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# Occurrence of cellobiose residues directly linked to galacturonic acid in pectic polysaccharides

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ABSTRACT

The study carried out in this work concerns the structural characterization of pectic polysaccharides from plum (*Prunus domestica* L.) and pear (*Pyrus communis* L.) cell walls and commercial pectic polysaccharides, obtained from *Citrus*. The α-(1 → 4)-D-galacturonic acid backbone was submitted to a selective hydrolysis with *endo*-polygalacturonase (EPG) and the fractions with low molecular weight (<1 kDa) obtained by size-exclusion chromatography were analysed by mass spectrometry using electrospray ionisation (ESI-MS). The ESI-MS spectra obtained revealed the presence of several $[M+Na]^+$ ions of pectic oligosaccharides identified as belonging to different series, including oligosaccharides constituted only by galacturonic acid residues ($GalA_n$, $n = 1–5$) and galacturonic acid residues substituted by pentose residues ($GalA_3Pent_n$, $n = 1–2$). Surprisingly, it was also observed the occurrence of galacturonic acid residues substituted by hexose residues ($GalA_nHex_m$, $n = 2–4$, $m = 1–2$). The fragmentation of the observed $[M+Na]^+$ ions, obtained under ESI-MS/MS and $MS^n$ allowed to confirm the proposed structures constituent of these pectic oligosaccharides. Furthermore, the ESI-$MS^n$ spectra of the ions that could be identified as $GalA_nHex_m$ ($n = 2–4$, $m = 1–2$) confirmed the presence of Hex or $Hex_2$ residues linked to a GalA residue. Methylation analysis showed the presence, in all EPG treated samples, of terminally linked arabinose, terminally and 4-linked xylose, and terminally and 4-linked glucose. The occurrence of GalA substituted by Glc, and Glc-β-(1 → 4)–Glc are structural features that, as far as we know, have never been reported to occur in pectic polysaccharides.



## 1. Introduction

Pectic polysaccharides are a group of associated polysaccharides present in plant cell wall. These polymers have an important role in plant growth, development, morphogenesis, defence, cell–cell adhesion, cell wall structure, signalling, cell expansion, cell wall porosity, binding of ions, and fruit development (Caffall & Mohnen, 2009; Voragen, Coenen, Verhoef, & Schols, 2009). Furthermore, it is of great importance to the food and cosmetic industries as it is used as a gelling and stabilizing additive (Stephen, 1995). In order to improve our understanding of their biological function and to explain their gelling and stabilizing properties, knowledge of detailed structural features is essential.

Pectic polysaccharides are constituted by different associated polysaccharides, such as homogalacturonans (HG), xylogalacturonans (XGA), type I rhamnogalacturonans (RG-I), type II rhamnogalacturonans (RG-II), arabinans, and arabinogalactans (Caffall & Mohnen, 2009; Mohnen, 2008; Ridley, O'Neill, & Mohnen, 2001; Stephen, 1995; Voragen et al., 2009). The most abundant pectic polysaccharides are HG, linear homopolymers of α-1,4-linked galacturonic acid (Gal*p*A) that comprise 60–70% of total pectic polysaccharides amount. HG could be partially methylesterified at the C-6 carboxyl group and/or *O*-acetylated at O-2 and/or O-3. XGA also have a HG backbone, but 25–75% of the Gal*p*A residues are substituted at C-3 with β-D-Xyl*p* residues. Xylose is mainly present as single residues but occasionally an additional β-2-linked or β-4-linked Xyl*p* to form a disaccharide have been identified (Le Goff, Renard, Bonnin, & Thibault, 2001; Oechslin, Lutz, & Amadò, 2003; Zandleven, Beldman, Bosveld, Schols, & Voragen, 2006). Furthermore, the Gal*p*A residues comprising the XGA backbone can be methylesterified and the methyl esters seem to be equally distributed among the substituted and unsubstituted Gal*p*A residues. The backbone of RG-I is composed of [→2)-α-L-Rha*p*-(1 → 4)-α-D-Gal*p*A-(1→] repeating units. The residues of Rha can be substituted (20–80%) at O-4 with Gal and/or Ara residues. In these side chains it has been identified single residues of β-D-Gal*p* as well as linear or branched polymers of arabinogalactans and/or arabinans.

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Other sugar residues could be also found in the side chains, such as α-L-Fuc*p*, β-D-Glc*p*A, and 4-O-Me-β-D-Glc*p*A (Strasser & Amadò, 2001). The Gal*p*A residues in the RG-I backbone may be highly acetylated on position O-2 and/or O-3 (Ridley et al., 2001). The most structurally complex pectic polysaccharide is RG-II, which consists of a HG backbone of at least eight 1 → 4-linked-α-D-Gal*p*A residues with branching chains consisting of 12 different types of sugars in over 20 different linkages. Four different side chains have been described in the RG-II structure, with peculiar sugar residues, such as apiose (Api), aceric acid, 3-deoxy-lyxo-2-heptulosaric acid (DHA), and 3-deoxy-manno-2-octulosonic acid (KDO) (Caffall & Mohnen, 2009; Mohnen, 2008; Ridley et al., 2001; Stephen, 1995; Voragen et al., 2009).

The high sensitivity and capacity of mass analyzers to analyse mixtures without any manipulation/derivatisation of sample, led to the emergent use of mass spectrometry for oligosaccharides structural characterization (Zaia, 2004), in particular, with the use of ionization techniques like matrix assisted laser desorption (MALDI) and electrospray (ESI). This technique allowed the identification of detailed structural features, namely sugars composition, sequence and branching of oligosaccharides (Fernández, 2007; Reis, Coimbra, Domingues, Ferrer-Correia, & Domingues, 2002; Zaia, 2004).

