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# Structural diversity of pectins isolated from the Styrian oil-pumpkin (*Cucurbita pepo* var. *styriaca*) fruit

Zuzana Košťálová*, Zdenka Hromádková, Anna Ebringerová

*Institute of Chemistry, Center for Glycomics, Slovak Academy of Sciences, Dúbravská cesta 9, SK-845 38 Bratislava, Slovak Republic*

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ABSTRACT

To evaluate the seeded fruit biomass of the Styrian oil-pumpkin in view of its pectin component, a series of acidic polysaccharides were isolated by a six-step sequential extraction using hot water, EDTA, dilute HCl (twice) and dilute and stronger NaOH solutions. Chemical, physicochemical and spectroscopy analyses revealed that the first four fractions comprised partially methyl-esterified and acetylated pectins with varying proportions of rhamnogalacturonan regions ramified with galactose- and arabinose-containing side chains and showed considerable polymolecularity. The alkali-extracted polysaccharides contained lower amounts of pectins with homogalacturonan and arabinose-rich rhamnogalacturonan regions next to hemicelluloses prevailing in the last polysaccharide. Using $^{1}H$–$^{13}C$ HSQC and HMBC spectroscopy, the resonances of free and methylesterified galacturonic acid residues in the purified acid-extracted pectin were unambiguously established and various diads formed by both residues identified. The results might serve as a basis for searching technological conditions to produce pectin from the oil-pumpkin fruit biomass.



## 1. Introduction

Nowadays, there is an increasing interest of food, pharmacy and other industries in new plant sources of pectin. The term 'pectin' covers a group of acidic polysaccharides with distinct structural features which are currently suggested to be composed of at least five different elements (Thibault & Ralet, 2001; Voragen, Coenen, Verhoef, & Schols, 2009). The main element consists of linear homogalacturonan chains which might be partly methylesterified and/or acetylated forming 'smooth' domains or might carry single xylosyl or apiosyl branches forming xylogalacturonan and apiogalacturonan elements. The linear homogalacturonan sections might be interspersed by rhamnogalacturonan elements. The RG I-type bears galactosyl, arabinosyl and arabinogalactosyl side chains of different length, whereas, RG II-type contains various rare sugars and complex oligosaccharide chains. Both types form complex 'hairy' domains in the pectin molecules. Pectin is located in the middle lamella and primary cell wall of higher plants, and constitutes around 40% of the cell walls of fruits and vegetables (Brett & Waldron, 1996). Currently, apple pomace, citrus peels and some extent sugar beet pulp are used as sources for commercial pectin production (Willats, Knox, & Mikkelsen, 2006). Potential alternative sources are the various pumpkin species, their pectin component were reported to exhibit useful functional properties and important biological effects (Adams et al., 2011; Caili, Huan, & Quanhong, 2006; Caili, Haijun, Tongyi, Yi, & Quanhong, 2007; Yadav, Jain, Tomar, Prasad, & Yadav, 2010). Several studies described extraction procedures used for the isolation of pectin from the pumpkin fruit (Shkodina, Zeltser, Selivanov, & Ignatov, 1998) and peel (Jun, Lee, Song, & Kim, 2006). The isolated pectic polysaccharides were characterized by composition and physicochemical properties (Zhemerichkin & Ptitchkina, 1995). The structural features are assumed to essentially contribute to the functional and biological properties of pectin polymers (Ralet, Bonnin, & Thibault, 2005). To our best knowledge, reports on the structural features of the pumpkin pectins in contrast to pectins from other non-traditional sources are not available.

In continuation of our search for novel pectin resources, we evaluated the fruit biomass – a by-product of oil processing from the seeds of the Styrian oil-pumpkin (*Cucurbita pepo* var. *styriaca*) which is an economically important horticultural plant in Austria and adjacent countries. In previous studies, the overall composition of the pumpkin biomass and its tissues has been described (Košťálová, Hromádková, & Ebringerová, 2009) and pectic polysaccharides isolated from the oil-pumpkin biomass were

*Abbreviations:* GH1 and GC1, anomeric proton and carbon of non-esterified Gal*p*A residue (G); EH1 and EC1, anomeric proton and carbon of methylesterified Gal*p*A residue (E); DE, degree of methylesterification; $DS_{ac}$, degree of acetylation; EE, diad of two methylesterified Gal*p*A residues; GG, diad of two non-esterified Gal*p*A residues; EG and GE, diads of both methylesterified and non-esterified galacturonic acid residues.

* Corresponding author. Tel.: +421 2 59410284; fax: +421 2 59410222.
*E-mail address:* chemzuko@savba.sk (Z. Košťálová).

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reported to exhibit antioxidant activity (Košťálová, Hromádková, & Ebringerová, 2010) and cough-suppressing effects (Nosál'ová, Prisenžňaková, Košťálová, Ebringerová, & Hromádková, 2011). This study is focused on the isolation, separation and chemical characterization of pectic polysaccharides present in the oil-pumpkin biomass and a more detailed NMR study of acid-extracted pectin.

## 2. Materials and methods

### 2.1. Plant material and chemicals

The ripe fruits of the oil-pumpkin (*Cucurbita pepo* L. var. *styriaca*) were harvested in September 2005 at the local fields belonging to the scholar enterprise Kolíňany in vicinity of Kolíňany (Slovakia). The fresh fruits were cleaned with tap water and then rinsed with distilled water. After halving the fruits, the seeds were carefully removed and the resulting biomass was dried on air yielding the free-dried biomass (DOP). After grinding into small particles (0.6–0.8 mm), it was used as starting material in this study. The dewatering process and analytical evaluation of DOP was described in detail in a previous paper (Košťálová et al., 2009). Gallic acid and D-galacturonic acid were obtained from Fluka (Germany). Folin–Ciocalteu's phenol reagent was from Merck (Germany). 3-Hydroxydiphenyl, 3-(trimethylsilyl)propionic acid (TSP) and bovine serum albumin (≥96%) were purchased from Sigma–Aldrich (Germany). The reagent Coomassie Brilliant Blue G-250 was obtained from Serva and ethylenediaminetetraacetic acid (EDTA) from Lachema Brno (Czech Republic). All other used chemicals were of analytical grade.

