ISOLATION AND CHARACTERIZATION OF ACETYLATED LM-PECTINS EXTRACTED FROM OKRA PODS

K. Alba\textsuperscript{1}, A. P. Laws\textsuperscript{2} and V. Kontogiorgos\textsuperscript{1*}

\textsuperscript{1}Department of Biological Sciences, University of Huddersfield, HD1 3DH, UK
\textsuperscript{2}Department of Chemistry, University of Huddersfield, Queensgate, HD1 3DH, UK

*Corresponding author
Tel.: +44 1484 472488
e-mail: v.kontogiorgos@hud.ac.uk
Abstract

Pectin was isolated by aqueous extraction at pH 6.0 or 2.0 from okra (Abelmoschus esculentus L.) pods. An isolation protocol was designed to extract pectin and to study the influence of the extraction pH on their composition and physicochemical properties. The extracted pectin was assessed using sugar compositional analysis (neutral sugars, galacturonic acid, acetyl and methyl contents). FT-IR and NMR spectroscopy, size exclusion chromatography (SEC) and dilute solution viscometry were also used to determine the macromolecular characteristics of isolated pectin. The extraction protocols resulted in the isolation of pectin of high purity as evidenced by their high total carbohydrate (70.0 – 81.8%) and low protein (4.3 – 6.3%) contents. Samples contained between 46-56% galacturonic acid, had broad molecular weight distributions, a low degree of methylation (40.0 and 24.6 %) and high degree of acetylation (52.2 and 37.6 %). Neutral sugar analysis showed that the pectin extracted at pH 6.0 contained more neutral sugars, particularly, galactose (21.7 – 25.7 mol%), rhamnose (10.1 – 13.2 mol%) and arabinose (7.1 – 7.3 mol%) than that extracted at pH 2.0 indicating variations in fine structure. In addition, molecular parameters of the isolated pectins, such as intrinsic viscosity (2.8 – 4.4 dL g⁻¹), critical concentration (0.15 – 0.45 dL g⁻¹) and coil overlap parameter (0.66 – 1.51), showed that extraction conditions resulted in pectin with different chain morphology. The yield and physico-chemical characteristics of the extracted pectin from okra pods were influenced by the extraction conditions.

Keywords: pectin; okra; NMR; acetylation; characterization; isolation
1. Introduction

Pectins are described as acidic heteropolysaccharides composed mainly of $\alpha$-(1→4) linked $\alpha$-D-galacturonic acid (GalA) residues. Three major structural units of pectic polysaccharides are recognised, all containing various amounts of GalA residues. Homogalacturonan (HG) is mainly composed of $\alpha$-(1→4) linked $\alpha$-D-galacturonic acid (GalA) residues, whereas rhamnogalacturonan (RG-I) backbone consists of repeating units of $\alpha$-(1→4) linked $\alpha$-D-galacturonic acid and $\alpha$-(1→2) linked $\alpha$-L-rhamnose monomers attached to the arabinan, galactan and/or arabinogalactan side chains (Vincken, Schols, Oomen, Beldman, Visser & Voragen, 2003). Rhamnogalacturonan II (RG-II) has a backbone similar to RG-I, composed of $\alpha$-(1→4) linked $\alpha$-D-galacturonic acid residues with side chains consisting of various sugars. The okra pectin obtained by sequential extraction are described as acidic random coil heteropolysaccharides containing $\alpha$-(1→2)-rhamnose and $\alpha$-(1→4)-galacturonic acid residues with disaccharide side chains composed of galactose attached to O-4 of half of the rhamnose residues (Tomada, Shimada, Saito & Sugi, 1980). It has been also reported that okra extracts contain high amounts of RG-I segments and acetylation on rhamnose residues something that is uncommon for pectin from other sources (Sengkhamparn, Bakx, Verhoef, Schols, Sajjaanantakul & Voragen, 2009).