The analysis of pectic polysaccharides in their native form is difficult due to their complexity. In order to know more about their fine structure, they need to be degraded into oligosaccharides by controlled chemical and/or enzymatic hydrolysis. After partial fractionation of the degradation products, isolated structural elements can then be analysed by mass spectrometry. Therefore, mass spectrometry, in combination with the use of glycolytic enzymes, has been extensively used in the last few years to provide extensive structural information including monomer composition, branching, substituent type, and linkage information (Ralet, Lerouge, & Quéméner, 2009).

This work describes the structural features of pectic polysaccharides extracted from plum cell walls. In order to confirm the presence of these structural features in overall pectic polysaccharides, a pectic polysaccharide-rich extract from pear and a commercial pectin (obtained from *Citrus*) were also analysed. The oligosaccharides from these pectic polysaccharide sources were obtained by an enzymatic hydrolysis of the GalA backbone by an *endo*-polygalacturonase, followed by elution of the resultant oligosaccharides through a size-exclusion chromatography. The fractions with lower molecular weight were analysed by mass spectrometry using electrospray ionisation (ESI-MS and ESI-MS$^n$) and by methylation analysis.

## 2. Experimental

### 2.1. Samples origin and preparation

Pectic polysaccharides were extracted from plum (*Prunus domestica* L.) and pear (*Pyrus communis* L.) cell walls, as described by Ferreira, Barros, Coimbra, and Delgadillo (2001) and Nunes, Saraiva, and Coimbra (2008), fractions CA snCR and f:CR-spt, respectively. The commercial pectic polysaccharides were purchased from Riedel-de Haën (Sigma–Aldrich).

### 2.2. Enzymatic hydrolysis

The pectic polysaccharides were submitted to a selective hydrolysis of the α-(1 → 4)-D-galacturonic acid backbone with *endo*-polygalacturonase M2 (*Aspergillus niger*, EC 3.2.1.15, Megazyme, Ireland). The sample (50 mg) was dissolved in 10 mL phosphate buffer (0.1 M, pH 5.5) and the enzyme was added (100 U). The enzymatic hydrolysis was performed during 1 h at 25 °C with stirring. After hydrolysis, the material was concentrated on rotary evaporator (40 °C) until 1 mL.

To allow the cleavage of galacturonic acid residues by the *endo*-polygalacturonase (EPG), the commercial pectic polysaccharides were saponified by the addition of 2 M NaOH (80 mL). The reaction time was of 1 h (at 5 °C) and was finished by the addition of 2 M HCl (80 mL). After saponification, the precipitate was collected by centrifugation (4000 rpm for 10 min at 4 °C). This procedure was not necessary to perform on the plum and pear samples because they are resultant of an alkali treatment.

### 2.3. Size-exclusion chromatography

The oligosaccharides obtained after enzymatic hydrolysis were separated by size-exclusion chromatography using Bio-gel P2 for plum pectic polysaccharides and Bio-gel P6 for pear and commercial pectic polysaccharides, both gels from Bio-Rad Laboratories, CA, USA. Plum sample was eluted with water at a flow rate of 0.3 mL/min and fractions of 2.5 mL were collected, whereas pear and commercial pectic polysaccharides were eluted with acidified water (pH 2–3) at a flow of 0.6 mL/min and 1.5 mL fractions were collected. All fractions were assayed for sugars with the phenol–$H_2SO_4$ method and for uronic acids with m-phenylphenol colorimetric method (Blumenkrantz & Asboe-Hansen, 1973; Coimbra, Delgadillo, Waldron, and Selvendran, 1996). Elution profile was also monitored using an evaporative light scattering detector (ELSD). Exclusion and total volume were determined with blue dextran and glucose, respectively. The appropriated fractions were concentrated until dryness by centrifugal evaporation (Univapo 100 ECH, UniEquip, Munich, Germany) under vacuum.

### 2.4. Mass spectrometry

Positive-ion ESI-MS and ESI-MS/MS were carried out on a Micromass (Manchester, UK) Q-TOF2 hybrid tandem mass spectrometer. Fractions obtained after the size-exclusion chromatography with low molecular weight (<1 kDa) oligosaccharides were dissolved in 100 μL Milli-Q high-purity water and the excess of sodium was removed by exposing the solution to Dowex® 50W-X8 cation exchange resin (Bio-Rad Laboratories, CA, USA). Samples were diluted in methanol/water (50:50, v/v) containing formic acid 1% (v/v).

For ESI analysis, samples were introduced at a flow rate of 10 μL/min into the ESI source. In the MS and MS/MS experiments, the time-of-flight (TOF) mass resolution was set to approximately 9000. The cone voltage was 35 V and the capillary voltage was 3 kV. The source temperature was 80 °C and the desolvation temperature was 150 °C. MS/MS spectra were obtained using argon as the collision gas with the collision energy set between 20 and 45 eV (Gonçalves et al., 2009). The data was processed using MassLynx software (version 4.0).