### 2.2. Sequential extraction of polysaccharides

The extractives of DOP were removed by refluxing (95 g) with ethanol–chloroform (1:4, v/v). The insoluble residue was dried on air and subjected to a sequential extraction process. In the first step this material (86.3 g) was extracted with distilled water (2 L) at 60 °C for 2 h. The insoluble residue separated by filtration and dried on air was further treated in the second step with 2 L of 0.05 M EDTA in 0.33 M $KH_2PO_4$ (pH 4) at 25 °C for 2 h. The insoluble residue, separated as described in the previous step, was successively extracted in the following third and fourth steps with 0.003 M HCl (2 L) at 60 °C for 30 min. The pH of the first and second acidic extracts separated by filtration was adjusted to pH 4.9 and 4.8, respectively, with 1 M NaOH. The acid-insoluble fiber residue from the second step was further extracted in succession with 1 L of 0.25 M NaOH (1 h, 35 °C) and 1 L of 1.32 M NaOH (1 h, 60 °C). Both alkaline extracts were neutralized to pH ~7 with 1 M acetic acid. Each of the six extracts was exhaustively dialyzed using cellulose membrane (MWCO, 3.5 kDa, Serva). The retentates were concentrated in vacuum and freeze-dried yielding polysaccharide fractions DP1–DP6.

### 2.3. Ion-exchange chromatography

Polysaccharide DP4 (200 mg) was dissolved in distilled water (4 mL) and the solution was centrifuged to remove the insoluble portion. The supernatant was applied to a column (30 × 3 cm) of DEAE Sephadex A25 ($Cl^-$ form) and eluted first with distilled water and then stepwise with 0.1 M, 0.3 M, 0.5 M and 1 M NaCl. The subfractions were collected and those eluted with NaCl solutions were dialyzed. After concentration in vacuum they were freeze-dried to yield five subfractions: DP4/A (4.1%), DP4/B (3.4%), DP4/C (32.8%), DP4/D (1.8%), and DP4/E (5.9%).

### 2.4. General methods

Polysaccharide fractions were hydrolyzed with 2 M TFA under reflux for 2 h. The composition of the neutral sugars was determined in form of alditol trifluoroacetates by gas chromatography as described in a previous paper (Kardošová et al., 2004). Acidic sugar components of the hydrolyzate were identified by paper chromatography (PC) in the ethyl acetate:acetic acid:water:formic acid system (18:3:4:1, v/v). The total carbohydrate content was estimated by the phenol–$H_2SO_4$ assay (Saha & Brewer, 1994) and the uronic acid content by the 3-hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973). In both assays galacturonic acid was used as a standard. The content of total phenolic compounds was estimated by the Folin–Ciocalteu assay (Yu et al., 2002) using gallic acid as a standard. The content of protein was determined by a modified Bradford method (Sedmak & Grossberg, 1977) using bovine serum albumin as a standard.

HPGPC measurement was performed on a system equipped with injector LCH 01 and high-pressure pump LCP 4020 (ECOM s.r.o., Prague, Czech Republic) and two, in series connected columns (250 × 8 mm) packed with HEMA BIO 300 and HEMA BIO 1000 sorbent (Tessek Ltd., Prague, Czech Republic). The separation process was monitored with a differential refractometric detector RIDK-102 (Laboratórní přístroje, Prague, Czech Republic). As a mobile phase, 0.1 M $NaNO_3$ was used, the flow rate was 0.4 mL $min^{-1}$, 25 °C. A set of pullulan standards (Polymer Laboratories, Ltd., Varian Inc., UK) was used for molecular weight calibration. The polysaccharide fractions were analyzed after removal of oligomers by dialysis (MWCO 3.5 kDa).

Infrared spectra of the samples (2 mg/200 mg KBr) were collected on a NICOLET Magna 750 spectrometer with DGTS detector and OMNIC 7 software using 128 scans at a resolution of 4 $cm^{-1}$. NMR spectra were acquired on a Varian 400 MR spectrometer equipped with a 5 mm PFG AutoX Probe and operating at 400 MHz for $^1H$ and 100 MHz for $^{13}C$. Before analysis samples were freeze-dried two times from 95% $D_2O$ and after they were dissolved in 99.98% $D_2O$. Measurements were performed at 60 °C and in case of DP1 at 40 °C. For both $^1H$ and $^{13}C$ NMR spectra chemical shifts are referenced to 3-(trimethylsilyl)propionic acid (TSP) as internal standard ($\delta$, 0.00). Multiplicity edited $^1H$–$^{13}C$ HSQC spectrum was recorded in phase-sensitive pure absorption mode, $^1H$–$^1H$ COSY spectra with gradient selection and $^1H$–$^{13}C$ HMBC spectra were measured in absolute intensity mode. The spectral widths employed in 2D NMR experiments were typically 6000 Hz ($^1H$) and 20,000 Hz ($^{13}C$), respectively. Data processing was performed using the MestReNova 7.0 software.

### 2.5. Statistics

Measurements of the content of phenolic components, total carbohydrates and uronic acids were performed (at least) in triplicate. The values were averaged and reported along with the standard deviation (mean ± SD).

## 3. Results and discussion

### 3.1. Chemical composition of the polysaccharide fractions

The seeded pumpkin biomass (DOP) composed of 27% skin and 73% flesh tissues was reported to contain approximately 14.4% pectin, 11.3% protein and 8.6% phenolic compounds based on oven dried DOP (Košťálová et al., 2009). Sequential extraction procedure were used in various studies to isolate pectins from the cell walls, such as from apple and sugar beet (Renard & Thibault, 1993), opuntia fruit skin (Habibi, Heyraud, Mahrouz, & Vignon, 2004) and

**Table 1**
Yield and compositional data of acidic polysaccharide fractions isolated from the oil-pumpkin biomass.

| Polysaccharide fraction | DP1 | DP2 | DP3 | DP4 | DP5 | DP6 |
|---|---|---|---|---|---|---|
| Yield (wt%)[a] | 3.7 | 2.4 | 1.0 | 1.1 | 0.9 | 2.10 |
| Total phenolics (wt%)[b] | 2.2 ± 0.5 | 0.9 ± 0.3 | 0.8 ± 0.3 | 0.6 ± 0.1 | 0.8 | 1.6 ± 0.1 |
| Protein (wt%)[b] | 3.1 | 2.1 | 2.1 | 0.8 | 4.2 | 7.6 |
| Total carbohydrates (wt%)[b,c] | 69.7 ± 3.7 | 57.1 ± 1.8 | 57.9 ± 1.7 | 77.7 ± 0.4 | 71.1 ± 3.9 | 58.7 ± 3.1 |
| Uronic acid (w%)[b,c] | 39.5 ± 2.1 | 54.3 ± 0.6 | 47.7 ± 2.0 | 63.1 ± 3.0 | 33.3 ± 2.0 | 6.1 ± 0.8 |
| Sugar composition (mol%)[d] | | | | | | |
| Galacturonic acid | 56.7 | 95.1 | 82.4 | 81.2 | 47.5 | 10.4 |
| Rhamnose | 1.2 | 0.1 | 0.4 | 3.8 | 9.2 | 4.7 |
| Fucose | 4.5 | 0.2 | 1.2 | 1.5 | 2.5 | 2.6 |
| Arabinose | 15.9 | 0.7 | 3.2 | 4.4 | 22.1 | 23.6 |
| Xylose | 4.7 | 0.1 | 0.6 | 0.9 | 4.1 | 49.0 |
| Mannnose | 1.4 | 0.5 | 0.9 | 1.3 | 0.7 | 0.4 |
| Glucose | 5.9 | 2.7 | 8.5 | 3.3 | 4.8 | 3.0 |
| Galactose | 9.7 | 0.5 | 2.8 | 3.6 | 9.1 | 6.3 |

