Commonly the AMMS is used prior to fraction collection for purification of oligosaccharide mixtures, before further characterization and identification using other analytical techniques including mass spectrometry. Off-line analysis has been reported by fast atom bombardment (FAB)[3,4] which still is a powerful technique in structure elucidation.[5] The analysis of higher oligosaccharides has been reported with matrix-assisted laser desorption[6,7] up to a mass range of 100 000 Da. The on-line coupling of HPAEC with mass spectrometry using thermospray (TSP) ionization was first described by Niessen *et al.*[8] Oligosaccharides up to a degree of polymerization (DP) of 14 for mixtures of α-1,4-glucose oligomers could be detected as sodiated and doubly charged disodiated molecules. Sodium acetate gradients up to a total concentration of 0.4 M of sodium ions could be desalted with two AMMS suppressors in series down to sodium concentrations below 1 mM, which makes on-line mass spectrometric detection possible. However, the method is hampered by the overall sensitivity and high background signal due to sodium acetate clusters ions to masses above 1000 Da. Another disadvantage of using the AMMS together with a TSP interface is the need for a booster pump between these two devices, to prevent damage of the AMMS from the high pressure built up by the TSP probe.[1] A similar design with an ion spray interface in conjunction with a booster pump was described by Conboy and Henion.[9] Although damage of the AMMS is prevented in this way, unavoidable peak broadening is observed which reduces both the sensitivity and the chromatographic resolution.

The method of cationization can be used for rapid screening of oligosaccharide mixtures from different sources. Tinke *et al.*[10] described the characterization of oligosaccharides, obtained by enzymatic degradation of plant cell wall polysaccharides, using electrospray ionization (ESI). Recently Torto *et al.*[11] described the coupling of a microdialysis system with HPAEC/MS for the characterization of oligosaccharides in bioprocesses.

*Correspondence to: U. R. Tjaden, Division of Analytical Chemistry, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands.
† On leave from: Department of Chemistry, University of Botswana, P/Bag 0022, Gaborone, Botswana.

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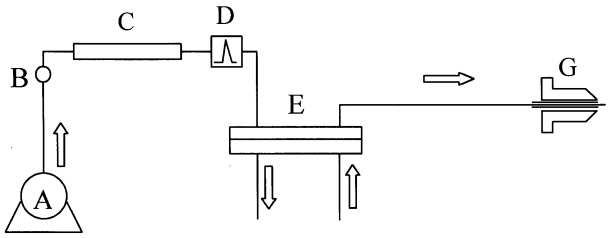

Figure 1. Schematic diagram of the experimental arrangement. A = pump module; B = injection valve with 6.3 μL loop; C = 250 mm × 2 mm i.d. CarboPac PA1 column; D = PAD; E = MMD; G = IS probe. (The missing component F was the booster pump required for thermospray operation).

The aim of this study was to extend the possibilities with respect to sensitivity and robustness of coupling HPAEC with MS. Using an ion spray interface the ion source is prevented from intense contamination, due to the fact that the ionization process takes place at atmospheric conditions, this reducing the involatile sodium acetate cluster ions from entering the ion source via the heated sampling capillary. Moreover, the booster pump can be omitted from the system because the back pressure built up by the ion spray probe remains within the operation range of the suppressor. To broaden the applicability of the method a different kind of membrane suppressor, a membrane desalting device (MDD), is used instead of the earlier described AMMS. The high capacity continuous desalting of sodium containing effluents by the MDD, accomplished by a combination of water electrolysis or electrolysis in combination with pneumatically supplied sulphuric acid, enables the use of gradients up to a total sodium concentration of 0.6 M.

The developed system was evaluated with respect to sensitivity by a series of oligomers of α-1,4-glucose, down to 20 ng per compound detected by single ion monitoring. The applicable mass range was investigated with several maltodextrine samples. Furthermore, the robustness of the total system with respect to the total sodium loadability was tested with a series of unsaturated galacturonic acid oligomers.