A LXQ linear ion trap (LIT) mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for sequence analysis. The heated capillary was kept at 275 °C. In each experiment, the ion transmission parameters were optimised automatically in order to improve the detection of the analytes of interest. The flow rate was set to 8 μL/min and the voltage applied was 5.0 kV. Nitrogen was used as nebulising and drying gas. Full scan mass spectra ranging from *m*/*z* 100 to 1500 were acquired in the positive mode. In the MS$^n$ experiments, collision energy varied between 15 and 25 of normalised collision energy (Gonçalves et al., 2009). Standard hexose disaccharides were also used for comparison with samples fragmentation using LIT. Isomaltose, cellobiose, sophorose, kojibiose, and lactose were acquired from Sigma–Aldrich (Madrid, Spain), laminaribiose and galactobiose

**Table 1**
Sugar composition of the pectic polysaccharide samples used.

| Samples | Sugars (mol%) | | | | | | | | Total (mg/g) |
|---|---|---|---|---|---|---|---|---|---|
| | Rha | Fuc | Ara | Xyl | Man | Gal | Glc | GalA | |
| Plum[a] | 2 | – | 32 | 1 | – | 23 | *t* | 42 | 767 |
| Pear[b] | – | – | 44 | 12 | – | 19 | 3 | 21 | 908 |
| Commercial | – | – | – | – | 2 | 1 | 3 | 94 | 366 |

*t* – traces amounts (less than 0.5 mol%).
[a] Nunes, Saraiva, et al. (2008).
[b] Ferreira et al. (2001).

were from Megazyme (Wicklow, Ireland), maltose from Riedel (Buchs, Switzerland), gentiobiose from Fluka (Buchs, Switzerland), nigerose from Hayashibara (Okayama, Japan), and mannobiose from Dextra (Reading, United Kingdom).

### 2.5. Sugar composition and linkage analysis

Neutral sugars were determined by gas chromatography (GC) as alditol acetates (Coimbra et al., 1996). The hydrolysis was performed with TFA 2 M at 120 °C for 1 h. Monosaccharides were reduced with $NaBH_4$ (15% in $NH_3$ 3 M) for 1 h at 30 °C and subsequently acetylated with acetic anhydride (3 mL) in the presence of 1-methylimidazole (450 μL) for 30 min at 30 °C. Alditol acetate derivatives were separated with dichloromethane and analysed by GC with a FID detector and equipped with a 30 m column DB-225 (J&W Scientific, Folsom, CA, USA) with i.d. and film thickness of 0.25 mm and 0.15 μm, respectively. The oven temperature program used was: initial temperature 200 °C, a rise in temperature at a rate of 40 °C/min until 220 °C, standing for 7 min, followed by a rate of 20 °C/min until 230 °C and maintain this temperature 1 min. The injector and detector temperatures were, respectively, 220 and 230 °C. The flow rate of the carrier gas ($H_2$) was set at 1.7 mL/min.

Uronic acids (UA) were determined colorimetrically according to a modification (Coimbra et al., 1996) of the method of Blumenkrantz and Asboe-Hansen (1973). Samples were prepared by pre-hydrolysis in 0.2 mL of 72% $H_2SO_4$ for 3 h at room temperature followed by hydrolysis 1 h in 1 M $H_2SO_4$ at 100 °C. A calibration curve was made with D-galacturonic acid.

Linkage analysis was carried out by methylation as described by Ciucanu and Kerek (1984). Oligosaccharides (1–2 mg) were dissolved in 1 mL of anhydrous dimethylsulfoxide (DMSO), then powdered NaOH (40 mg) was added and samples were methylated with $CH_3I$ (80 μL) during 20 min. The methylated fractions were also carboxyl-reduced by a modification of the method described by Coimbra et al. (1996). The methylated oligosaccharides were hydrolyzed with 2 M TFA at 121 °C for 1 h, and then reduced and acetylated as previously described for neutral sugar analysis. The partially methylated alditol acetates (PMAA) were separated and analysed by gas chromatography–mass spectrometry (GC–MS) on an Agilent Technologies 6890 N Network. The GC was equipped with a DB-1 (J&W Scientific, Folsom, CA, USA) capillary column (30 m length, 0.25 mm of internal diameter and 0.15 μm of film thickness). The samples were injected in splitless mode (time of splitless 5 min), with the injector operating at 220 °C, and using the following temperature program: 45 °C for 5 min with a linear increase of 10 °C/min up to 140 °C, and standing for 5 min at this temperature, followed by linear increase of 0.5 °C/min up to 170 °C, and standing for 1 min at this temperature, followed by linear increase of 15 °C/min up to 280 °C, with further 5 min at 280 °C. The helium carrier gas had a flow rate of 1.7 mL/min and a column head pressure of 2.8 psi. The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range *m*/*z* 40–500 in a 1 s cycle in a full scan mode acquisition.

**Table 2**
Oligosaccharides identified in the ESI-MS spectra, with the indication of the respective $[M+Na]^+$ *m*/*z* value.

| Oligosaccharide ion | Plum | Pear | Commercial |
|---|---|---|---|
| $[GalA+Na]^+$ | 217 | – | 217 |
| $[GalA_2+Na]^+$ | 393 | 393 | 393 |
| $[GalA_3+Na]^+$ | 569 | 569 | 569 |
| $[GalA_4+Na]^+$ | 745 | 745 | 745 |
| $[GalA_5+Na]^+$ | 921 | – | – |
| $[GalA_3Pent+Na]^+$ | 701 | 701 | 701 |
| $[GalA_3Pent_2+Na]^+$ | 833 | – | – |
| $[GalA_2Hex+Na]^+$ | 555 | – | 555 |
| $[GalA_2Hex_2+Na]^+$ | 717 | – | 717 |
| $[GalA_3Hex+Na]^+$ | 731 | 731 | 731 |
| $[GalA_3Hex_2+Na]^+$ | 893 | – | – |
| $[GalA_4Hex+Na]^+$ | 907 | 907 | – |

## 3. Results and discussion

### 3.1. Pectic polysaccharide samples

The pectic polysaccharides used in this study were obtained from plum, pear, and from a commercial preparation of *Citrus* fruits. The sugar composition of the pectic polysaccharide samples is presented in Table 1. The sample obtained from plums contained 42 mol% of uronic acids, attributed to galacturonic acid (GalA). This sample contained also arabinose (Ara, 32 mol%) and galactose (Gal, 23 mol%). The sample obtained from pear was composed mainly by Ara (44 mol%), corresponding the GalA and Gal to 21 and 19 mol%, respectively. The relatively higher proportion of Ara and Gal in relation to GalA allows inferring the occurrence of highly branched pectic polysaccharides in these fractions. On the contrary, the higher relative content of GalA (94 mol%) of commercial pectic polysaccharides shows the presence of mostly unbranched pectic polysaccharides in this sample.

