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Introducing porous graphitized carbon liquid chromatography with evaporative light scattering and mass spectrometry detection into cell wall oligosaccharide analysis

Y. Westphal, H.A. Schols, A.G.J. Voragen, H. Gruppen*

Wageningen University, Laboratory of Food Chemistry, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

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ABSTRACT

Separation and characterization of complex mixtures of oligosaccharides is quite difficult and, depending on elution conditions, structural information is often lost. Therefore, the use of a porous-graphitized-carbon (PGC)-HPLC-ELSD-MS^n-method as analytical tool for the analysis of oligosaccharides derived from plant cell wall polysaccharides has been investigated. It is demonstrated that PGC-HPLC can be widely used for neutral and acidic oligosaccharides derived from cell wall polysaccharides. Furthermore, it is a non-modifying technique that enables the characterization of cell wall oligosaccharides carrying, e.g. acetyl groups and methylesters. Neutral oligosaccharides are separated based on their size as well as on their type of linkage and resulting 3D-structure. Series of the planar β-(1,4)-xylo- and β-(1,4)-gluco-oligosaccharides are retained much more by the PGC material than the series of β-(1,4)-galacto-, β-(1,4)-manno- and α-(1,4)-gluco-oligosaccharides. Charged oligomers such as α-(1,4)-galacturonic acid oligosaccharides are strongly retained and are eluted only after addition of trifluoroacetic acid depending on their net charge. Online-MS-coupling using a 1:1 splitter enables quantitative detection of ELSD as well as simple identification of many oligosaccharides, even when separation of oligosaccharides within a complex mixture is not complete. Consequently, PGC-HPLC-separation in combination with MS-detection gives a powerful tool to identify a wide range of neutral and acidic oligosaccharides derived from various cell wall polysaccharides.

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1. Introduction

Plant cell wall polysaccharides play an important role in the structure and functionality of the whole plant [1,2]. The elucidation of their biosynthesis as well as a better understanding of their functional properties and enzymatic degradability is essential for the use of these polysaccharides by the food and non-food industry. Therefore, detailed analysis of their precise structure is essential [3].

Since monomeric sugar composition analysis alone cannot deliver any structural information of the parental polysaccharide, the analysis of these polysaccharides is mostly done after partial degradation by chemical or enzymatic treatment into oligosaccharides. Nowadays, high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is frequently used for oligosaccharide analysis [4,5]. However, the high pH eluent used for separation and detection will result in elimination of methylesters and acetyl groups of the oligomers under investigation. In addition, HPAEC-PAD is not fully MS-compatible due to the high salt content used within the eluents. As methyl- and acetyl-substitutions are important for the function and the properties of polysaccharides, it is necessary to have analytical methods that leave the structural features intact [6].

Recently, oligosaccharide analysis using capillary electrophoresis (CE) coupled to laser induced fluorescence (LIF) and mass spectrometry (MS) detection has been described [7–9]. This technique is very powerful and provides good separation of many oligosaccharides derived from plant cell wall polysaccharides in a short time but may not be commonly available. In addition, sample preparation includes time-consuming labeling of the oligosaccharides with a fluorescent chromophore by which non-reducing sugars are excluded from analysis. Furthermore, acidic oligosaccharides derived from pectin are being eluted rather rapidly due to the additional charges and an adequate separation is rather difficult to obtain [9].

Another available technique for the separation and identification of neutral oligosaccharides is the gradient elution using RP-HPLC coupled to an evaporative light scattering (ELS) detector and a mass spectrometer (MS) as described by Kabel et al. [10]. However, due to the high polarity no retention could be achieved for acidic oligosaccharides derived from pectins. Recently, Hem-

* Corresponding author. Tel.: +31 317 483211; fax: +31 317 484893.
E-mail address: harry.gruppen@wur.nl (H. Gruppen).

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ström and Irgum [11] reviewed the use of hydrophilic interaction chromatography (HILIC) for the separation of certain classes of oligosaccharides. Although the use of HILIC within oligosaccharide analysis seems to be promising, solubility of higher oligomers in the starting eluent (e.g. 80% organic modifier) might be a problem.

Porous graphitized carbon (PGC)-HPLC columns have been shown to be able to separate maltodextrins, fructo-oligosaccharides, human milk oligosaccharides, oligosaccharides derived from glycoproteins and even very polar sugar phosphates and glucosinolates [12,13]. The ability of PGC material in retaining very polar components is based on the polar retention effect of graphite, which enables electron transfer between the carbon material and the acidic analytes as described by Pereira [14].