[a] Based on the dry biomass DOP.
[b] Based on the polysaccharide fraction.
[c] Expressed as galacturonic acid.
[d] Expressed as anhydrounits.

pumpkin (*Cucurbita moschata* Duch) peel (Jun et al., 2006). In this study, the DOP deprived of extractives (9%) was subjected to a sequential extraction procedure using hot water, aqueous EDTA solutions acting as chelating agent, dilute HCl (in two steps) and dilute and stronger NaOH solutions. The yields of the polysaccharide fractions DP1–DP6 separated from the extracts (based on dried DOP) and their chemical composition data are listed in Table 1. The total yield of the isolated fractions was 9.2%, whereby the yields of the individual fractions ranged between 3.7 and 0.9%.

The uronic acid content of the isolated polysaccharides varied from ~40% in DP1 up to 63% in DP4 what is comparable with pectic polysaccharides isolated from other sources as the skin of *Opuntia* pears (Habibi et al., 2004) or mango and lime peels (Koubala et al., 2008). In pectin fractions isolated from other pumpkin species the UA content ranged between 4.9 and 60% (Jun et al., 2006; Shkodina et al., 1998). The alkali extracted DP5 and DP6 contained lower amounts of UA. Whereas, in the hydrolyzates of DP1–DP4 only galacturonic acid was identified (by PC), DP5 and DP6 contained also 4-O-methylglucuronic acid. The presence of rhamnose indicated that all pectin fractions contain 'hairy' RG I regions ramified with side chains composed of arabinose and galactose, similarly as in pectins from other sources (Habibi et al., 2004; Thibault & Ralet, 2001).

Fucose-containing xyloglucans known to occur in pumpkin primary cell walls (Kato, Ito, & Mitsuishi, 2004) prevailed in DP1, occurred in fewer proportions in fractions DP3–DP6, and the least in DP2. DP6 had the lowest content of uronic acids with 4-O-methylglucuronic acid prevailing over galacturonic acid (identified by PC) and showed xylose as the main neutral sugar indicative of glucuronoxylan. The results suggested the presence of pectin polymers in all fractions.

Whereas, the protein content of the separated polysaccharide fractions decreased continually during the first four extraction steps, it increased in fractions DP5 and DP6. The relatively high content of proteinaceous materials in DP1 could be ascribed to co-precipitated intracellular proteins, but might also origin from water-extractable glycoproteins rich in arabinose. Recently, such glycoprotein was isolated by hot water from the fruit of another pumpkin species (*Cucurbita moschata*) (Caili et al., 2007). The pectic polysaccharide fractions contained minor amounts of phenolic substances which were the most accumulated in DP1. They might be strongly associated with the pectin component, either by physical entrapments during the separation of the polysaccharide from the extracts and/or by native chemical bonds. Phenolic acids esterified to arabinose and galactose residues of the RG I side chains were found in sugar beet cell-wall polysaccharides (Ralet, Andre-Leroux, Quemener, & Thibault, 2005).

As expected, the use of extraction agents selective to certain structural pectin types (Davis, Derouet, Herve Du Penhoat, & Morvan, 1990; Habibi et al., 2004; Renard & Thibault, 1993) enabled the separation of low methyl-esterified pectins by the chelating agent in fraction DP2. In the previous paper (Nosál'ová et al., 2011), a similar EDTA-extracted pectin fraction was isolated by the same extraction procedure from the ethanol-dried oil pumpkin biomass. Its main subfraction containing 75.3% uronic acids was shown to comprise a homogalacturonan free of methoxyl and acetyl groups with 21% RG I regions. Ion-exchange chromatography of DP4 afforded 5 fractions (DP4/A–DP4/E) differing in the uronic acid content and composition of neutral sugars (Table 2). The main subfraction DP4/C representing 68% of all subfractions had the highest uronic acid content (62.3%), which practically coincided with that of the crude DP4 (Table 1), similarly as the dominance of rhamnose, arabinose and galactose in neutral sugars.

### 3.2. Molecular properties of water soluble pectin fractions

HPGPC was used for the gross characterization of the pectin molecular size using pullulan standards for calibration. The chromatograms of pectin fractions DP1–DP4 and subfraction DP4/C illustrated in Fig. 1 were cut off according to the MWCO of the dialysis membrane (3.5 kDa). The elution profiles indicated a broad distribution of molecular weight. Two overlapping high molecular weight populations (I, II) eluting at the lowest elution volume (8.5–10.5 mL) were observed in high proportions for all pectin fractions. They occurred in DP1 and DP3 as a broad peak with peak molecular weight (MW) of 543 kDa. In fraction DP2 population I showed a distinct peak with MW of 714 kDa, whereas, in DP4 and DP4/C both populations were observed with MW values of 714 (I) and 400 kDa (II), respectively. Further populations eluted at medium elution volumes (10.5–12.8 mL). Population III with MW of 231 kDa can be distinguished in DP3 and occurred non-separated in DP1 and DP2. Fractions DP1and DP4 contained well separated populations with MW of 60 (IV) and 96 (V), respectively. The subfraction DP4/C showed at low elution volumes (12.8–14 mL) a large population VI with MW of 25 kDa. Populations with very low MW (6–3.5 kDa) were present mainly in DP1 and in smaller amounts in DP3 and DP4.

Large variations in molecular weight were reported also for the water-, EDTA- and alkali extracted pectins isolated from peels of pumpkin (*Cucurbita moschata* Duch) containing populations with

**Table 2**
Sugar composition of DEAE-Sephadex subfractions of DP4.

| Subfraction | Eluent | UA | Neutral sugar composition (mol%) | | | | |
|---|---|---|---|---|---|---|---|
| | | % | Rha | Ara | Xyl | Glc | Gal |
| DP4/A | Water | 0 | 0[a] | 23.1 | 10.3 | 38.2 | 28.4 |
| DP4/B | 0.1 M NaCl | 0 | 18.3 | 31.0 | Tr | 22.1 | 28.6 |
| DP4/C | 0.3 M NaCl | 62.3 | 19.0[a] | 40.4 | 13.5 | 5.6 | 21.4 |
| DP4/D | 0.5 M NaCl | 32.8 | 26.1 | 32.5 | 0 | 13.2 | 28.2 |
| DP4/E | 1 M NaCl | 1.3 | 11.4 | 8.4 | 4.0 | 53.3 | 22.9 |

[a] Traces of fucose present; Tr, traces.