EXPERIMENTAL

The HPAEC/MS system consisted of a Dionex (Sunnyvale, CA, USA) DX-300 chromatography microbore system coupled to a Finnigan MAT (San Jose, CA, USA) TSQ-700 mass spectrometer equipped with a custom made electrospray interface,[12] which was operating in the ion spray (IS) mode. The experimental arrangement is depicted in Fig. 1.

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 poly ether ether ketone (PEEK) injector with a 6.3 μL loop, and a pulsed amperometric detector (PAD) with a gold electrode. A membrane desalting device, introduced by Dionex as carbohydrate membrane desalter, which is a cation-exchange micromembrane system to remove the sodium ions from the effluent stream prior to introduction into the MS, was connected after the chromatographic column. 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 at 500 mA. The water reservoir was pressurized by 50 kPa of helium resulting in a water flow rate of 2 mL/min when the current of the Dionex SRS controller was on.

A 250 mm × 2 mm ID Dionex CarboPac PA1 column was used at a flow-rate of 0.2 mL/min. Linear gradient elution was performed using (A) water, (B) 0.5 M sodium hydroxide, (C) 0.625 M sodium acetate. The flow rate through the column was 0.2 mL/min.

Due to the negligible back pressure in the electrospray interface, the outlet of the MDD could be coupled directly to the MS. For optimal performance and electrical contact a liquid sheath flow rate of 10 μL/min, consisting of a mixture of isopropanol 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, were used. In order to prevent electrical breakdown by means of superfluous solvents the atmospheric pressure region is pumped by a Leybold 12 m^3/hr rotary pump. Pumping is performed with a leaking system keeping the pressure in the interface at 1 bar. The ion source itself is pumped by an Edwards 28 m^3/hr rotary pump. In the pipeline to this pump a ball-valve is installed to reduce the pumping capacity in the ion source, thus keeping the ion source pressure at 400 Pa. The mass spectrometer was optimized with respect to the temperature of the sampling capillary, which appeared to be 200 °C, and optimal repeller voltage, which appeared to be mass dependent and varies between 50 and 100 V in positive ionization mode for DP2 and DP7, respectively. The mass spectra were acquired in both positive and negative ionization modes, either by scanning from *m/z* 200 to 1500 in 3 s, or in selective ion monitoring (SIM) mode.

Chemicals

Isopropanol, sulphuric acid and sodium hydroxide were purchased from Baker (Deventer, Netherlands). Water was purified with a Milli-Q apparatus (Millipore, Bedford, MA, USA). Sodium acetate was obtained from Merck (Darmstadt, Germany). The maltodextrine MD-20 was supplied by Roquette (Lille, France). The α-1,4-glucose oligomers were obtained from Boehringer Mannheim (Mannheim, Germany). The mixture of unsaturated galacturonic acid oligomers was prepared by degrading polygalacturonic acid with endopectate lyase from *Pseudomonas GK5.*[13]

RESULTS

Ion spray

In the case of TSP, evaporation as well as ion focusing takes place within the TSP ion source. Due to the residual sodium ions after evaporation and to the presence of an increasing concentration of acetic acid during sodium acetate gradients, cluster ions will be formed. They will be stabilized in the low pressure region in the ion source, introducing in this way a high chemical background. This hampers full scan mass spectrometric detection, which influences the sensitivity unfavourably due to space–charge effects.

However, using electrospray (ESI) or ion spray (IS), the ionization takes place in atmospheric conditions. The involatile sodium acetate cluster ions probably will be formed, but will be dissociated and/or neutralized before entering the heated sampling capillary.

Although not performed by Conboy and Henion,[9] HPAEC coupled with IS is well suited for a design without

a booster pump, and the outlet of the MDD can be coupled directly to the MS due to the negligible back pressure in the IS probe. In this way the loss of sensitivity caused by diffusion in the total chromatographic system is limited to the MDD alone. Due to the high background of cluster ions using TSP this method has a limited capacity, capable of only confirming molecular masses in SIM. The absence of cluster ions in IS makes this ionization technique suitable for full scan mass spectrometric detection.