As analysis of neutral and acidic oligosaccharides still lacks a rapid, versatile and widely available technique, this research aimed at the separation and identification of a broad range of neutral and acidic oligosaccharides within complex mixtures derived from plant cell wall polysaccharides. To this end, the use of PGC-HPLC in combination with ELS- and MS-detection was explored.

2. Experimental

2.1. Materials

2.1.1. Neutral and acidic homoglycan oligosaccharides

The following components have been used as such: maltodextrins (α-1,4-gluco-oligomers) with an average degree of polymerization (DP) of 5 (AVEBE, Veendam, The Netherlands) and cellulodextrins (β-1,4-gluco-oligomers) with DP 2–7 (a kind gift of Dr. Vladimir Farkas, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia).

α-1,5-Arabino-oligomers (DP 1–8) were produced by partial enzymatic degradation of linear arabinan with α-1,5-arabinanase (*Aspergillus aculeatus*) [15]. A mixture of acetylated β-1,4-xylo-oligomers derived from *Eucalyptus* wood has been produced as described by Kabel et al. [16] (fraction Euc NI A). β-1,4-Manno-oligomers were produced by partial enzymatic degradation of β-1,4-mannan derived from Ivory Nut (Megazyme, Bray, Ireland) with β-1,4-mannananase (*Aspergillus niger* [17]). β-1,4-Galacto-oligomers were produced by partial enzymatic degradation of pectic potato galactan (Megazyme, Ireland) with β-1,4-galactanase (*A. niger* [18]).

Purified fractions of α-1,4-galacturonic acids (DP 2–10) as described by Van Alebeek et al. [19] have been used.

2.1.2. Neutral and acidic heteroglycan oligosaccharides

Xyloglucan oligosaccharides derived from tamarind were obtained after digestion with xyloglucan specific endo-glucanase (XEG, *A. aculeatus*) as described by Hilz et al. [8].

Partially esterified α-(1,4)-galacturonic acid oligosaccharides were obtained by digestion of low methylesterified pectin (DM 30) with α-(1,4)-endo-polygalacturonase (*Kluyveromyces fragilis marxianus* [20]).

Oligosaccharides derived from rhamnogalacturonan I were obtained by saponification of pectic modified hairy regions (MHR-B; [21]) with 0.1 M sodium hydroxide followed by treatment with rhamnogalacturonan hydrolase in overdose [22].

Xylogalacturonan oligomers were produced by treating xylo-galacturonan from *gum tragacanth* [23] with an overdose of xylogalacturonan hydrolase (XGH [24]).

2.2. HPLC-ELSD-MS^n

Liquid chromatography was performed on a Thermo Accela UHPLC system (Waltham, MA, USA) equipped with a Hypercarb column (PGC, 100 mm × 2.1 mm; 3 μm, Thermo Electron Corporation, San José, USA) in combination with a Hypercarb guard column (10 mm × 2.1 mm; 3 μm, Thermo Electron Corporation). Elution was performed with a flow of 0.4 mL/min and a column oven temperature of 70 °C. The injection volume was set to 10 μL. The following eluents were used: Millipore water (A), acetonitrile (B) and 0.2% (v/v) trifluoroacetic acid in water (TFA, C), to all eluents 25 μM sodium acetate was added to ensure sodium adducts of all components. The following gradient was used: 0–1 min, isocratic 100% A, 1–15 min, linear from 0 to 27.5% (v/v) B, 15–28 min linear from 27.5 to 60% B and concomitant linear from 0 to 10% (v/v) C, 28–31 min linear from 60 to 80% B and from 10 to 20% C, 31–35 min isocratic 80% B and 20% C, 35–36 min from 80% B and 20% C to 100% A, 36–41 min equilibration with 100% A.

The PGC-column was coupled to a 1:1-splitter (Accurate, Dionex, Sunnivale, USA) directing the eluent both to the evaporative light scattering detector (ELSD 85, Sedere, Alfortville Cedex, France) and to the ESI-MS^n-detector (LTQ XL MS, ion trap, Thermo Scientific, San Jose, CA, USA). The ELSD micro flow nebulizer (Sedere, Alfortville Cedez, France) had a gas pressure of 3.5 bar and a gas flow of 1.75 L/min. The drift tube temperature of the ELSD was set to 50 °C and the gain to 12. MS-detection was performed in positive mode using a spray voltage of 4.5 kV and a capillary temperature of 260 °C and auto-tuned on malto-pentaose (m/z 851). The MS^n-experiments were performed based on dependent scan-settings (Xcalibur software, Thermo Electron Corporation, San José, USA). The addition of sodium acetate (25 μM) in combination with the use of TFA introduces a significant amount of noise due to the polymerization of sodium trifluoroacetate ($\Delta m/z$ 136). The m/z-values originating from the sodium-TFA-complexes are excluded from the online-MS^2 measurements by means of Xcalibur software (Thermo Electron Corporation, USA). The column was regenerated with 70 column volumes of tetrahydrofuran after each series of analysis [25].