MW at 2680–1860, 250–205 and 13–16 kDa, respectively (Jun et al., 2006). However, the MW values of the largest populations were 3–4 times higher. The high molecular weight populations were suggested to result from diferulic acid cross-linking (bridging) of arabinans and/or arabinogalactan side chains ramifying the RG I domains in pectins (Ralet, Andre-Leroux, et al., 2005). However, this population dominating in DP2 can be ascribed to the aggregation tendency of pectins (Ralet, Bonnin, & Thibault, 2002)

The large varieties of molecular populations can be explained by cleavage of larger molecules which have taken place enzymically already during the ripening process (Rose, Hadfield, Labavitch, & Bennett, 1998) of the pumpkin fruits and the further treatments (dewatering and dialysis). However, chemical effects during the extraction and separation processes cannot be ruled out (Oosterveld, Beldman, Schols, & Voragen, 2000).

### 3.3. Structural properties of the acidic polysaccharide fractions

FTIR spectroscopy was used to support the presented chemical analysis data of the acidic polysaccharide fractions and replenish them with some structure information. The most informative is the spectral region 1800–800 $cm^{-1}$ illustrated for fractions DP1–DP6 in Fig. 2. The spectra were examined and compared with spectral data from IR studies of pectins and other plant polysaccharides (Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000; Synytsya, Čopíková, Matějka, & Machovič, 2003). In the region featuring the state of carboxylic groups, the band at 1744 $cm^{-1}$ corresponded to protonated or esterified carboxylic groups, whereas the couple of bands at 1611 and 1413 $cm^{-1}$ were attributed to asymmetric and symmetric stretching modes of the $COO^-$ group. Since samples DP1–DP4 were carefully neutralized before the measurements, the presence of both esterified and ionized forms can be identified. The increasing intensity of the band at 1740 $cm^{-1}$ following the same order of the samples can be correlated with the degree of methylesterification of carboxylic groups (DE). The fingerprint region (FPR) of these fractions showed the characteristic pattern of pectins with bands at 1145 1103, 1077, 1050 and 1017 $cm^{-1}$. Typical bands of arabinogalactans at 1139, 1074 and 1034 $cm^{-1}$ (Kačuráková et al., 2000) were the strongest in DP1 and in accord with its neutral sugar composition (Table 1). The FTIR spectra of the alkali-extracted fractions DP5 and DP6 showed quite different patterns. Due to the high protein content, the carboxyl region was overlapped by the amide I (1657 $cm^{-1}$) and amide II (1543 $cm^{-1}$) bands. The 'pectin' pattern of DP5 was strongly disturbed by the bands of arabinogalactan and xyloglucan (1153, 1118, 1078, 1041, 945 $cm^{-1}$). The DP6 fraction showed bands typical of glucuronoxylans (1147 and 1047 $cm^{-1}$) which prevailed, as former suggested, in this fraction (Table 1). The overlapping effect of proteins broadened also the carboxylate region of DP1.

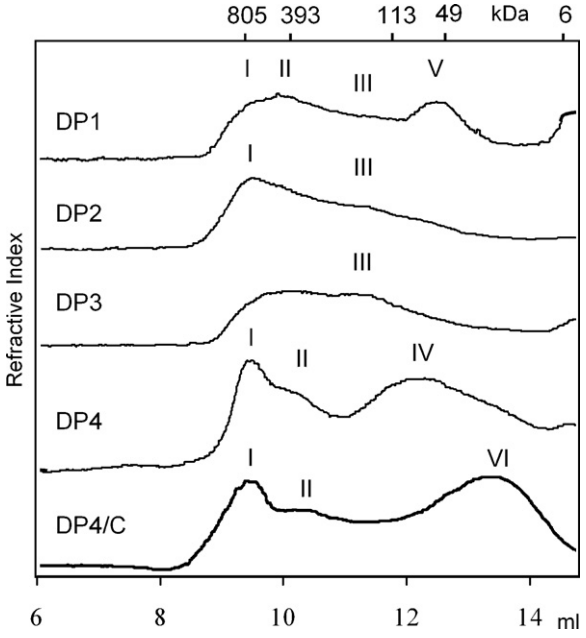



**Fig. 1.** HPGPC chromatograms of pectin fractions isolated from oil-pumpkin biomass. The chromatograms are cut off according to the MWCO of the dialysis membrane (3.5 kDa). Pullulan molecular weight markers (kDa) used as a calibration scale are shown at the top. Numbers I–VI indicate different molecular populations.

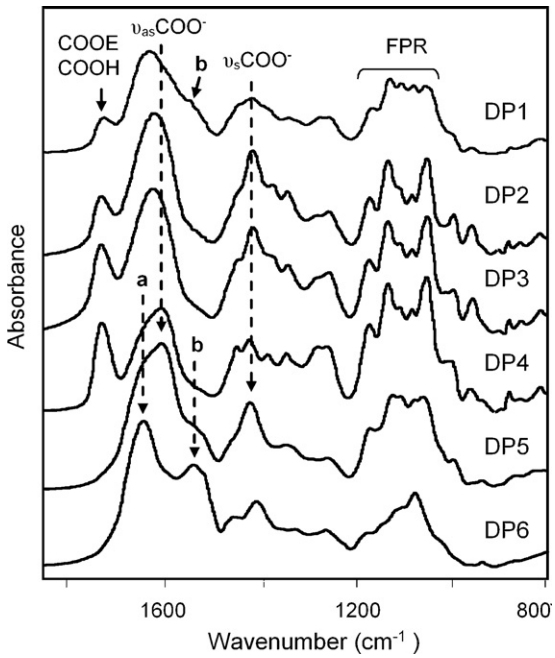



**Fig. 2.** Partial FTIR spectra of pectic polysaccharide fractions DP1–DP6. (a) Amide I; (b) Amide II; FPR, Fingerprint region.