Anion-exchange membrane suppressor

The incompatibility of anion-exchange chromatography with mass spectrometry can be overcome partly by using an anion micromembrane suppressor. With 0.15 M sulphuric acid as regenerating solvent and a flow rate of 10–15 mL/min, sodium ions could be displaced by hydronium ions to an extent sufficient for application up to a total sodium concentration of 0.4 M. This means that, with a base concentration of 0.1 M sodium hydroxide, sodium acetate gradients up to 0.3 M could be used. However, when using a CarboPac PA-1 column, oligomers of e.g. galacturonic acid and arabanes elute at higher sodium acetate concentrations starting at DP 3 and 6.

To extend the applicability of HPAEC/MS to even higher total sodium concentrations a recently developed anion membrane suppressor or so-called membrane desalting device,[14] was used. With respect to the AMMS the MDD differs by the design of the compartment, the connection of the electrodes and the polymer used as membrane. The MDD exchanges sodium ions for hydronium ions from the effluent stream by means of electrolysis or a combination of electrolysis and pneumatically supplied sulphuric acid at a flow rate of 1–2 mL/min. The system was tested with both settings, using an α-1,4-glucose mixture up to DP7. Sulphuric acid is known to pass the membrane, thus partly entering the effluent chamber resulting in a pH below 2. In this way negative ionization can be promoted by forming deprotonated molecules, $[M\text{-}H]^-$, together with several cluster ions with acetic acid as well as with sulphuric acid, such as $[M+OAc]^-$, and $[M+HSO_4]^-$. However, signal intensities are thus distributed over more then one *m/z* value,[15] which makes it less attractive to perform SIM experiments. Another drawback of passage of sulphuric acid through the membrane is suppression of the cationization of the oligosaccharides, in this way decreasing the sensitivity in positive ionization mode.

Comparison between positive and negative ionization, using the combination of electrolysis and pneumatically supplied sulphuric acid, showed that almost the same performance was obtained in the two modes when comparing sodiated molecules $[M+Na]^+$ with sulphuric acid cluster ions $[M+HSO_4]^-$, which appeared to be the most abundant ion using negative ionization. Full scan experiments in negative ionization showed also the presence of $[M\text{-}H]^-$, $[M+H_2SO_4+HSO_4]^-$ and $[M+2H_2SO_4+NaSO_4]^-$, which was anticipated from earlier experiments.

However, use of the MDD only performing electrolysis of water (no sulphuric acid) showed an increase of pH to 3–4, together with an increase in signal in positive ionization mode by a factor of 10. Using negative ionization the sensitivity was not influenced drastically, but the ions observed were changed from cluster ions with sulphuric acid to acetic acid. It was decided to perform further experiments with the MDD in the electrolysis mode, paying most attention to positive ionization.

HPAEC/MS

The experimental arrangement depicted in Fig. 1 shows the PAD in-line with the MDD and the IS probe. However, the PAD used appears to have a dead volume of about 130 μL,