Isolation of polysaccharides can be performed on a laboratory scale by extractions of the cell-wall material, which involve the use of calcium-chelating agents, dilute alkali or dilute acid (Levigne, Ralet & Thibault, 2002). Alternatively, degrading enzymes can be employed in order to release polysaccharide fragments. One of the drawbacks of the extraction with chelating agents is that it is laborious to remove the residual chelates. Alkaline extraction contributes to the reduction of length
and degree of acetylation and methylation by $\beta$-elimination (Rombouts & Thibault, 1986). It has been reported that the highest yields of pectic substances are generally obtained by hot acid extractions which is also the most convenient approach for industrial extraction of pectin (May, 1990; Pagan, Ibarz, Llorca & Coll, 1999). Previous studies reported that the temperature, pH and time could modify the quantity as well as the quality of the extracted pectins (Levigne, Ralet & Thibault, 2002; Pagan, Ibarz, Llorca & Coll, 1999). Furthermore, it was shown that the variations in the number of methyl-esterified groups and composition of neutral sugars of the isolated fractions are primarily governed by the extraction protocol (Kjøniksen, Hiorth & Nyström, 2005; Turquois, Rinaudo, Taravel & Heyraud, 1999). The extracted materials typically are polydisperse heteropolymers having diverse chemical structures and molecular sizes (MacDougall & Ring, 2004).

Okra polysaccharides are potentially a new source of natural polysaccharides, which can be used as functional ingredients (thickeners, viscosity enhancers, gelling agents and texture modifiers) by the food industry (Georgiadis, Ritzoulis, Sioura, Kornezou, Vasiliadou & Tsiptsias, 2011). Recent studies have mainly focused on characterization of okra polysaccharides obtained with sequential extractions, starting with acidic hot buffers followed by chelating agents and dilute alkali buffers. Nevertheless, the effect of extraction pH on the physicochemical characteristics and therefore functional properties of okra isolates has not been extensively studied (Georgiadis, Ritzoulis, Sioura, Kornezou, Vasiliadou & Tsiptsias, 2011; Kontogiorgos, Margelou, Georgiadis & Ritzoulis, 2012; Ndjouenkeu, Akingbala & Oguntimein, 1997; Sengkhamparn, Verhoef, Schols, Sajjaanantakul & Voragen, 2009). The aims of the present work were to extract okra pectins at different pH
values and examine the effect of the extraction conditions on their molecular and compositional characteristics.

2. Materials and Methods

2.1 Materials

Okra pods of Abelmoschus esculentus L. were purchased from the local market. Pods were frozen and kept at –20 °C until use. Sodium azide, all buffer salts, acetic acid, phenol, 3-phenylphenol, sodium tetraborate, sulfamic acid, 1.25 M hydrogen chloride-methanol solution, anhydrous pyridine, acetic anhydride, anhydrous ethyl acetate, ethanol (96% w/w) (all analytical grade reagents) and petroleum ether (bp 40-60°C) were obtained from Sigma-Aldrich (Poole, UK). De-ionized water was used throughout the extraction experiments. Dextrans (Mₚ, 270, 410 × 10³ kDa), D-galacturonic acid monohydrate, D-galactose, L-rhamnose, L-arabinose, D-glucose, D-xylose, pectins from citrus fruit (esterified 55-70% and 20-34% potassium salts) and dialysis membranes (molecular weight cut-off 12000) were purchased from Sigma–Aldrich (Poole Dorset, UK).

2.2 Isolation of okra pectins

The isolation of pectins from okra pods was carried out according to the experimental design shown in Figure 1. The extraction protocol resulted in the isolation of two pectin samples namely OP2 and OP6 for isolates extracted at pH 2.0 and pH 6.0, respectively.