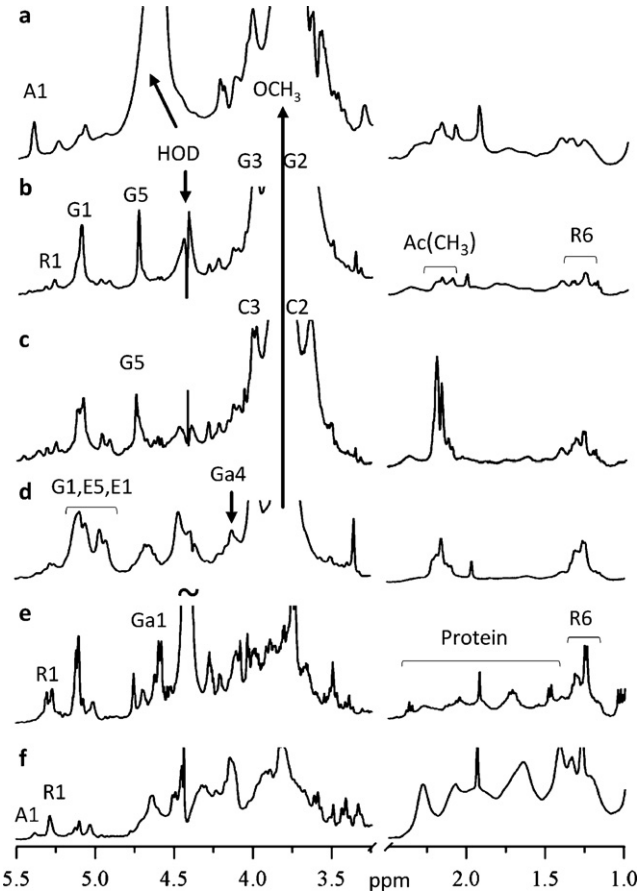



**Fig. 3.** $^{1}$H NMR spectra of pectic polysaccharide fractions (a) DP1, (b) DP2, (c) DP3, (d) DP4, (e) DP5 and (f) ws-DP-6. Sugar abbreviations: G; free and E; methyl-esterified α-D-Gal*p*A; A; α-L-Ara*f*; Ga; β-D-Gal*p*; R; α-L-Rha*p*. The attached numbers denote the corresponding protons.

### 3.4. $^{1}$H NMR spectroscopy analysis of acidic polysaccharide fractions

The $^{1}$H NMR spectra of fractions DP1–DP6 are displayed in Fig. 3. The dominant signals in all spectra corresponded to Gal*p*A residues of the pectin molecules in their free (G) and methoxylated (E) forms denoted according to Grasdalen, Bakøy, and Larsen (1988). The region at $\delta$ 5.20–4.20 contained the H-1, H-5 and H-4 resonances of both G and E units (Table 3). Well detectable are the protons of the methyl ester group at $\delta$ 3.82 and of acetyl groups in the region $\delta$ 2.10–2.22 (Renard & Jarvis, 1999). As seen, the spectral pattern of DP2, DP3 and DP4 considerably differed.

Based on reports from $^{1}$H NMR studies on pectins differing in DE (Andersen, Larsen, & Grasdalen, 1995; Grasdalen, Andersen, & Larsen, 1996; Renard & Jarvis, 1999), it can be deduced that the pattern of DP3 and DP4 resembled that of medium methylesterified pectins, whereas, that of DP2 indicated a very low DE typical of chelate-extracted pectins. According to Grasdalen et al. (1988), the DE of these fractions were calculated by the relationship of area integrals (1) DE (%) = $[(I_A - I_B)/(I_A + I_B)] \times 100$, where A comprises the lines from protons H-1 of G and E (GH-1, EH-1) and H-5 of E (EH-5) residues at $\delta$ 5.15–4.87, and B stands for lines from H-5 of G residues (GH-5) at $\delta$ 4.75–4.68. The obtained DE values of fractions DP2, DP3 and DP4 were 28, 34 and 48%, respectively. The presence of RG I domains in fractions DP1–DP4 was documented by two doublets in the region $\delta$ 1.26–1.31 assigned to methyl protons of 2- and 2,4-linked Rha*p* residues (Renard & Jarvis, 1999; Huisman et al., 2001; Sun, Cui, Tang, & Gu, 2010). The $^{1}$H NMR spectra served also for gross estimation of the proportion of RG I regions and degree of acetylation ($DS_{ac}$) for fractions DP2–DP4. It was calculated using the area integrals of methyl protons from Rha*p* ($I_{Rha}$) and acetyl groups ($I_{Ac}$) in relation to the above mentioned ($I_A + I_B$) values which included both G and E units. The equation (2) $DS_{ac}$ (mol/mol Gal*p*A) = $2(I_{Ac})/3(I_A + I_B)$ gave respective $DS_{ac}$ values 0.16, 0.32 and 0.16 mol/mol Gal*p*A. From equation (3) Rha*p* (mol/mol Gal*p*A) = $2(I_{Rha})/3(I_A + I_B)$, the proportion of the RG I regions was calculated by doubling the amount of Rha*p*, thus, yielding the respective estimated values 16, 26 and 16 mol%. These results suggested the prevalence of homogalacturonan regions in fractions DP2, DP3 and DP4 (Scheme 1).

In both DP5 and DP6 fractions the upfield region is overlapped by resonance of proteins. However, in DP5 with a high proportion of rhamnose and lower Gal*p*A content the anomeric protons of Rha*p* showed well-resolved signals at $\delta$ 5.22–5.27. From the neutral side chains of the RG I region, the Gal*p* units detectable by protons H-1 and H-4 at $\delta$ ~4.61 and 4.17, respectively, pointed to the presence of β-1,4-D-galactan chains. The Ara*f* units usually attached as monomer or oligomers to the galactan chains were detected by the anomeric proton at $\delta$ 5.3–5.1 ppm. The assignments were in accord with published data (Habibi et al., 2004; Huisman et al., 2001; Sun et al., 2010) in fractions DP1 and DP6. In DP1, phenolic substances gave several weak signals in the region $\delta$ 6.5–7.5 (not shown) indicative of aromatic and unsaturated structures.

### 3.5. Structural features of purified pectin fraction DP4/C

A combination of 1D and 2D NMR spectroscopy techniques was used to characterize the main structural features of DP4/C. As shown in Fig. 4a, the $^{1}$H NMR spectrum (top) resembled strongly to that of the parent polysaccharide DP4 (Fig. 3). Estimation of the DE and $DS_{ac}$ and proportion of RG I regions applied in Section 3.3 revealed that DP4/C contained 12 mol% of RG I, had a medium DE (45%) and was slightly acetylated ($DS_{ac}$ 0.05 mol/mol GalA).