The oligosaccharides resultant from the EPG hydrolyses of each sample were fractionated by size-exclusion chromatography and the main low molecular weight oligosaccharides fractions (<1 kDa) were used for further characterization, both by ESI-MS and ESI-$MS^n$ and by methylation analysis.

The ESI-MS spectra obtained for oligosaccharide-rich fractions resultant from EPG treated plum, pear, and commercial pectic polysaccharides revealed the presence of several sodium adduct ions corresponding to oligosaccharides constituted only by galacturonic acid residues ($GalA_n$, $n$ = 1–5), at *m*/*z* 217, 393, 569, 745, and 921, respectively (Table 2). Also, it was observed the presence of ions at *m*/*z* 701 and 833, that can be attributed to the sodium ions of galacturonic acid residues substituted by pentose residues ($GalA_3Pent_m$, $m$ = 1–2). Fig. 1 shows the ESI-MS spectrum of one size-exclusion chromatography fraction obtained from enzyme treated plum polysaccharides where it is possible to observe some of these ions. Surprisingly, it is possible to observe in this spectrum a major ion at *m*/*z* 731, and an ion at *m*/*z* 555 that can correspond to a galacturonic acid series composed by three and two GalA residues respectively, both linked to one hexose residue. Also, the ion at

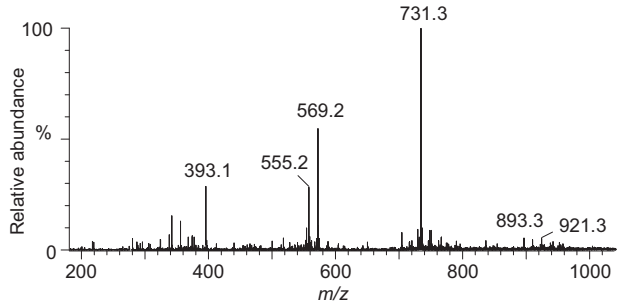


**Fig. 1.** ESI-MS spectra, obtained on a Q-TOF2 instrument, of the plum oligosaccharides with the main *m/z* value of the $[M+Na]^+$ identified.

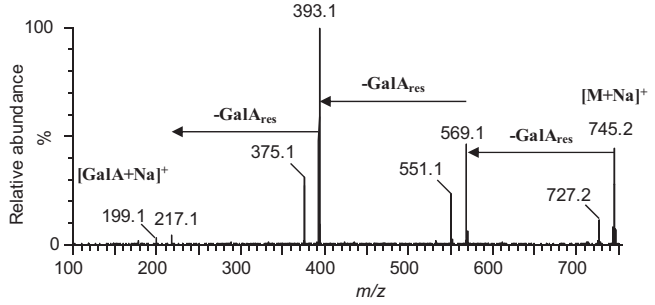


**Fig. 2.** Product ion spectra, obtained on a Q-TOF2 instrument, for the oligosaccharide at *m/z* 745 ($[GalA_4+Na]^+$).

*m/z* 893 can be attributed to a pentasaccharide composed by three galacturonic acid residues and two hexose residues. Also in minor amounts it was possible to observe in plum EPG resultant oligosaccharides the presence of the ions at *m/z* 717 and 907, attributed to $[GalA_2Hex_2+Na]^+$ and $[GalA_4Hex+Na]^+$, respectively (Table 2). The analysis of the oligosaccharides fractions obtained from pear and commercial pectic polysaccharides allowed also to obtain ions that can be attributed to galacturonic acid residues substituted by hexose residues, namely, for pear, the *m/z* at 731, $[GalA_3Hex+Na]^+$, and 907, $[GalA_4Hex+Na]^+$, and for commercial pectic polysaccharides, the *m/z* at 555, $[GalA_2Hex+Na]^+$, 717 $[GalA_2Hex_2+Na]^+$, and 731, $[GalA_3Hex+Na]^+$ (Table 2).

To confirm these assignments, ESI-MS/MS was performed for all ions shown in Table 2. Since ESI-MS$^n$ provides a sensitive means for structural analysis of oligosaccharides, ESI-MS$^n$ experiments were also performed for all ions.

### 3.2. Characterization of galacturonic acid oligosaccharides

The oligosaccharides constituted only by galacturonic acid residues ($GalA_n$, $n = 1–5$) were identified in ESI-MS/MS spectra obtained for all the samples shown in Table 2. As an example, the product ion spectrum of the ion at *m/z* 745, attributed to $[GalA_4+Na]^+$, obtained on a Q-TOF2 instrument, is presented in Fig. 2. The occurrence of the product ions at *m/z* 569, 393, and 217, attributed to $[GalA_3+Na]^+$, $[GalA_2+Na]^+$, and $[GalA+Na]^+$, respectively, due to the loss of 176, 352, and 528 Da, correspondent to $GalA_{res}$, $GalA_{2res}$, and $GalA_{3res}$, and the product ions at *m/z* 727, 551, 375, and 199, attributed to $[GalA_4\text{-}H_2O+Na]^+$, $[GalA_3\text{-}H_2O+Na]^+$, $[GalA_2\text{-}H_2O+Na]^+$, and $[GalA\text{-}H_2O+Na]^+$, respectively, due to the loss of 18, 194, 370, and 546 Da, correspondent to $H_2O$, GalA, $GalA_2$, and $GalA_3$, confirms the $GalA_4$ structure assigned by ESI-MS for this oligosaccharide.