2.3 Chemical characterization

Total carbohydrates were determined by the phenol-sulphuric method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Protein content was established using
Bradford assay (Bradford, 1976). The galacturonic acid (anhydrous) content of pectins was estimated colorimetrically by the \( m \)-hydroxydiphenyl method (Filisetti-Cozzi & Carpita, 1991). The methoxyl (-OCH\(_3\)) content of pectins was determined by titration (Schultz, 1965). The method is based on a titration of free carboxyl groups present followed by de-esterification and titration of the carboxyl groups that have been made available. A correction was made for the acetic acid liberated due to the cleavage of the \( O \)-acetyl groups. The degree of methyl esterification (DM) was calculated from the galacturonic acid and methoxyl content values determined above according to the following equation (Schultz, 1965):

\[
DM(\%) = \frac{176 \times \text{methoxyl content (\%w/w)}}{31 \times \text{GA content (\%w/w)}} \times 100
\]  

where 176 and 31 are the molecular weights of anhydrous galacturonic acid (GA) and methoxyl, respectively. The acetyl content was determined with the hydroxamic acid method (McComb & McCready, 1957). The degree of acetylation (DA) was calculated from the galacturonic acid and acetyl content values determined above according to the following equation:

\[
DA(\%) = \frac{176 \times \text{acetyl content (\% (w/w))}}{43 \times \text{GA content (\% (w/w))}} \times 100
\]

where 176 and 43 are the molecular weights of anhydrous galacturonic acid (GA) and acetyl, respectively. Neutral sugars were determined using methanolysis conducted with 1M methanolic HCl solution at 85 °C for 24 h, as described previously (Bleton, Mejanelle, Sansoulet, Goursaud & Tchapla, 1996). Sugar derivatives were analysed using an Agilent 7890A GC system (Santa Clara, CA, USA) coupled to an Agilent 5675C quadrupole MS. The samples were eluted from a HP-5 column (30 m x 0.25 mm, 0.25 µm film) using helium as carrier at a flow rate of 1 mL min\(^{-1}\) by applying
the following temperature settings: start temperature 140 °C, hold time 1 min and
final column temperature 220 °C with 2.5 °C min\(^{-1}\) gradient.

Calculations on sugar composition were performed using molar ratios formulated
specifically for pectic substances (Houben, Jolie, Fraeye, Van Loey & Hendrickx,
2011). The molar percentage of homogalacturonan (HG) and rhamnogalacturonan-I
(RG-I) were also calculated according to the following equations (M’sakni et al.,
2006):

\[
\text{HG (mol\%)} = \text{GalA (mol\%)} - \text{Rha (mol\%)} \\
\text{RG-I (mol\%)} = 2\text{Rha (mol\%)} + \text{Ara (mol\%)} + \text{Gal (mol\%)}
\]

2.4 FT-IR spectroscopy

Spectra were obtained between 400-4000 cm\(^{-1}\) in Attenuated Total Reflection
(ART) mode at a resolution of 4 cm\(^{-1}\) using 128 scans (Nicolet 380, Thermo
Scientific, UK). Spectral smoothing was applied using instrument software (OMNIC
3.1).

2.5 \(^1\)H-NMR and \(^{13}\)C-NMR spectroscopy

NMR spectroscopy was performed with a Bruker AV 500 spectrometer
(Bruker Co., Switzerland) at 500 MHz \(^1\)H and 125.76 MHz \(^{13}\)C using a 5 mm probe.
In order to record \(^{13}\)C-NMR spectra samples were dispersed (5% w/v) in D\(_2\)O (99.9% D, Goss Scientific Instruments Ltd., Essex) by continuous stirring overnight. Proton-
decoupled spectra were recorded at 70°C with 19000 scans by applying 12800 pulses
with a delay time of 2 s and 30°C pulse angle.

\(^1\)H-NMR spectra were recorded for 640 scans at the same temperature. Prior to
scanning, samples were sonicated (QSonica 1375, QSonica LL, Newtown) for 9 min
in order to assist in aggregate dissociation. Sets of \(^1\)H-NMR spectra were recorded at
various okra pectin concentrations (1%, 2%, 4% and 5% w/v) with and without sonication in order to investigate how sonication affects the primary structure of the polymers. Preliminary data (not shown) demonstrated that sonication for 9 min does not contribute to the structural modifications as evidenced by inspection of $^1$H-NMR spectra of sonicated and non-sonicated samples at various concentrations. Chemical shifts were expressed in $\delta$ (ppm) relative to the resonance of internal standard: spectra were referenced to internal or external acetone ($^{13}$C $\delta = 31.55$ ppm and $^1$H $\delta = 2.225$ ppm).