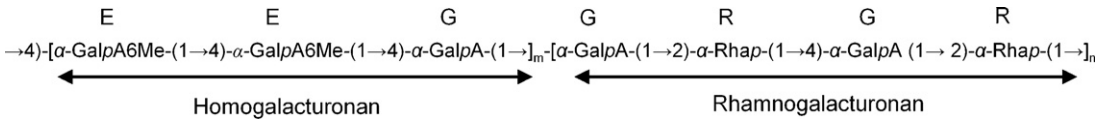
As already stated in earlier studies (Andersen et al., 1995; Denes, Baron, Renard, Pean, & Drilleau, 2000; Grasdalen et al., 1988; Renard & Jarvis, 1999), the chemical shifts of EH-1 and GH-1 units are strongly dependent on the DE of the pectin, the distribution of G and E units along the macromolecular chains forming diads, triads and larger groups, and the proximate neighbors at both sites of the E and G units in these groups. Because the signal of EH-5 is downfield shifted into the anomeric zone, ambiguity arose in its assignment. As a result, there exists a large discrepancy of the chemical shifts of EH-1, GH-1 and EH-5 protons, and their corresponding $^{13}$C chemical shifts reported in later studies (Perrone et al., 2002; Petersen, Meier, Duus, & Clausen, 2008; Rosenbohm, Lundt, Christensen, & Young, 2003; Winning, Viereck, Nørgaard, Larsen, & Engelsen, 2007). Therefore, the identification of these protons in DP4/C was a crucial task.

From the very crowded COSY spectrum (not shown) it was not possible to discern the spin systems of G and E units and, moreover, the H-4 signals visible in the proton spectrum as two adjacent peaks without baseline separation were in part obscured by the HOD signal. Therefore, $^{1}$H–$^{13}$C HSQC and $^{1}$H–$^{13}$C HMBC NMR spectra were used to assign the chemical shifts of both G and E units. To get a better overview about the protons in the anomeric region of the $^{1}$H NMR spectrum (Fig. 4a, top of HSQC spectrum), the proton peaks were denoted as 1–5 covering diads and triads according to Grasdalen et al. (1988): (1) EG and GE, (2) GG + GE and EG, (3) EEE, GEE (4) EE, (5) EG. As seen in Fig. 4a, two broad not well-resolved cross-peaks dominated in the anomeric region of the HSQC spectrum, centered at $\delta$ 5.13/102.0 and 4.96/102.9 (region of proton peaks 1–5). A further couple of two well-separated cross-peaks centered at $\delta$ 5.11/73.4 and 4.76/74.2 confirmed that the signal at $\delta$ 5.11

**Table 3**
Chemical shifts of the glycosyl residues of DP4/C from $^{1}H$–$^{13}C$ HSQC and HMBC NMR spectra.

| Glycosyl residues | Chemical shifts, $\delta$ (ppm) | | | | | |
|---|---|---|---|---|---|---|
| | H1/C1 | H2/C2 | H3/C3 | H4/C4 | H5/C5 | H6/C6 |
| →4)-α-Gal*p*A-(1→4 | 5.15/102.2 (GG) | 3.76/71.2 | 4.00/71.4 | 4.44/81.0 | 4.76/74.2 | 177.6 |
| | 5.12/102.0 (GE) | 3.76/71.2 | 4.00/71.4 | 4.42/81.0 | 4.73/74.5 | 177.7 |
| →4)-α-6MeGal*p*A-(1→4 | 4.96/102.9 (EE) | 3.75/71.0 | 4.01/71.6 | 4.47/81.3 | 5.11/73.4 | 173.8 |
| | 4.92/102.9 (EG) | –/71.0 | 3.97/– | 4.47/81.4 | 5.06/74.1 | 173.8 |
| →2)-α-Rha*p*-(→4 | 5.26/101.6 | 4.12/79.6 | 3.89/72.5 | 3.42/75.1 | 3.85/71.9 | 1.26/19.3 |
| →2,4)-α-Rha*p*-(→4 | –/99.4 | –/80.3 | –/73.7 | –/83.6 | 3.50/71.2 | 1.30/19.5 |
| →4)-α-Gal*p*-(1→4 | 4.64/107.2 | 3.66/74.9 | 3.77[a]/76.1 | 4.77/80.1 | 3.71/77.5[a] | 3.82/3.78/63.9 |
| | 4.64/107.4 | 3.70/74.9 | –/76.8 | –/80.9 | – | 3.71/64.1/64.1 |
| t-α-L-Ara*f*-(1→ | 5.09/110.5 | 4.14/83.6 | 3.98/79.3 | –/86.0 | – | |
| →5)-α-L-Ara*f*-(1→ | –/112.2 | – | – | 4.23/85.2 | 3.80/71.6 | |

–, not determined.
[a] Tentatively assigned.