The fragmentation pathway reported for the ion at *m/z* 745 was also observed for the ions at *m/z* 393, 569, and 921 (data not shown) identified in Table 2 as GalA oligosaccharides, confirming the proposed structures. These oligosaccharides are expected to occur in *endo*-polygalacturonase M2 digests (Cameron, Luzio, Savary, Nuñez, & Goodner, 2009; Luzio & Cameron, 2008), derived from the linear α-1,4-linked galacturonic acid residues of the homogalacturonan backbone.

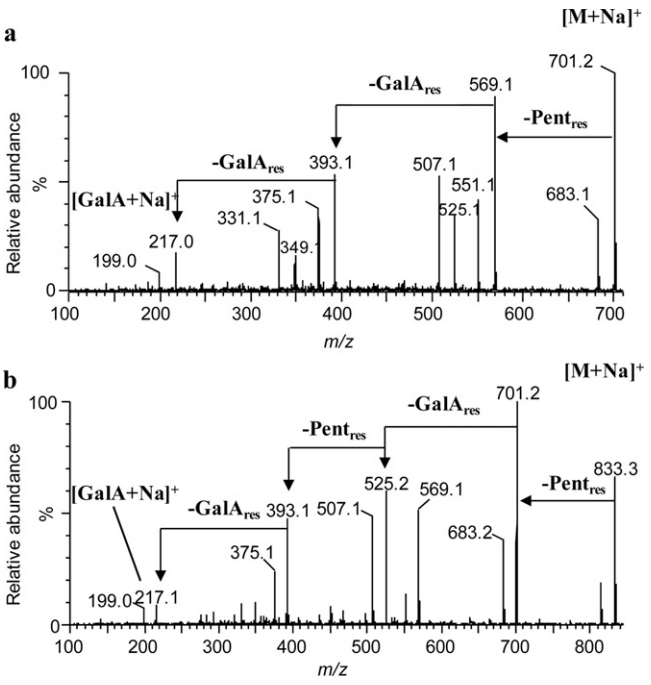


**Fig. 3.** Product ion spectra, obtained on a Q-TOF2 instrument, for the oligosaccharides at (a) *m/z* 701 ($[GalA_3Pent+Na]^+$) and (b) *m/z* 833 ($[GalA_3Pent_2+Na]^+$).

### 3.3. Characterization of oligosaccharides constituted by galacturonic acid and pentoses

The samples analysed from the three different origins, all revealed the presence of the ion at *m/z* 701 in their ESI-MS spectra (Table 2), attributed to $[GalA_3Pent+Na]^+$. The ESI-MS/MS spectra obtained for this ion (Fig. 3a) show the product ion at *m/z* 569, attributed to $[GalA_3+Na]^+$, due to the loss of 132. A loss of 132 Da confirms the presence of a pentose residue ($Pent_{res}$) (Nunes, Reis, Silva, Domingues, & Coimbra, 2008). The ions at *m/z* 525, 393, 349, and 217, attributed to $[GalA_2Pent+Na]^+$, $[GalA_2+Na]^+$, $[GalAPent+Na]^+$, and $[GalA+Na]^+$, respectively, due to the loss of 176, 308, 352, and 484 Da, correspondent to $GalA_{res}$, $GalAPent_{res}$, $GalA_{2res}$, and $GalA_2Pent_{res}$, were also observed. Also present were the ions at *m/z* 683, 551, 375, 331, and 199, attributed to $[GalA_3Pent\text{-}H_2O+Na]^+$, $[GalA_3\text{-}H_2O+Na]^+$, $[GalA_2\text{-}H_2O+Na]^+$, $[GalAPent\text{-}H_2O+Na]^+$, and $[GalA\text{-}H_2O+Na]^+$, due to the loss of 18, 150, 326, 370, and 502 Da, correspondent to $H_2O$, Pent, GalAPent, $GalA_2$, and $GalA_2Pent$. This fragmentation confirms the occurrence of a pentose linked to the GalA residues, which corresponds to an oligosaccharide possibly arising from the xylogalacturonan (XGA) regions of pectic polysaccharides (Le Goff et al., 2001; Ralet et al., 2009; Voragen et al., 2009).

The ion at *m/z* 833, observed only in the ESI-MS spectra of plum (Table 2) was identified as a pentasaccharide constituted by three galacturonic acid residues and two pentose residues $[GalA_3Pent_2+Na]^+$. The ESI-MS/MS spectrum of this ion (Fig. 3b) shows the formation of the ion at *m/z* 701, corresponding to $[GalA_3Pent+Na]^+$, showing the loss of a $Pent_{res}$ (−132 Da) and the ion at *m/z* 683, corresponding to $[GalA_3Pent\text{-}H_2O+Na]^+$, showing the loss of a Pent (-150 Da). Also, the observation of the ions at *m/z* 569, 525, 507, 393, 375, 217, and 199, attributed, respectively,