2.6 Molecular weight determination

To evaluate the average molar masses ($M_w$, weight average molar mass; $M_n$, number-average molar mass) samples were analyzed by size exclusion chromatography (SEC). Pectins were solubilized in 50 mM NaNO$_3$ solution (3 mg mL$^{-1}$) at room temperature under magnetic stirring overnight. Samples were injected onto an analytical SEC system comprising of three columns Aquagel-OH 40, 50 and 60 (15$\mu$m particle size, 25cm $\times$ 4mm, Agilent, Oxford, UK) connected in series. Pectins were eluted with 50 mM NaNO$_3$ (containing 0.02% NaN$_3$ as a preservative) at a flow rate of 1 mL min$^{-1}$ and detected with an RI detector (differential index of refraction (dn/dc) equal to 0.1470 ml g$^{-1}$). Molecular parameters (Mw, Mn, Rg, Mw/Mn) were measured with a multiangle laser light scattering (MALLS) detector (mini-DAWN, Wyatt, Santa Barbara, CA, USA).

2.7 Dilute solution rheology

Okra pectin was dispersed at 0.01 – 5.0 % g dL$^{-1}$ at pH 7.0 in Sorensen’s phosphate buffer or pH 3.0 citric buffers in the presence of 0.1 M NaCl with 0.02 g dL$^{-1}$ NaN$_3$ as a preservative. Pectins were placed in sealed glass-vials and left
overnight under continuous stirring to ensure complete solubilisation. Intrinsic viscosity $[\eta]$ of okra pectins was determined at 20 °C with an Ubbelohde capillary viscometer (PSL, UK). Calculations were obtained by extrapolation of viscometric data to zero concentration according to the Huggins equation: 

$$\eta_{sp}/c = [\eta] + k_H[\eta]^2c$$

where $\eta_{sp}=([\eta]_{solution}/[\eta]_{buffer}) - 1$ and $k_H$ is the Huggins constant. Zero shear viscosity measurements were carried out at 20 °C using a Bohlin Gemini 200HR Nano rotational rheometer (Malvern Instruments, Malvern, UK) equipped with cone-and-plate geometry (55 mm diameter, cone angle 2°) and a Peltier temperature controller. All measurements were performed in a steady shear mode in the range 1–1000 s$^{-1}$.