**Scheme 1.** Schematic structure of pectin. For sugar abbreviation see Fig. 3.

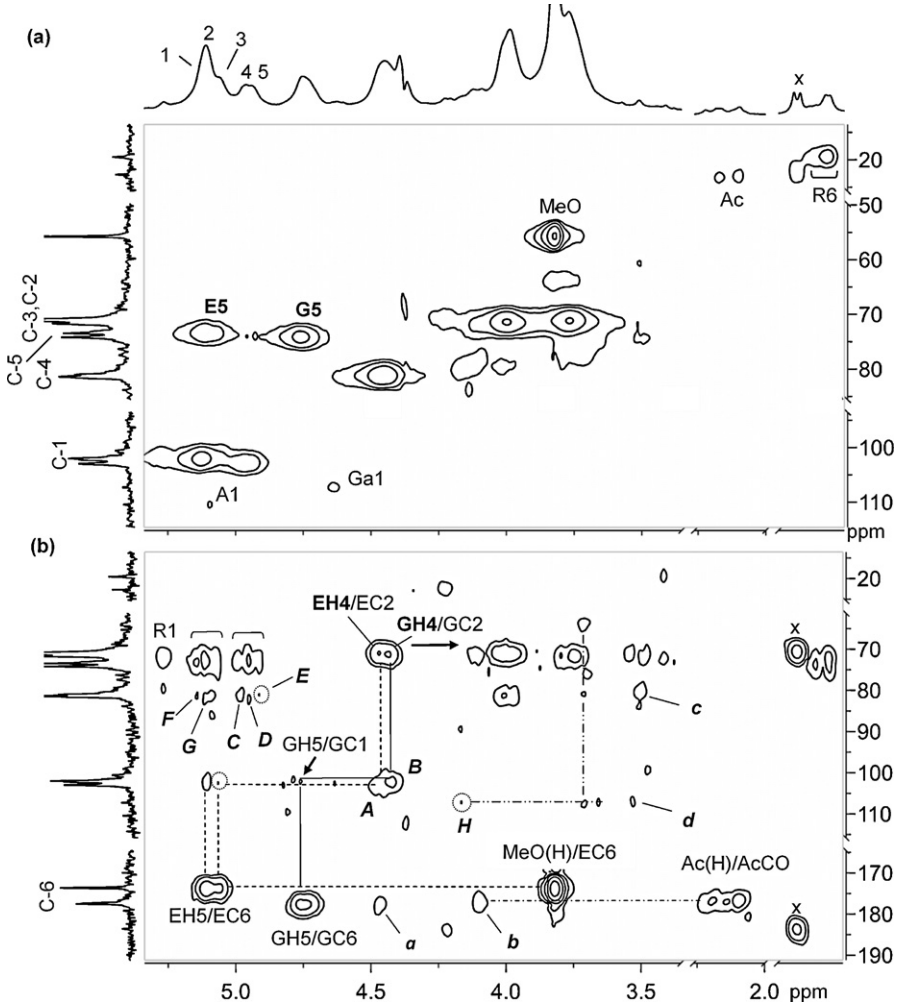



**Fig. 4.** 2D NMR spectra of DP4/C in $D_2O$ at 60 °C. (a) $^{1}H$–$^{13}C$ HSQC NMR spectrum and the $^{1}H$ NMR spectrum (top) and (b) $^{1}H$–$^{13}C$ HMBC spectrum. The numbers on the $^{1}H$ NMR spectrum denote the proton peaks of diads and triads (1) EG and GE, (2) GG + GE and EG, (3) EEE, GEE (4) EE, (5) EG according to Grasdalen et al. (1988). Letters ***a*** and ***b*** correspond to intra-residue H5/C6 cross-peaks of α-Gal*p*A and β-Gal*p*A reducing end units, respectively. Letters ***c*** and ***d*** denote the intra-residue cross-peak R′H5/R′C2 and tGaH2/tGaC1, respectively; *x* indicates contaminating low molecular weight impurities. The capitals *A*–*H* refer to inter-residue cross-peaks (summarized in Table 4). For sugar abbreviations see footnotes to Fig. 2.

corresponds to EH-5 which, as expected, is downfield shifted from the H-5 of G units (~0.35 ppm). Both H-5/C-5 resonances of G and E units were determined in a partially methylesterified xylogalacturonan fraction from apple pectin (Schols, Bakx, Schipper, & Voragen, 1995). The proportion of the integrated areas of both cross-peaks used to estimate the DE yielded a value of 42%, close to that derived from the $^{1}$H NMR spectrum (45%). The HSQC spectrum showed also two broad, non-resolved cross-peaks centered at $\delta$ 4.00/71.4 and 3.76/71.2 corresponding to C-3 and C-2 atoms of both α-Gal*p*A residues (E and G) (Catoire, Goldberg, Pierron, Morvan, & Hervé du Penhoat, 1998).

The differentiation of the anomeric cross-peaks of E and G units (shown in Fig. 4a) was resolved using the HMBC NMR spectrum (Fig. 4b). The G units gave intra-residue couplings between two non-resolved H-5 protons ($\delta$ 4.76 and ~4.73) and the carboxyl carbon ($\delta$ 177.6), whereas, the E units showed intra-residue contacts of two EH-5 protons ($\delta$ 5.11 and 5.06) with the carboxyl carbon at $\delta$ 173.8 correlating further with protons of the methoxyl group ($\delta$ 3.82). The splitting of both GH-5 and EH-5 protons indicated different locations of G and E units in the pectin chains what accorded with the splitting of the C-1, C-4 and C-5 signals in the $^{13}$C NMR spectrum (Fig. 4a and b). The EH-5/EC-6 cross-peak of E units showed connectivity with EC-1 ($\delta$ 102.9) and further one with EH-4 ($\delta$ 4.47) yielding the inter-residue cross-peak ***A*** between E units. The GH-5/GC-6 cross-peak at $\delta$ 4.76/177.6 had connectivity with C-1 at $\delta$ 102.2 which correlated with the H-4 proton at $\delta$ 4.44. This inter-residue cross-peak ***B*** confirmed the 1,4-glycosidic bonding in homogalacturonan regions. The weaker GH-5/GC-6 cross-peak at $\delta$ 4.73/177.7 might belong to α-Gal*p*A residues from the RG regions and showed no further interactions, very probably due to the low proportion of RG I elements. Both *A* and *B* cross-peaks gave further correlations with C-2 carbons at $\delta$ ~71.2.

The HMBC spectrum contained three protons at $\delta$ 5.15, 5.12 and 5.08 which gave broad, non-resolved cross-peaks covering the region of C-2, C-3 and C-5 carbons. The first two protons corresponded to H-1 of G units giving with C-4 carbons at $\delta$ 81.0 (GC-4) and 81.4 (EC-4) inter-residue cross-peaks ***F*** and ***G***, respectively. They confirmed the 1,4-glycosidic bonding between the G and E units in diads GG and GE (see proton peaks 1, 2 in Fig. 4a). However, the third proton corresponded to EH-5 (proton peak 3 in Fig. 4a) showing an intra-residue cross-peak with EC-4 at $\delta$ 81.4.

In the anomeric region of the HMBC spectrum, there were further three protons at $\delta$ 4.98, 4.96 and 4.92. Based on the aforementioned assignments, they were attributed to H-1 of E units which gave, similarly as the GH-1 protons, broad, non-resolved cross-peaks in the region of C-2, C-3 and C-5 resonances. The second and third protons showed connectivity with the signals at $\delta$ 81.8 and 81.0 assigned previously to EC-4 and GC-4 carbons, respectively. The formed inter-residue cross-peaks ***D*** and ***E*** corresponded to the diads EE and EG (Fig. 4a, proton peaks 4 and 5, respectively). The results confirmed unambiguously the assignments of GH-1, EH-1 and EH-5 protons of partially methyl-esterified pectins earlier derived from 1D and 2D NMR spectroscopy analyses (Andersen et al., 1995; Renard & Jarvis, 1999; Denes et al., 2000; Westerlund, Aman, Andersson, Andersson, & Rahman, 1991). The anomeric proton at $\delta$ 4.98 showed a contact with the carbon signal at $\delta$ 81.0 what might be attributed to an inter-residue coupling (cross-peak ***C***) between H-1 of an E unit and C-4 of α-Gal*p*A reducing end group of oligomers containing such EG diad on their reducing end. This assignment was deduced from the NMR data reported for galacturonic acid oligomers (Perrone et al., 2002). Accordingly, the weak intra-residue H-5/C-6 cross-peaks (Fig. 4b, ***a*** and ***b***) at $\delta$ 4.47/177.9 and 4.10/176.9 corresponded to α-and β-reducing Gal*p*A end units, respectively, of such oligomers. Their presence was substantiated by the former-mentioned large amount (~50%) of a very low-molecular weight population in DP4/C. The chemical shifts derived from the NMR spectra are summarized in Table 3 and the inter-residue cross-peaks presented in Table 4.