3. Results and discussion

Various mixtures of oligosaccharides have been analyzed with a PGC-HPLC column in order to investigate the potential of porous graphitized carbon material in the analysis of oligosaccharides derived from a broad range of neutral and acidic oligosaccharides and to understand the mechanism involved in this separation.

3.1. Optimization of elution conditions for separation of various cell wall derived oligosaccharides

Different conditions including various compositions of organic and acidic modifiers as well as different temperatures have been investigated for their ability to elute and separate the broad range of cell wall derived neutral and acidic oligosaccharides.

Water–acetonitrile (ACN)-gradients have been described before to be effective to separate oligosaccharides of various sources [13,26]. During this research the steepness of the water–ACN-gradient has been varied and an increase of 2% ACN/min has been selected as a suitable compromise for the separation of all neutral oligosaccharides under investigation in reasonable run times. In Fig. 1A–C typical chromatograms of α-1,4-linked maltodextrins (Fig. 1A), β-1,4-linked cellulodextrins (Fig. 1B) and α-1,5-linked arabinan oligomers (Fig. 1C) are presented.

α-(1,4)-Galacturonic acid oligomers are strongly retained by the PGC material and an acidic modifier is needed for elution. During this research three different acidic modifiers have been investigated, namely acetic acid, formic acid and TFA. The use of acetic acid did not result in an elution of all charged oligomers under investigation, whereas formic acid and TFA enable the elution of

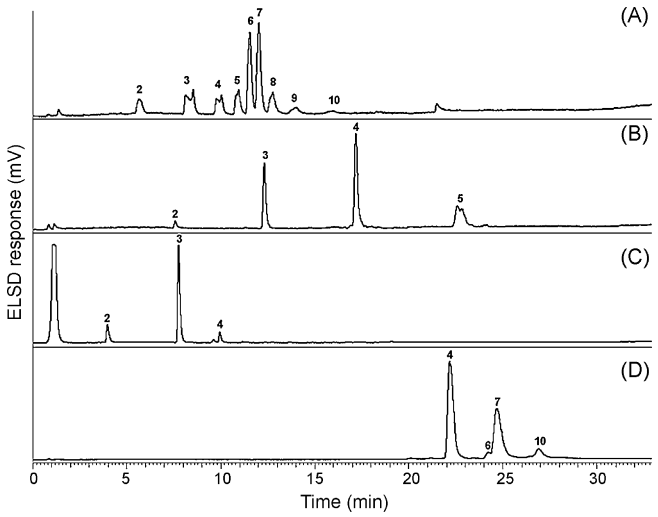

Fig. 1. Elution pattern of oligosaccharides on a PGC column using ELS-detection: (A) maltodextrins, (B) cellulodextrins (DP 2–5), (C) linear arabinan after digestion with endo-arabinanase and (D) mixture of galacturonic acids (tetra-, hepta- and decamer).

all oligomers. For a quick overview of all oligomers being present in a complex mixture, TFA is more suitable than formic acid due to less retention of the acidic oligomers when using TFA as acidic modifier. Nevertheless, for separation of specific series of oligosaccharides, the use of formic acid may provide a good alternative due to reduced elution strength. In Fig. 1D the chromatogram of α-(1,4)-galacturonic acid oligomers (DP 4, 7 and 10) by using a water–acetonitrile-gradient followed by a second gradient of trifluoroacetic acid (TFA) is presented.

Due to the fact that the addition of TFA is only started after 15 min, first neutral and afterwards acidic oligosaccharides are eluted (Fig. 1A–D). This elution behavior simplifies the identification of unknown components because the retention time already provides an indication about the nature of the component.