3. Results and discussion

3.1 Isolation and compositional analysis

An isolation protocol was designed to study the influence of pH on extraction yield and the molecular characteristics of pectic substances from fresh okra pods. Extraction with petroleum ether (bp 40-65 °C) was performed as a first step in order to obtain a lipid-free material, which was subsequently used in aqueous extractions at pH 2.0 and 6.0 with 100 mM citric and phosphate buffer, respectively. The highest yields of pectic substances are usually obtained at high temperatures and low pH values in order to facilitate the cleavage of strong bonds between protopectin and other cell wall materials (Voragen, Rolin & Marr, 2003). It has been also reported that temperature has significant impact on the extraction yield of okra polysaccharides (Samavati, 2013). The isolation of the present okra polysaccharides was performed at 80 °C in order to facilitate the solubilisation of insoluble pectic substances (protopectin). Polysaccharides with different compositional characteristics can be isolated depending on the pH, time and temperature of extraction. It has been reported
that pectic substances extracted at pH 3.0 have similar compositional characteristics to water-soluble pectin but result in low yield values. Extraction at pH values below 3.0 leads to higher yields with pectins rich in rhamnogalacturonans (Levigne, Ralet & Thibault, 2002). Therefore, the extractions of pectic substances from okra were performed at two different pH values in order to obtain polysaccharides with distinct molecular characteristics. Pectic substances from okra pods could not be quantitatively recovered in a single extraction step and a second extraction was required (Figure 1). Similar findings have been reported for the extraction of pectins from other raw materials (Samavati, 2013; Sudhakar & Maini, 2000). The final stage, which can significantly affect the yield and chemical characteristics of pectins, is precipitation with ethanol. In the present work, precipitation was performed with ethanol at a 1:2 (v/v) supernatant to ethanol ratio and resulted in higher yields of pectic substances in comparison to preliminary 1:1 (v/v) ratio. It has been also reported that there is a pronounced effect of ethanol volume used in precipitation step on DM of isolated pectic substances (Faravash & Ashtiani, 2007). This occurs as the interaction between water, the carboxylic groups of pectin and the hydroxyl groups of ethanol facilitates cleavage of methyl ester linkages. Following alcohol precipitation, the pectin was washed with isopropanol and extensively dialysed against distilled water. Extraction with citric buffer adjusted to pH 2.0 resulted in slightly lower yield compared to extraction at pH 6.0. Furthermore, these extraction protocols result in pectin isolates of high purity as evidenced by low protein content (Table 1). The highest yields of pectin are typically obtained by hot acid extraction in the pH range 1.5 to 3.0. Studies on pectin from other sources such as sugar beet pulp and banana peels also showed that the pectin yield increases significantly with a decrease in the pH of the extraction and the highest yields were obtained at pH around 1.5 (Happi
Emaga, Ronkart, Robert, Wathelet & Paquot, 2008; Levigne, Ralet & Thibault, 2002; Yapo, Robert, Etienne, Wathelet & Paquot, 2007). These discrepancies with present data could be attributed to the origin of the initial material and the extraction conditions applied. The lower pectin yield at pH 2.0 could be attributed to partial acid hydrolysis that occurs at elevated temperatures as will be discussed later.

The chemical composition of okra pectins is shown in Table 1. The GalA content of the okra isolates varied from 46.8 to 56.9 % (Table 1). The GalA content was found to be significantly higher than has been previously reported for okra hot buffer soluble solids (HBSS, 35%) (Sengkhamparn, Verhoef, Schols, Sajjaanantakul & Voragen, 2009) and was close to that of sugar beet pectins (29.5-52.8 %) (Levigne, Ralet & Thibault, 2002). Furthermore, the highest GalA content and pectin yield were obtained when okra pectins were extracted at pH 6.0. The results strongly indicate, that the pectin extraction yield is related to the content of GalA reinforcing that partial degradation of pectic substances can take place under extraction conditions at pH 2.0.

Both okra pectins were found to be low methoxyl (LM) pectins with DM of 40.0% and 24.6% for OP2 and OP6, considering that DM represents methoxyl content per galacturonic acid unit. The differences in DM of pectin samples could be attributed to the de-esterification process caused by β-eliminative degradation of the esterified homogalacturonan backbone that leads to the removal of methyl esters resulting in pectin with lower degree of methylation and consequently lower molecular weight (Kurita, Fujiwara & Yamazaki, 2008). Previous studies on okra extracts obtained by sequential extraction also revealed the presence of low methoxyl pectins (Sengkhamparn, Verhoef, Schols, Sajjaanantakul & Voragen, 2009). Okra extracts also exhibited high acetyl content with marginal differences for 6.0 (OP2) and 5.2 % (OP6) (Table 1). Highly acetylated pectins have been previously isolated from sugar
beet where acetyl content varied in the range of 2.2–9.0% (Dea & Madden, 1986; Endreß & Rentschler, 1999). Previous studies on okra polysaccharides obtained by sequential extraction reported DA in the range of 18–58% and also revealed uncommon acetylation patterns. It has been previously reported that not only galacturonosyl residues, but also rhamnosyl residues were acetylated in the RG-I segments (Sengkhamparn, Bakx, Verhoef, Schols, Sajjaanantakul & Voragen, 2009). It should be stressed, that in the present study, DA is expressed to a first approximation as acetyl content per galacturonic acid (GalA) unit assuming acetylation only on galacturonosyl residues.