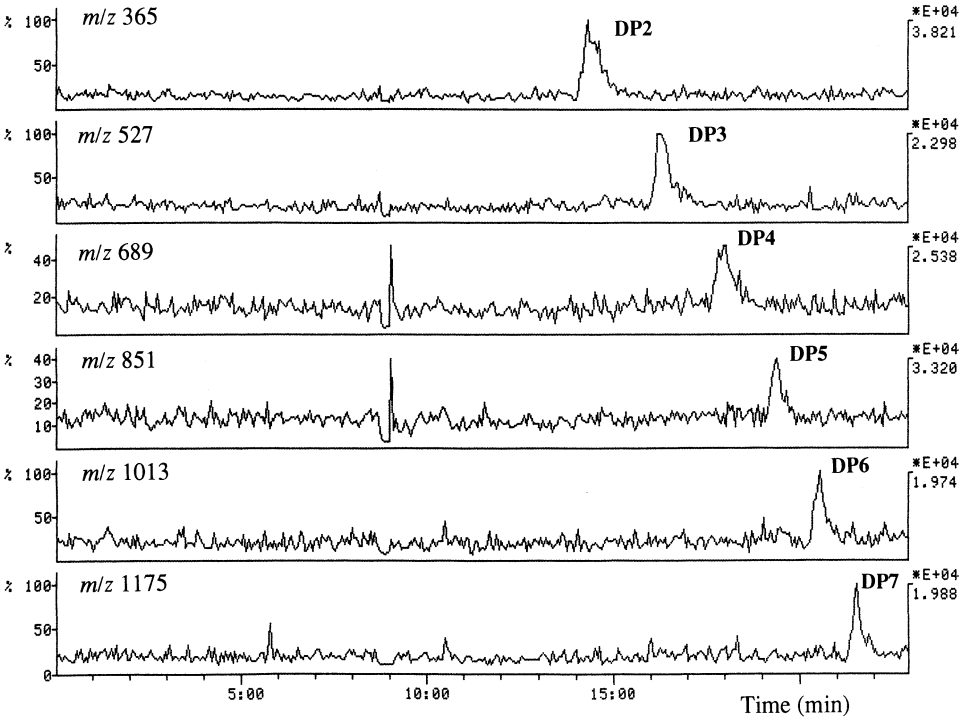

Figure 2. HPAEC/MS of α-1,4-glucose oligomers, each 3 μg/mL, up to DP7. The ions indicated are sodiated molecules. The linear gradient started at 0.1 M sodium hydroxide, up to 0.325 M sodium acetate + 0.1 M sodium hydroxide in 25 min.