The PGC-HPLC system is very versatile due to the possibility of using various organic and acidic modifiers. Thus, many adjustments can be performed, which are tailored for separating specific oligosaccharides. For strongly retained neutral oligosaccharides, such as xylo- and cellulo-oligosaccharides, either the steepness of the water–ACN-gradient can be adjusted or a stronger organic modifier such as isopropanol can be used for a faster elution. The use of ammonium acetate (pH 8) for elution has been investigated as well as it has been shown to be efficient in separating oligosaccharides derived from glycoproteins [25]. Indeed, separation of neutral and even acidic oligosaccharides could be achieved, although MS^n-spectra obtained from the NH_4^+-adducts of the oligomers did not include any structural information due to the absence of internal ring cleavages as has been described earlier [27].

Due to the tendency of the PGC-material to separate anomeric structures, which is not desired as this would not give any additional information, the separation has been carried out at elevated temperature (70 °C). Elevated temperatures have been described earlier to significantly reduce anomeric peak-forming for dimers [28]. In this study this behavior was seen for oligomers as well.

3.2. Influence of the degree of polymerization on retention time

To illustrate the influence of the DP, the structural conformation and the charge of the different oligosaccharides on the retention time under the conditions used for this research, the retention time has been plotted against the DP (Fig. 2).

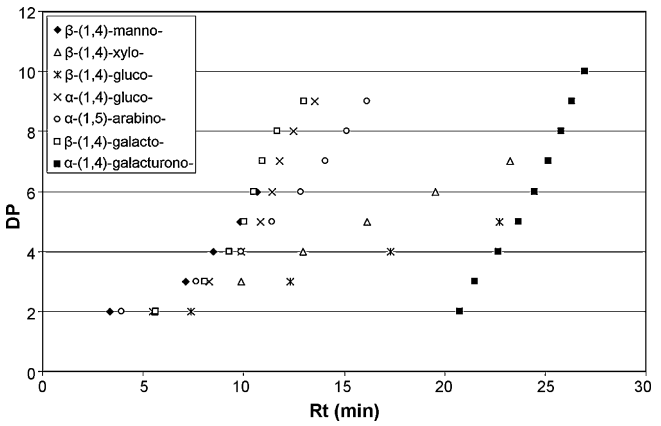

Fig. 2. Retention time (min) plotted against the degree of polymerization (DP) for different neutral and acidic oligosaccharides: (♦) β-(1,4)-manno-, (△) β-(1,4)-xylo-, (∗) β-(1,4)-gluco-, (×) α-(1,4)-gluco-, (○) α-(1,4)-arabino-, (□) β-(1,4)-galacto- and (■) α-(1,4)-galacturono-oligosaccharides.

3.2.1. Neutral oligosaccharide series

The series of β-(1,4)-gluco-oligosaccharides and β-(1,4)-xylo-oligosaccharides elute significantly later compared to other neutral oligosaccharide series such as β-(1,4)-galacto-, β-(1,4)-manno- and α-(1,4)-gluco-oligosaccharides. This elution behavior is in good agreement with the elution mechanism based on both hydrophobicity and planarity of the molecule [14]. A more planar structure will have a greater number of points of interaction with the graphite surface. While all hydroxyl-groups within the β-(1,4)-gluco- and the β-(1,4)-xylo-oligosaccharides are oriented in equatorial direction, in the β-(1,4)-manno- and β-(1,4)-galacto-oligosaccharides one hydroxyl-group is oriented in axial direction, O-2 for β-(1,4)-manno- and O-4 for β-(1,4)-galacto-oligosaccharides, respectively.

The series of α-(1,4)-gluco-oligosaccharides elutes significantly earlier in comparison to the β-(1,4)-gluco-oligosaccharides. Whereas the β-(1,4)-glucose-linkage creates a flat structure of the oligosaccharides, the α-(1,4)-glucose-linkage results in a helical structure which has fewer points to interact with the column material resulting in earlier elution. The retention of α-(1,5)-arabino-oligosaccharides by the graphite surface is stronger when compared to β-(1,4)-manno-, β-(1,4)-galacto- and α-(1,4)-gluco-oligosaccharides.

3.2.2. Acidic oligosaccharide series

PGC material has been described to have a so called 'polar retention effect on graphite' where charge-induced interactions