The main neutral sugars present in OP2 and OP6 were galactose (17.0 – 26.1%), rhamnose (7.1 – 12.1%) and arabinose (4.5 – 6.0%). The presence of 4 – 6% of the proteinaceous components may indicate that galactose and arabinose could also originate from arabinogalactans forming arabinogalactan-protein complexes (Immerzeel, Eppink, de Vries, Schols & Voragen, 2006). Very low levels of glucose (2.2 – 2.4%) and xylose (2.0% in OP2) were also detected in the okra pectins extracted at pH 2.0 suggesting the presence of rhamnogalacturonan II (RG-II) or xylogalacturonan regions. The total neutral sugar content was expressed as the sum of the individual neutral sugars and revealed that the highest neutral sugars yield was obtained with extraction at pH 6.0 (46.4%) that corresponds to milder extraction conditions which avoids degradation of pectin side chains. In addition, the content of GalA in OP2 was lower than in OP6. It seems that extraction at pH 2.0 also induces a breakdown in the smooth region composed primarily of homogalacturonan. Degradation of glycosidic linkages is usually observed at low pH values and elevated temperatures with different degree of stability (GalA – GalA > GalA – Rha > neutral sugar – neutral sugar) (Thibault, Renard, Axelos, Roger & Crépeau, 1993).
The ratios of constituent sugars were used in order to investigate the structure of the extracted pectins at the molecular level. According to the sugar composition data expressed as sugar molar ratios (Table 2) some interesting characteristics of the extracted polysaccharides were observed. The molar ratio of rhamnose to galacturonic acid is indicative of the presence of RG-I segments within the pectin population. The RG-I backbone is typically composed of alternating units of rhamnose and galacturonic acid and therefore the molar ratio of Rha/GalA is virtually 1:1 (Yapo, 2011). The contribution of RG-I to the pectin population was 0.18 and 0.25 for OP2 and OP6, respectively (Table 2). Therefore, OP2 and OP6 contained high amounts of RG-I regions with higher values for OP6 (59.4%) in comparison to OP2 (49%) indicating the prevalence of RG-I segments within the pectin population especially in OP6 (Table 2). The HG/RG-I ratio varied from 0.9 (OP2) to 0.7 (OP6), suggesting the presence of approximately equal proportions of HG and RG-I segments. These data suggest structural dissimilarities of our samples compared to common pectins isolated from apple or sugar beet, where RG-I segments constituted ~16.2 or ~31.9% of the pectin populations (Leroux, Langendorff, Schick, Vaishnav & Mazoyer, 2003). However, more than 50% of RG-I has been previously reported for hot water-extracted pectins from soybean and green tea leaves and almost as the only pectic component in okra polysaccharides obtained by hot buffer sequential extraction and linseeds mucilages (Ele-Ekouna, Pau-Roblot, Courtois & Courtois, 2011; Muralikrishna, Salimath & Tharanathan, 1987; Nakamura, Furuta, Maeda, Nagamatsu & Yoshimoto, 2001; Sengkhamparn, Verhoef, Schols, Sajjaanantakul & Voragen, 2009). The molar ratio of (Ara+Gal)/Rha is indicative for the degree of branching of RG-I segments. The molar ratio for OP2 was 2.9 and 2.5 for the OP6 suggesting shorter side chains of RG-I regions in OP6 than in OP2. Generally, OP2 and OP6
demonstrated remarkably higher degree of branching of side chains than was previously reported for okra polysaccharides obtained by sequential extractions (1.3–1.4) (Sengkhamparn, Verhoef, Schols, Sajjaanantakul & Voragen, 2009). In addition, the (Ara+Gal)/Rha ratio indicates the presence of galactan and arabinan side chains in the RG-I segments (Table 2).