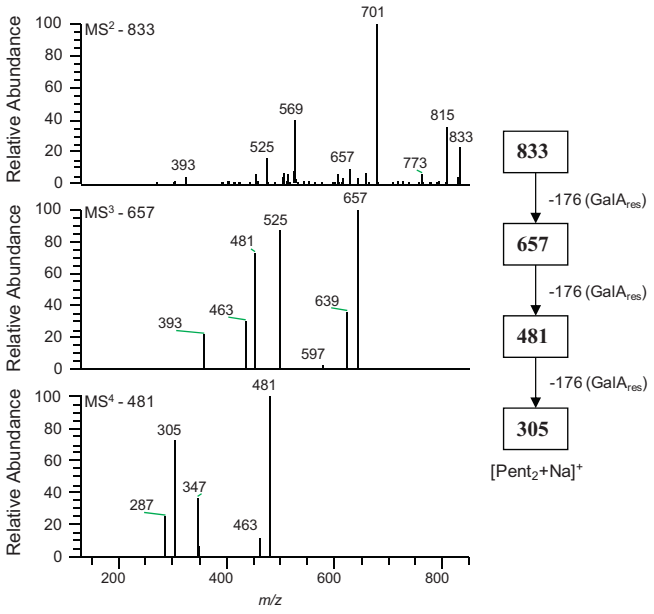


**Fig. 4.** Fragmentation pathways for the product ion spectra for the ion at *m/z* 833 ([$GalA_3Pent_2$+Na]$^+$), obtained on a LIT instrument.

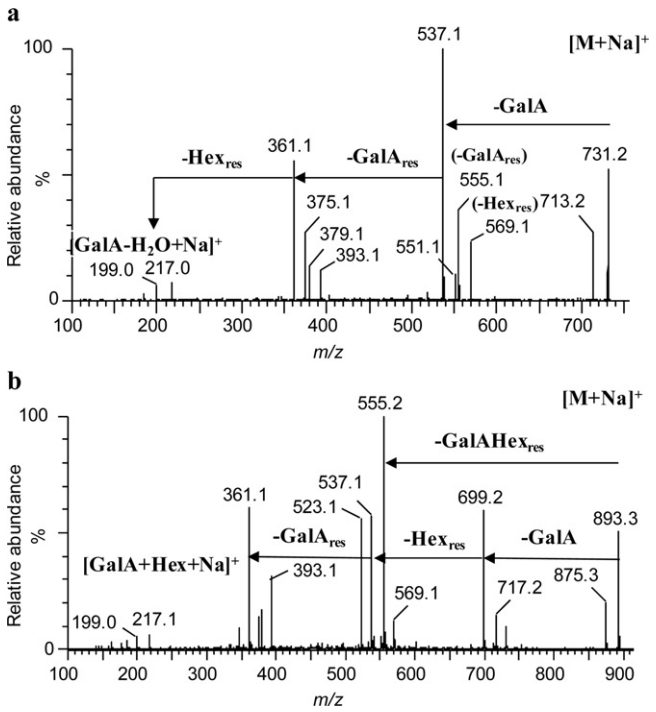


**Fig. 5.** Product ion spectra, obtained on a Q-TOF2 instrument, for the oligosaccharides at (a) *m/z* 731 ([$GalA_3Hex$+Na]$^+$) and (b) *m/z* 893 ([$GalA_3Hex_2$+Na]$^+$).

to [$GalA_3$+Na]$^+$, [$GalA_2Pent$+Na]$^+$, [$GalA_2Pent$-$H_2O$+Na]$^+$, [$GalA_2$+Na]$^+$, [$GalA_2$-$H_2O$+Na]$^+$, [GalA+Na]$^+$, and [GalA-$H_2O$+Na]$^+$ confirm the occurrence of a $GalA_3Pent_2$ structure.

In order to obtain a more detail structural information, an ESI-$MS^n$ experiment was carried out for the ion at *m/z* 833 (Fig. 4) on a LIT mass spectrometer, since ion trap have the capacity to perform multiple stages of mass spectrometry ($MS^n$) (Fernández, 2007). The $MS^2$ spectrum of the ion at *m/z* 833 showed the presence of a product ion at *m/z* 657, due to the loss of a $GalA_{res}$ (−176 Da), and the $MS^3$ fragmentation of the ion at *m/z* 657 gave an ion at *m/z* 481 due to the loss of other $GalA_{res}$. $MS^4$ spectrum of ion at *m/z* 481 showed the presence of a product ion at *m/z* 305, corresponding to the [$Pent_2$+Na]$^+$. This fragmentation confirms that pentose residues are linked together and occurred as a disaccharide side chain of the trisaccharide unit constituted by GalA residues. This type of structure has been already described to occur in pectic polysaccharides, namely, β-1,2-linked or β-1,4-linked Xyl*p* in XGA (Le Goff et al., 2001; Oechslin et al., 2003; Zandleven et al., 2006).

### 3.4. Characterization of oligosaccharides constituted by galacturonic acid and hexoses

The ion at *m/z* 731, identified in the ESI-MS spectra of all samples as a oligosaccharide constituted by three galacturonic acid and one hexose residues [$GalA_3Hex$+Na]$^+$ (Table 2) was analysed by ESI-MS/MS (Fig. 5a). It is possible to observed the ions at *m/z* 537 and 361, corresponding to [$GalA_2Hex$-$H_2O$+Na]$^+$ and [GalAHex-$H_2O$+Na]$^+$, resultant from the loss of 194 Da (GalA) and 370 Da ($GalA_2$), and the ions at *m/z* 555 and 379, corresponding to [$GalA_2Hex$+Na]$^+$ and [GalAHex+Na]$^+$, resultant from the loss of 176 Da ($GalA_{res}$) and 352 Da ($2GalA_{res}$). The ions at *m/z* 569, 393, and 217, corresponding to [$GalA_3$+Na]$^+$, [$GalA_2$+Na]$^+$, and [GalA+Na]$^+$, resultant from the loss of 162 Da ($Hex_{res}$), 338 Da ($GalAHex_{res}$), and 514 Da ($GalA_2Hex_{res}$), and the ions at *m/z* 551, 375, and 199, resultant from the loss of 180 Da (Hex), 356 Da (GalAHex), and 532 Da ($GalA_2Hex$), confirm the occurrence of a $GalA_3Hex$ structure.