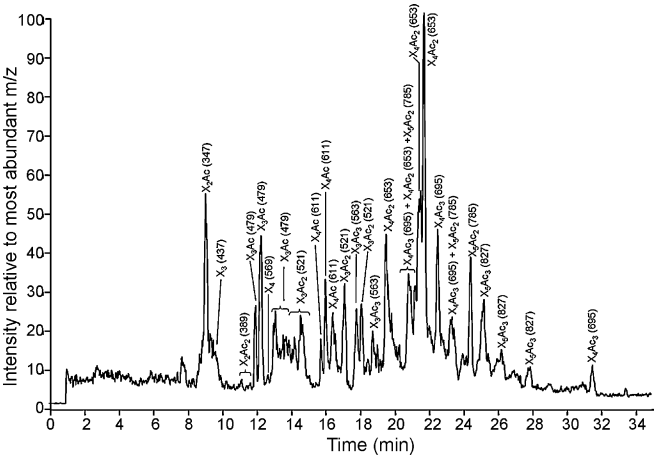

Fig. 3. PGC-HPLC-elution profile of an *Eucalyptus* wood xylan hydrolysate: mass base peak chromatogram; in brackets the *m*/*z*-values of sodium adducts are given.

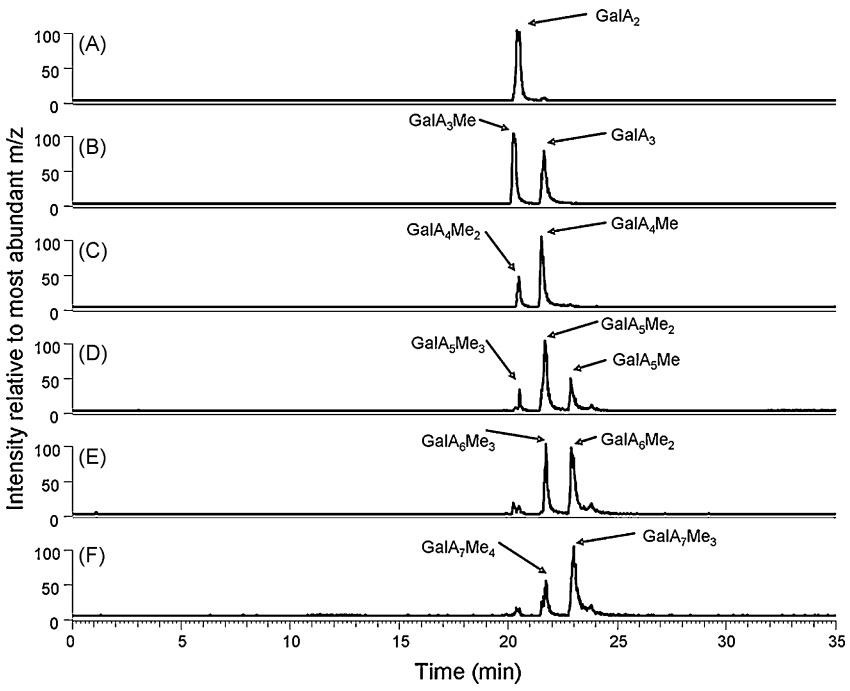

Fig. 4. PGC-HPLC-elution profile of partly methylesterified galacturonic acids derived from DM 30 pectin after treatment with endo-polygalacturonase (MS detection): (A) selected ion chromatogram (SIC) of $GalA_2$ (393); (B) SIC of $GalA_3$ (569), $GalA_3Me$ (583); (C) SIC of $GalA_4$ (745), $GalA_4Me$ (759), $GalA_4Me_2$ (773); (D) SIC of $GalA_5Me$ (935), $GalA_5Me_2$ (949), $GalA_5Me_3$ (963); (E) SIC of $GalA_6Me_2$ (1125), $GalA_6Me_3$ (1139), $GalA_6Me_4$ (1153); (F) SIC of $GalA_7Me_3$ (1325), $GalA_7Me_4$ (1339), $GalA_7Me_5$ (1353); in brackets the *m*/*z*-values of sodium adducts are given.

of the graphite surface with hydrophilic analytes determine the retention behavior [14]. The retention behavior of the analyzed α-1,4-galacturonic acid oligosaccharides supports this mechanism. By applying a gradient addition of TFA (0.002%/min), elution of α-1,4-galacturonic acid oligomers with good separation can be achieved (Fig. 2) while maintaining good peak shape as can be seen in Fig. 1. The separation of the galacturonic acid-oligomers (DP 2–10) seems to be predominantly based on their net charge.