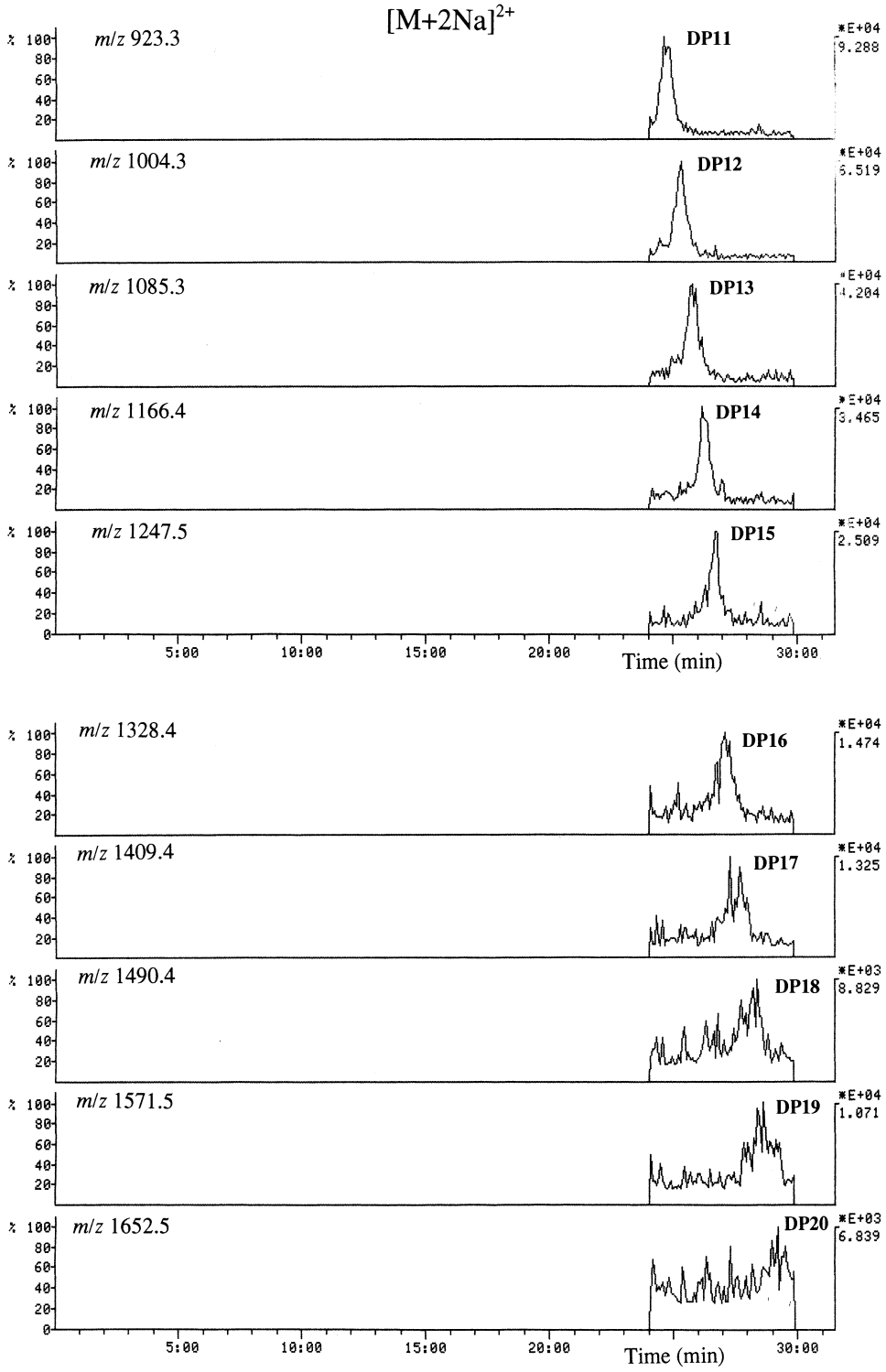

Figure 3. HPAEC/MS of maltodextrine (MD-20), 1 mg/mL. The ions indicated are doubly charged disodiated molecules. The linear gradient started at 0.1 M sodium hydroxide, up to 0.325 M sodium acetate + 0.1 M sodium acetate in 25 min, and then kept constant for 10 min.

leading to extra peak broadening in the chromatographic system using a flow rate of 0.2 mL/min. The MDD used appeared to be a 4 mm version, designed to be well suited for flow rates of 0.5–1.0 mL/min. However, using a flow rate of 0.2 mL/min the dead volume of 70 μL must be taken into account.

It was decided to omit the PAD from the system for sensitivity improvement. Using this configuration the detection of a series of α-1,4-glucose oligomers, each 3 μg/mL, is shown in Fig. 2, performing SIM experiments on the sodiated molecules. The gradient used was linear, starting from 0.1 M sodium hydroxide, up to 50% 0.625 M sodium acetate in 25 min, resulting in a maximum sodium concentration of 0.425 M. Sensitivity improvements, with respect to earlier published work, are at least one order of magnitude.

Up to DP8 the α-1,4-glucose oligomers can be detected as their sodiated molecules (M + 23), whereas above DP8 the