The ESI-MS/MS spectrum for the ions at *m/z* 555 and 907, attributed to [$GalA_2Hex$+Na]$^+$ and [$GalA_4Hex$+Na]$^+$ (Table 2) presented a similar fragmentation pattern as reported above (data not shown), confirming these assignments.

The ESI-MS/MS spectrum for the ion at *m/z* 893 (Fig. 5b), assigned by ESI-MS to [$GalA_3Hex_2$+Na]$^+$ (Table 2), shows product ions at *m/z* 717 and 699, corresponding to [$GalA_2Hex_2$+Na]$^+$ and [$GalA_2Hex_2$-$H_2O$+Na]$^+$, respectively, resultant from the loss of 176 Da ($GalA_{res}$) and 194 Da (GalA). Also, the ions at *m/z* 569, 555, 537, 523, 393, 361, 217, and 199, attributed, respectively, to [$GalA_3$+Na]$^+$, [$GalA_2Hex$+Na]$^+$, [$GalA_2Hex$-$H_2O$+Na]$^+$, [$GalAHex_2$-$H_2O$+Na]$^+$, [$GalA_2$+Na]$^+$, [GalAHex-$H_2O$+Na]$^+$, [GalA+Na]$^+$, and [GalA-$H_2O$+Na]$^+$ were present, confirming the occurrence of a $GalA_3Hex_2$ structure. The same fragmentation pathway was observed in the ESI-MS/MS spectrum of the ion at *m/z* 717, attributed to [$GalA_2Hex_2$+Na]$^+$ (data not shown).

In order to verify the monosaccharide sequence of the oligosaccharide structure, ESI-$MS^n$ experiment on a LIT mass spectrometer was performed for the ion [$GalA_2Hex_2$+Na]$^+$ with *m/z* 717 (Fig. 6). The $MS^2$ spectrum of this ion showed the presence of a product ion at *m/z* 541, corresponding to the loss of one GalA residue (−176 Da). The $MS^3$ fragmentation of the ion at *m/z* 541 revealed an ion at *m/z* 365 corresponding to the [$Hex_2$+Na]$^+$ ion and the $MS^4$ fragmentation of the ion at *m/z* 365 showed the presence of an ion at *m/z* 203, corresponding to the [Hex+Na]$^+$ ion. This fragmentation pathway confirmed the presence of a $Hex_2$ structure as a side chain of the GalA backbone. Moreover, the comparison of the relative intensity of the ions formed during the fragmentation in LIT is similar to the one observed for cellobiose (Fig. 7a) and lactose (Fig. 7b), with major ions at *m/z* 305 (100%), 347 (60%), and 203 (10%). These two disaccharides are composed by a Glc*p* residue β-1,4-linked to a Glc or Gal unit, respectively. The product spectra ion for maltose (Fig. 7c), composed by a Glc*p* residue α-1,4-linked to a Glc unit, showed a different relative intensity of the ions, namely the ions at *m/z* 347 (30%) and 203 (70%), a characteristic consistent with the mass spectrometry data previously presented by Simões et al. (2007) for reducing glucopyranosyl-glucose disaccharides using lithium ions. Also a different relative intensity of the ions were observed for the sodium adducts of β-1,4 galactobiose (data not shown). Based on these results, we can infer that the non-reducing end of the $Hex_2$ structure is composed by a β-1,4-Glc*p* residue. The ESI-$MS^n$ experiment

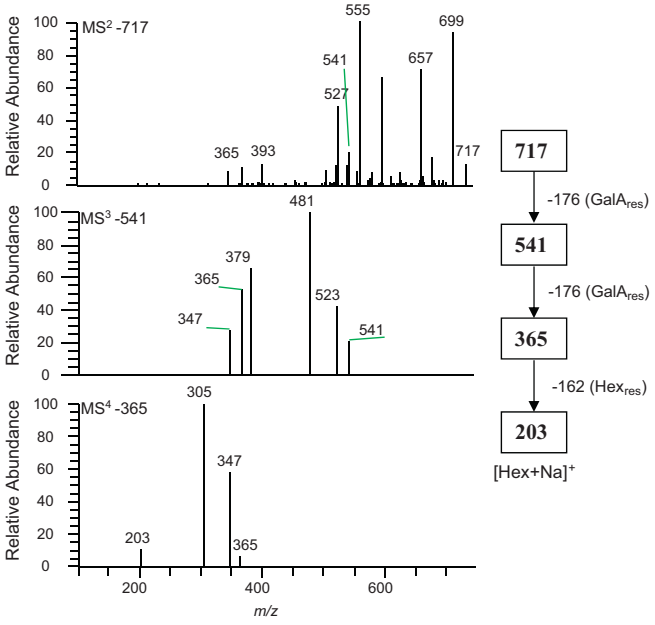


**Fig. 6.** Fragmentation pathways for the product ion spectra of ion at *m/z* 717 ([$GalA_2Hex_2$+Na]$^+$), obtained on a LIT instrument.