3.2.3. Retention behavior of complex neutral oligosaccharides

To investigate the influence of acetyl groups on the retention behavior of neutral oligosaccharides in more detail an *Eucalyptus* wood xylan hydrolysate [16] has been subjected to the PGC column. The mass base peak chromatogram including peak annotation as based on the interpretation of automated MS^{1-2}-spectra, is presented in Fig. 3. Highly acetylated xylo-oligosaccharides are present in the *Eucalyptus* wood xylan hydrolysate confirming the

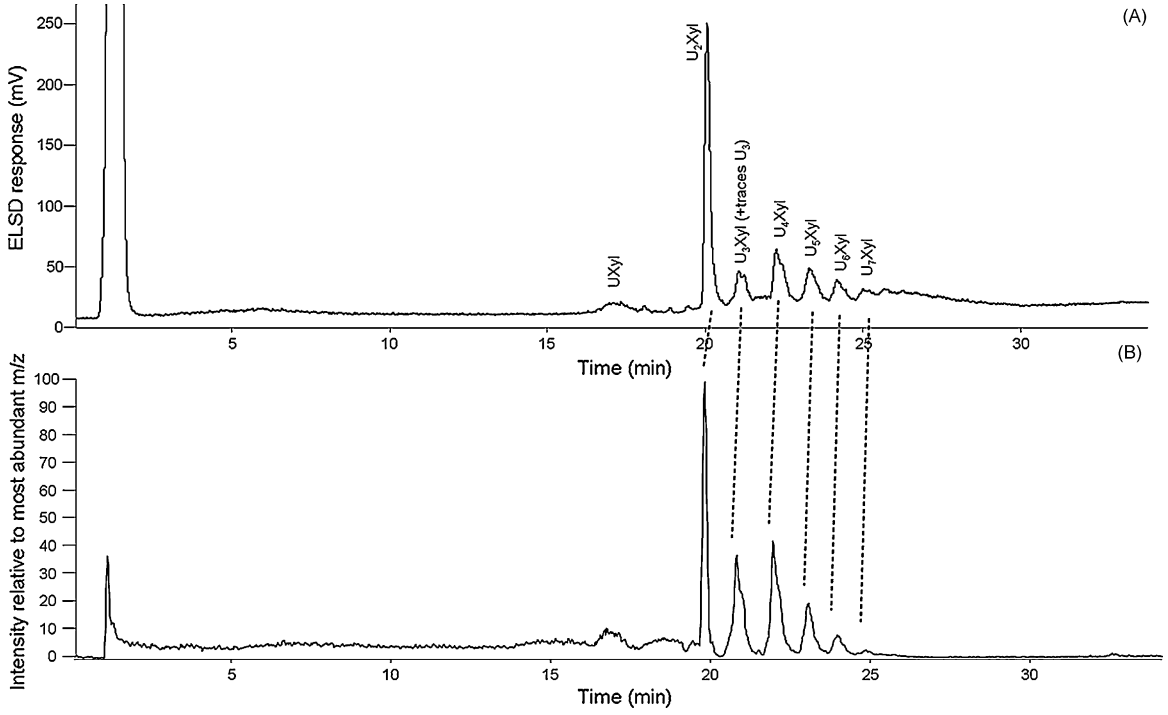

Fig. 5. PGC-HPLC chromatograms of a xylogalacturonan derived from *gum tragacanth* after digestion with xylogalacturonan hydrolase with (A) ELS-detection and (B) MS (base peak)-detection; annotation of peaks according to their *m*/*z*-values.

results of Kabel et al. [10]. With increasing number of acetyl-groups attached to the xylo-oligosaccharides of a given DP, the retention times increase significantly. Many peaks with the same *m*/*z*-values, corresponding to differently acetylated xylo-oligosaccharides, are present indicating that the PGC material is indeed able to separate different isomers.

The group of xyloglucan oligomers under investigation (XXXG, X(XL)G, XLLG, XLFG; nomenclature as described by Fry et al. [29]) elute between DP 5 and 6 of the β-(1,4)-gluco-oligosaccharides. This elution behavior is most likely due to the fact that the xyloglucan oligomers are mostly interacting with the column material through their glucan backbone. The side chains L and F are influ-