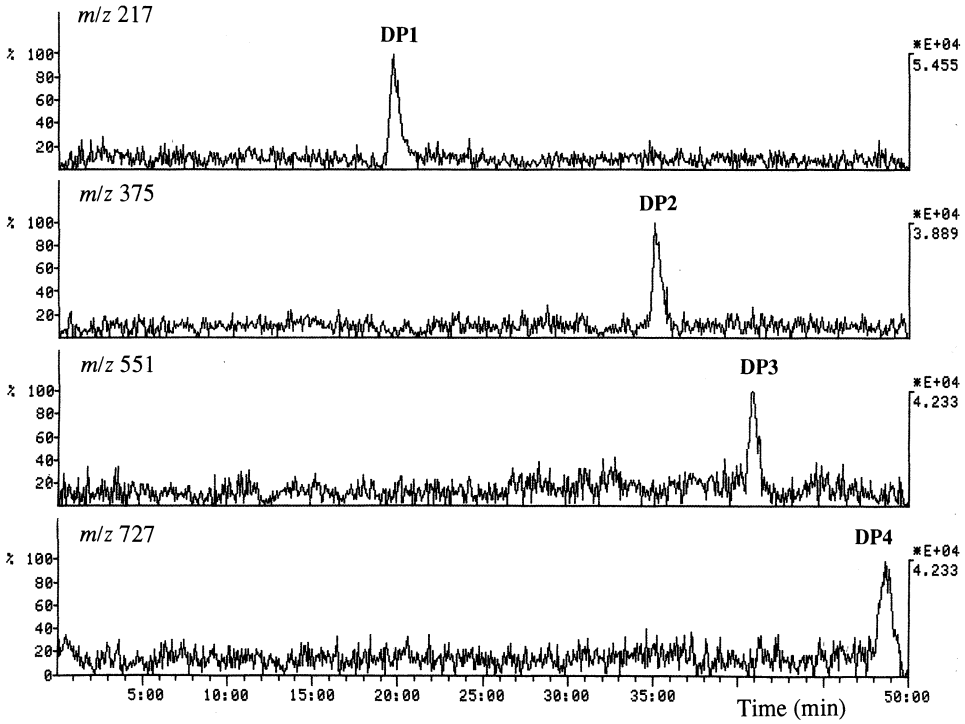

Figure 4. HPAEC/MS of unsaturated galacturonic acid oligomers, each 10 μg/mL, up to DP4. The ions indicated are sodiated molecules. The linear gradient started at 0.1 M sodium hydroxide, up to 0.5 M sodium acetate + 0.1 M sodium hydroxide in 30 min, and then kept constant for 30 min.

most dominant ions are the doubly charged disodiated molecules at $m/z = (M_r + 46)/2$. To show the applicable mass range the detection of DP11 to 20 is shown in Fig. 3, using different SIM procedures monitoring only the doubly charged disodiated molecules of a maltodextrine sample (MD-20) at a total concentration level of 2 mg/mL. The gradient used was linear, starting from 0.1 M sodium hydroxide, up to 50% solvent C in 25 min, and maintained constant for 10 min. Although the total concentration of the MD-20 is high it should be noted that the contribution of the higher oligomers is minor. (The definition of MD is the amount of reducing sugars per 100 gram sample, times 100 percent. This means that maltose for example, can be characterized as MD-50.)

The combination of the high capacity continuous desalting of sodium-containing effluents by the MDD by electrolysis of water, with IS as ionization technique, expanded the range of sodium gradients which could be used up to a level of 0.6 M total sodium concentration. No blockage of the interface has been observed using such high sodium concentrations. The sampling capillary was washed thoroughly with a mixture of water/methanol daily, before starting the experiments. Although the sampling capillary of the ESI interface turned black during the day, no severe loss of sensitivity has been observed.

Oligomers of galacturonic acid analysed with a CarboPac PA1 column elute at sodium acetate gradients above 0.3 M. Although with another stationary phase separations can be performed at lower sodium acetate gradients, a CarboPac PA1 column was used to demonstrate the application using a sodium gradient up to a total sodium concentration of 0.6 M. In Fig. 4 the SIM detection of a series of unsaturated galacturonic acids at a concentration of 10 μg/mL is shown. The gradient used was linear, starting from 0.1 M sodium hydroxide, increasing to 80% solvent C in 30 min, and kept constant for 30 min. This means that a maximum load of 0.6 M sodium is fed into the MDD for half an hour. Due to the almost total removal of the sodium ions in the MDD no decrease in signal for the sodiated molecules was observed for the linear range up to DP4. Although acidic oligosaccharides are expected to exhibit good sensitivity in negative ionization, this mode proved to be less reliable for the oligomers tested here. Only DP2 gave a better signal-to-noise ratio in negative ionization mode.

CONCLUSIONS

The combination of on-line coupling HPAEC with ion spray MS has improved both the sensitivity and robustness of the analysis of neutral and acidic oligosaccharides. On-line desalting is performed by a recently developed cation-exchange micromembrane system, a MDD, operating in the water electrolysis mode and thus avoiding the leakage of sulphuric acid to the effluent chamber, which appeared to be disadvantageous for mass spectrometric sensitivity. Using electrolysis alone, efficient exchange of sodium ions for hydronium ions can be obtained up to a total sodium concentration of 0.6 M, without affecting the mass spectrometric sensitivity. Using ion spray as ionization technique, which introduces negligible back pressure, a booster pump (required for thermospray operation) could be eliminated from the system.

Sensitivities comparable to those for a PAD could be obtained for both neutral and acidic oligosaccharides, e.g. detection limits of 20 ng for each component of a α-1,4-glucose oligomer mixture up to DP7.

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