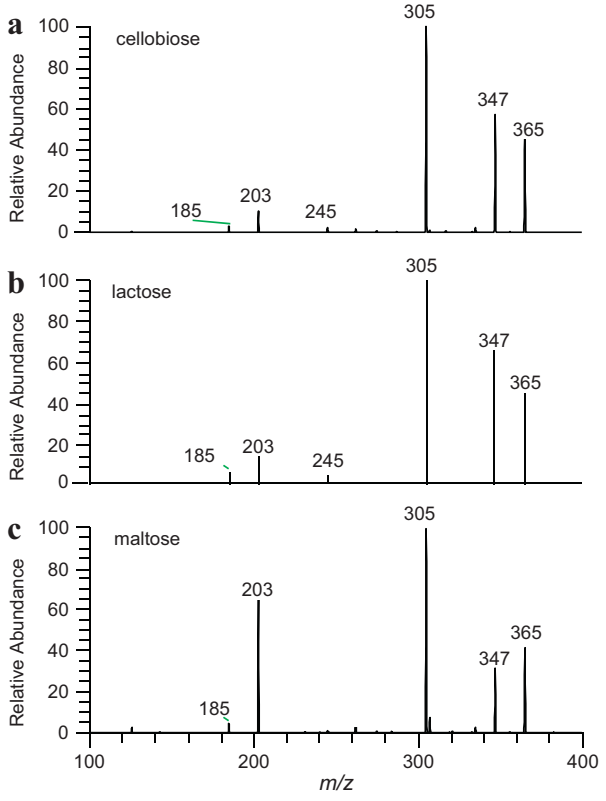


**Fig. 7.** Product ion spectra (MS$^2$), obtained on a LIT instrument, for the disaccharides of hexose ([$Hex_2$+Na]$^+$ ions): (a) cellobiose, (b) lactose, and (c) maltose.

was also performed for the ion at *m/z* 893 [$GalA_3Hex_2$+Na]$^+$ and the presence of $Hex_2$ structure, with the same fragmentation pattern was also confirmed for this oligosaccharide (data not shown).

The analysis of the ESI-MS/MS data obtained for the oligosaccharides of the series [$GalA_{2-4}Hex_{1-2}$] using the Q-TOF mass spectrometer and also MS$^n$ data using the LIT mass spectrometer allows the assignment of fragmentations showing the presence of never reported pectic oligosaccharide structures. Although the association of a Gal residue linked to the *O*-3 of Gal*p*A have postulated in the pectic polysaccharides isolated from the leaves of *Diospyros kaki* (Duan, Zheng, Dong, & Fang, 2004), the presence of a $Hex_2$ disaccharide glycosidically linked to a GalA residue, as a side chain of the homogalacturonan backbone, had never been described before for pectic polysaccharides.

**Table 3**
Glycosidic linkage composition (mol%) of the low molecular weight fractions obtained by EPG hydrolysis and size exclusion chromatography of pectic polysaccharide samples.

| Linkage | Plum | Pear | Commercial |
|---|---|---|---|
| T-Araf | 6.1 | 9.5 | 4.5 |
| T-Xyl*p* | 6.2 | 27.0 | 19.8 |
| 4-Xyl*p* | 32.4 | 18.4 | 9.1 |
| T-Gal*p* | 2.7 | – | – |
| 4-Gal*p* | 4.1 | – | – |
| T-Glc*p* | 12.9 | 8.0 | 29.9 |
| 2-Glc*p* | – | – | 9.1 |
| 4-Glc*p* | 35.5 | 37.2 | 23.7 |
| 6-Glc*p* | – | – | 3.4 |

### 3.5. Methylation analysis

In order to try to identify the type of linkages present in these pectic oligosaccharide structures, methylation analysis was performed. Table 3 shows the glycosidic linkage composition of the size exclusion chromatography fractions from the plum, pear, and citrus analysed by ESI-MS and ESI-MS$^n$. As carboxyl-reduction of uronic acid was not performed, this analysis only accounts for neutral sugars linkages.

Methylation analysis revealed the presence of terminal and (1 → 4)-linked Glc*p* in all samples (Table 3), ranging from 8.0 to 29.9 mol% and 23.7–37.2 mol%, respectively. Small amounts of (1 → 2)-linked Glc*p* and (1 → 6)-linked Glc*p* were also observed in the sample from commercial pectic polysaccharides and terminal and (1 → 4)-linked Gal*p* were observed in the sample from plum. Because in plum pectic polysaccharide the Glc content was only detected as trace amounts (Table 1), a sugar analysis was also performed to these oligosaccharide fractions (data not shown), revealing that Glc accounted for 49 mol% of all neutral sugars. These analyses allow to infer that the single residue of Hex observed linked to GalA residues in all samples should be Glc and that the $Hex_2$ residues observed in commercial and plum samples should have arisen from a Glc-β-(1 → 4)-Glc residue (cellobiose).

In all samples, methylation analysis also showed terminal and (1 → 4)-linked Xyl*p* residues, confirming the presence of oligosaccharides derived from xylogalacturonans containing single and (1 → 4)-linked Xyl*p* disaccharides, as already described for the XGA from other sources (Le Goff et al., 2001; Oechslin et al., 2003; Zandleven et al., 2006). The higher abundance of these residues in plum than in the other two samples could justify why this pentosyl disaccharide was only observed by mass spectrometry in plum.

## 4. Concluding remarks

Electrospray tandem mass spectrometry was used for the structural characterization of pectic polysaccharides from three different sources, plum, pear and a commercial pectic polysaccharide from *Citrus*. It allowed to confirm the presence, in all samples, of pectic oligosaccharides belonging to different series, constituted only by galacturonic acid residues ($GalA_n$, $n$ = 1–5), galacturonic acid residues substituted by pentose residues ($GalA_3Pent_m$, $m$ = 1–2), and galacturonic acid residues substituted by hexose residues ($GalA_nHex_p$, $n$ = 2–4, $p$ = 1–2). Methylation analysis

showed that these hexoses are Glc residues that can be present in pectic polysaccharides directly linked to GalA as single residues or as cellobiose residues. As this characteristic was observed in pectic polysaccharides from different origins and structural features, namely, different proportions of branching residues, it can be postulated that this is a common characteristic of pectic polysaccharides.

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