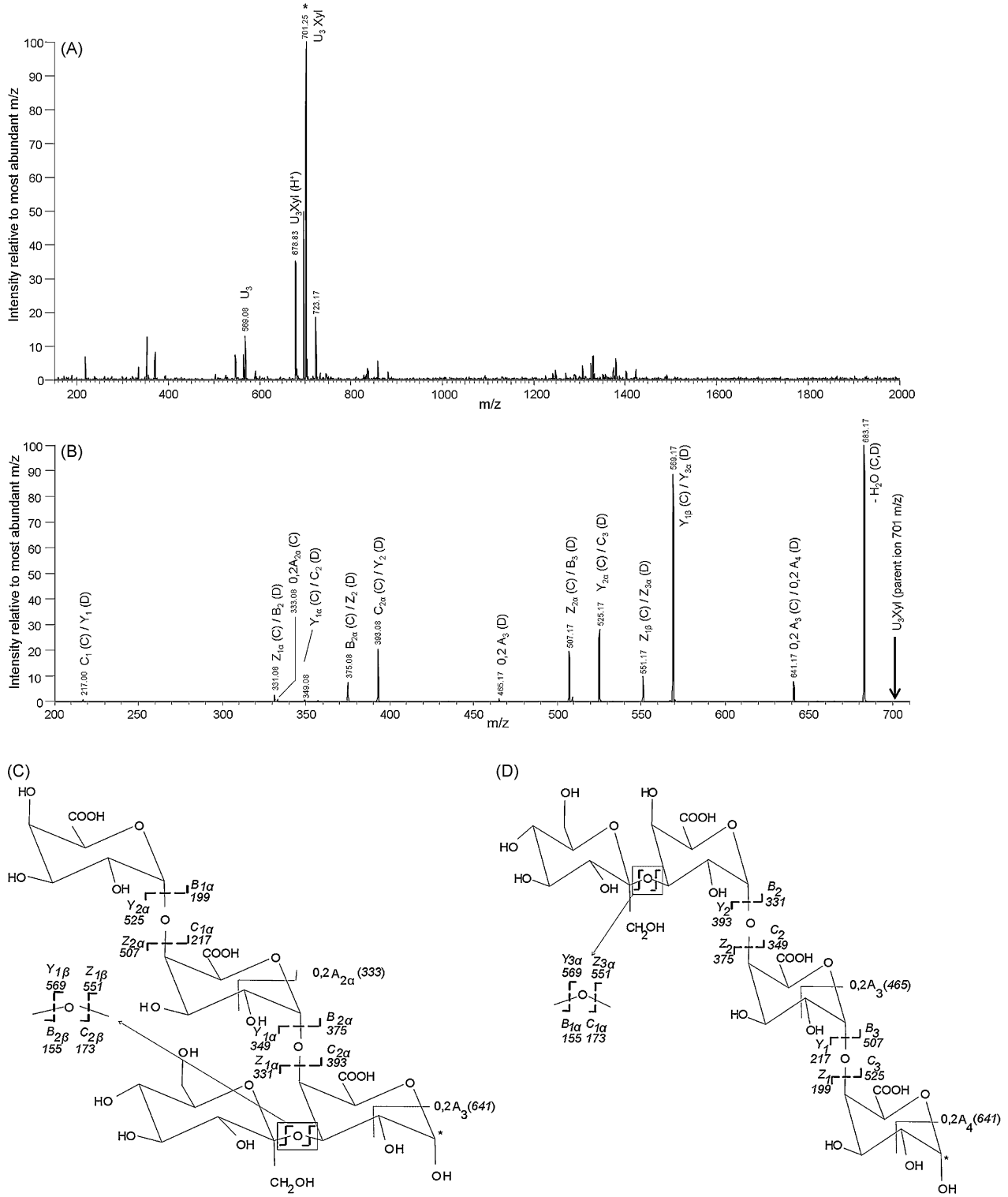

Fig. 6. MS^n spectra of the peak at 21.0 min in the ELSD chromatogram and 20.9 min in the mass base peak chromatogram (Fig. 5) of xylogalacturonan derived from *gum tragacanth* after digestion with xylogalacturonan hydrolase: (A) MS^1-spectrum at 20.9 min, (B) MS^2-spectrum of *m*/*z* 701 (marked with * in A), (C) chemical structure of U_3Xyl with xylose attached at the reducing end, (D) chemical structure of U_3Xyl with xylose attached at the non-reducing end (C and D: nomenclature of MS fragmentation pattern according to Domon and Costello [31]).

encing the retention mechanism based on planarity that much that separation between the xyloglucans occurs. The elution order of the xyloglucan oligosaccharides (XLLG < XLFG < XXXG < X(XL)G) is predominantly based on the extension or reduction of the planar system which is present in XXXG. However, further optimization of the elution conditions has to be performed for optimal separation, possibly by using a gradient including stronger organic modifiers, such as isopropanol.