**Table 4**
Inter-residue cross-peaks identified in the HMBC spectrum of DP4/C.

| Label | Cross-peak $\delta$H/$\delta$C (ppm) | Connected atoms |
|---|---|---|
| A | 4.47/102.9 | EH-4/EC-1 |
| B | 4.44/102.2 | GH-4/GC-1 |
| C | 4.99/81.0 | EH-1/αGC-4 |
| D | 4.96/81.8 | EH-1/EC-4 |
| E | 4.92/81.0 | EH-1/GC-4 |
| F | 5.15/81.0 | GH-1/GC-4 |
| G | 5.12/81.4 | GH-1/EC-4 |
| H | 4.17/107.2 | GaH-4/GaC-1 |

The acetyl groups showed four HMBC cross-peaks between the acetyl carbonyl at $\delta$ 177.2–176.9 and the methyl protons at $\delta$ 2.22, 2.18, 2.14 and 2.10. They indicated according to Renard and Jarvis (1999) as acetylation sites *O*-2, *O*-3, and both *O*-2 and *O*-3 of Gal*p*A units. Acetylated Gal*p*A residues were found in both homogalacturonan and RG regions (Ishii, 1997; Komalavilas & Mort, 1989). The spin systems reported for acetylated Gal*p*A residues revealed significant downfield shifts of both protons H-2 and H-3 from $\delta$ ~3.76 and 4.00 to about $\delta$ 5.10 and 5.40, respectively. However, these signals were not identified in the HMBC spectrum of DP4/C. The first signal might be overlapped by the EH-5 protons and the second one was not seen, even at a higher level of the correlation plot. The low content of acetyl groups and their various locations disabled the identification of the acetylation sites. Although, the weak cross-peak at $\delta$ 4.10/176.9 pretended connectivity to the acetyl carbon, it did not accord with published data for acetylated pectins. As described before, it was attributable to Gal*p*A β- end residues.

The 2D NMR spectra provided also information about the rhamnose, galactose and arabinose components of DP4/C. The $^{1}$H–$^{1}$H COSY spectrum showed the whole spin system for 2-linked Rha*p* residues at $\delta$ 5.26 (H-1), 4.12 (H-2), 3.89 (H-3), 3.42 (H-4), 3.85 (H-5) and 1.26 (H-6). The corresponding chemical shifts of C-1 to C-6 were assigned from the HSQC and HMBC spectra and presented in Table 3. The 2,4-linked Rha*p* residues showed a weak H-6/C-6 cross-peak at $\delta$ 1.30/19.5. The HMBC connectivity of proton H-5 at $\delta$ 3.50 with C-1, C-2 and C-4 at $\delta$ 99.4, 80.3 and 83.4, respectively, confirmed the substitution of Rha*p* at position O-4. These assignments accorded with literature data (Colquhoun, Ruiter, Schols, & Voragen, 1990; Huisman et al., 2001; Polle, Ovodova, Shashkov, & Ovodov, 2002; Sun et al., 2010).

The galactose component showed weak signals in the $^{13}$C NMR spectrum (Fig. 4a and b) at $\delta$ ~107.2, 77.4, 76.3 and 63.6 and shoulders at $\delta$ 80.1 and 74.9. They were attributed to carbons C-1, C-5, C-3, C-6, C-4 and C-2, respectively, based on the assignment of the corresponding proton shifts from the HSQC and HMBC spectra (Table 3). The inter-residue H-1/C-4 cross-peak ***H*** at $\delta$ 4.17/107.2 detected clearly after elevating the level of the correlation plot showed further connectivity with C-3 and C-6 (Fig. 4b) and confirmed the presence of 1,4-β-galactan chains. The results accorded with published data (Habibi et al., 2004; Polle et al., 2002). The existence of more than one chemical shift of carbons 1, 2, 3, 4 and 6 pointed to different chemical environments of the Gal*p* residues. The HMBC cross-peak (***d***) at $\delta$ 3.53/107.0 can be attributed to C-1 and H-2 of non-reducing Gal*p* residues (Bushneva, Ovodova, Shashkov, & Ovodov, 2002; Sun et al., 2010) which might occur as single side chains and/or terminate the galactan side chains. The arabinose component showed weak H-1/C-1 cross-peak at $\delta$ 5.09/110.5 and H-2/C-2 cross-peak $\delta$ 4.14/83.6 which are together with the C-4 signal at $\delta$ 86.0 typical of terminal α-L-Ara*f* residues. The anomeric carbon signal at $\delta$ 109.5 and H-4/C-4 cross-peak at $\delta$ 4.23/85.2 indicated the presence of 5-linked α-L-Ara*f* residues. The

data corresponded with reports concerning the arabinose moieties in pectic polysaccharides containing 1,4-β-galactan side chains (Habibi et al., 2004; Sun et al., 2010).

## 4. Conclusion

The applied sequential extraction procedure allowed a partial separation and isolation of various pectic polysaccharide components of the oil-pumpkin biomass. The hot water-extracted fraction represented a complex of high-methylesterified and acetylated pectin with a very large proportion of neutral sugar components, proteins and phenolic substances. The EDTA-extracted polysaccharide fraction contained pectin with a low DE (28%), whereas, the pectin fractions isolated by the dilute acid-treatments showed a medium DE (34–48%). All three pectins were slightly acetylated and contained RG I regions ranging between 16 and 26 mol%. The alkali-extracted pectins were to various extents accompanied with hemicellulosic polysaccharides – xyloglucan and glucuronoxylan. More detailed structural features were determined for the purified pectin fraction (DP4/C) isolated from the second acid extraction step. The application of $^{1}H$–$^{13}C$ HSQC and HMBC spectra allowed establishing unambiguously the $^{1}H$ and $^{13}C$ chemical shifts for atoms 1, 4, and 5 of the methyl-esterified and non-esterified GalpA residues, to identify the glycosidic linkages between them and to confirm various diads of these units in the pectin macromolecules. The RG-I regions were found to be branched with β-1,4-galactan and α-Ara*f*-derived side chains. The considerable molecular polydispersity referred to the native state of the pectic polysaccharides, however, effects of degradation processes due to enzymic activities and the used extraction conditions cannot be ruled out. This paper filled a gap of information on the structural features of pumpkin pectic polysaccharide components. The results might serve as a basis for searching and developing technological conditions to produce pectins differing in degree of methylesterification from the oil-pumpkin fruit biomass for potential industrial exploitation in food, pharmacy and other fields.

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