3.2.4. Retention behavior of complex acidic oligosaccharides

The influence of the presence of methylesters on the elution behavior of the α-(1,4)-galacturonic acid oligomers has been investigated. In Fig. 4A–F the selected ion chromatograms (SIC) of oligo-uronides derived from a 30% methylesterified pectin (DM 30) is shown, representing oligo-uronides of various degrees of polymerization (DP 2–7) with a diverse number of methylesters attached. It can be observed that the presence of methylesters decreases the retention time of the α-(1,4)-galacturonic acid oligosaccharides significantly due to the shielding of a charged carboxyl-group by the methylester. As demonstrated in Fig. 4A–C the dimer ($GalA_2$) is coeluting with the trimer carrying one methylester ($GalA_3Me$) and the tetramer carrying two methylesters ($GalA_4Me_2$). This behavior can be observed for every charge-level (z) as is demonstrated in Fig. 4A–F for $z = 2–4$. From this behavior it can be concluded that the elution pattern of galacturonic acid oligomers, partly present as methylesters, appears to be predominantly based on the net charge of the molecules.

By applying a gradient using formic acid instead of TFA, a partial separation of galacturonic acid oligosaccharides with methylesters attached becomes possible, however, elution time will be increased significantly (data not shown).

A similar behavior is observed for rhamnogalacturonan- and xylogalacturonan-oligosaccharides. The elution patterns of these structures within the TFA gradient appear to be predominantly based on the net charge of the molecule. Only neutral sugar side chains of acidic oligomers highly influencing the planarity of the whole molecule will change the elution behavior in such a rate that separation will occur based on these differences in 3D-structure as well.

3.3. Structural information using PGC-HPLC-MS^n

In order to demonstrate the efficiency of our PGC-HPLC-ELSD-MS-method to elucidate complex acidic structures, a digest of xylogalacturonan derived from *gum tragacanth* after xylogalacturonan hydrolase digestion has been subjected to analysis. XGA is a pectic polysaccharide having a backbone of α-(1,4)-galacturonic acids containing xylose residues attached to O-3 of the galacturonic acids [30]. Typical XGA degradation products after XGH digestion are single xylose substituted oligo-uronides [9,24]. In Fig. 5 an overlay of a ELSD-chromatogram (Fig. 5A) and simultaneously recorded mass base peak chromatogram (Fig. 5B) is presented. Good alignment for both chromatograms can be observed, which allows mass peak annotation in the ELSD chromatogram. The given annotation in Fig. 5 has been made based on the online-measured mass spectra and is in agreement with HPAEC- and CE-LIF measurements [24]. For the peak eluted at 20.9 min in the ELSD-chromatogram and at 21.0 min in the mass base peak chromatogram the MS^1- and MS^2-spectra are presented in Fig. 6 to substantiate the annotation. As can be seen in Fig. 6A the main m/z-value present in the MS^1-spectrum is m/z 701 corresponding to $GalA_3Xyl$. Furthermore, some minor peaks are present: m/z 569 belongs to a trimer of galacturonic acid, which is not separated from $GalA_3Xyl$ as explained before. m/z 679 represents the H^+-adduct of $GalA_3Xyl$ and m/z 723 represents $GalA_3Xyl$ with additional sodium attached. All other peaks could be considered as background peaks.

To verify the position of the xylose attached, the online measured MS^2-spectrum of m/z 701 has been evaluated. In Fig. 6B the MS^2-spectrum is shown with all fragments annotated following the nomenclature established by Domon and Costello [31]. According to the MS^2-spectrum the structure with xylose attached to the second galacturonic acid within the oligomer is not very likely as the fragments m/z 331, 349, 375 and 393 indicate the presence of a dimer of galacturonic acid. The other two chemical structures are depicted in Fig. 6C (reducing end) and D (non-reducing end). As the reducing end is not labeled, the m/z-values of the fragments originating from glycosidic bond cleavage are similar in both structures. Nevertheless, the expected internal ring cleavage in the middle galacturonic acid ring is different as depicted in Fig. 6C and D ($^{0,2}A_{2\alpha}$, $^{0,2}A_3$), respectively. As both fragments, in very small amounts, are present in the MS^2-spectrum, it is most likely that both structures are present, although Zandleven et al. [24] and Coenen et al. [9] have only supposed the xylose to be attached to the reducing end of the oligomer.

4. Conclusions

To our knowledge this is the first time that a PGC-HPLC-ELSD-MS method has been applied to such a broad range of neutral and acidic oligosaccharides derived from plant cell wall polysaccharides. Within a homologous oligosaccharide series separation is based on size for neutral and on charge for galacturonic acid oligosaccharides. Between different series of neutral oligosaccharides the type of linkage and their resulting 3D-structure is responsible for separation. Acetyl groups present within neutral oligosaccharides result in a significant increase of retention.

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