3.2 FT-IR spectroscopy

Polysaccharides extracted at pH 2.0 or pH 6.0 were analysed using FT-IR spectroscopy in the wavenumber region 900 – 4000 cm\(^{-1}\) and their spectra were compared to low- and high-methoxyl citrus pectin (Figure 2). The region between 3500 and 1800 cm\(^{-1}\) shows two major identical peaks for both samples corresponding to O-H stretching absorption due to inter- and intramolecular hydrogen bonding of the GalA backbone (3000 – 3500 cm\(^{-1}\)) and C-H absorption (2940 cm\(^{-1}\)), which typically includes CH, CH\(_2\) and CH\(_3\) stretching vibrations (Chatjigakis, Pappas, Proxenia, Kalantzi, Rodis & Polissiou, 1998; Gnanasambandam & Proctor, 2000). A second region of the FT-IR spectra below 1800 cm\(^{-1}\) indicates the ‘fingerprint’ region for carbohydrates and corresponds to the skeletal C-O and C-C vibration bands (ca. 900 – 1200 cm\(^{-1}\)) of glycosidic bonds and pyranose rings (Kamnev, Colina, Rodriguez, Ptitchkina & Ignatov, 1998). The spectral regions with three bands at around 1044, 1072 and 1147 cm\(^{-1}\) were assigned to pyranose ring vibrations and may indicate certain similarities in the monosaccharide composition of OP2 and OP6 (Figure 2). Also this region of FT-IR spectra demonstrates considerable differences in neutral sugars composition between commercial citrus and extracted okra pectin. While citrus pectin has typical bands at around 1004, 1022, 1047, 1072 cm\(^{-1}\), the okra pectin has only at 1044, 1072 and 1147 cm\(^{-1}\) with relatively higher abundance of each band. This difference was expected as citrus pectin typically contains low amounts of galactose
(2.4%) and arabinose (1.1%) as opposed to the OP2 and OP6 (Table 1) (Kravtchenko, Voragen & Pilnik, 1992). The chemical analysis of OP2 and OP6 also indicated the presence of proteins (Table 1), which were detected also by FT-IR with absorption bands appearing at around ca. 1500–1600 cm\(^{-1}\). Qualitative analysis of OP2 and OP6 FT-IR spectra revealed similarities with low-methoxyl citrus pectin in absorption bands corresponding to stretching vibration of free (ca. 1610 – 1630 cm\(^{-1}\)) and methyl-esterified (ca. 1730 cm\(^{-1}\)) carboxyl groups. In addition, FT-IR spectra for OP6 have demonstrated higher intensity of free carboxyl stretching band in comparison to OP2, which indicates lower degree of esterification for OP6 sample. These data further support chemical analysis, which revealed DM of 40.0 and 24.6% for OP2 and OP6, respectively.

### 3.3 \(^1\)H and \(^{13}\)C-NMR spectra

NMR spectroscopy was employed in order to obtain structural information about the isolated okra polymers. \(^1\)H-NMR spectra (Figure 3a, b) of both samples revealed similar resonance patterns suggesting similarities in compositional characteristics of OP2 and OP6. A large signal was detected at 3.84 ppm, which was attributed to methyl groups bonded to carboxyl groups of galacturonic acid (GalA) (Cheng & Neiss, 2012). Signals at around 2.10 ppm were assigned to acetyl groups. Previous work on okra extracts reported the acetylation of both galacturonosyl and rhamnosyl residues in the RG-I fractions. Signals at 1.27 and 1.36 ppm are from methyl groups of unbranched \(\alpha-(1\rightarrow2)\)-linked and branched \(\alpha-(1\rightarrow2)\) and \(\alpha-(1\rightarrow4)\)-linked rhamnose. Due to the complexity of \(^1\)H-NMR spectra in the low-field region, proton signals found at around 3.70–5.20 ppm were investigated with the aid of a COSY spectrum (data not shown), which provided the evidence of the presence of six major protons, which were assigned to \(D\)-galacturonic acid.
13C-NMR spectra OP2 and OP6 are presented in Figure 4 (a, b). The signals at around 172.00 ppm in the carbonyl region of the spectrum were attributed to the carbonyl group (C=O) of galacturonic acid whereas the next signal at around 175 ppm corresponds to the C-6 of esterified carboxyl groups of galacturonic acid (Tamaki, Konishi, Fukuta & Tako, 2008). In the 13C-NMR spectra of both pectins, two signals at around 21.87 ppm can be readily assigned to the methyl of acetyl groups. The presence of methyl groups bonded to carboxyl groups of galacturonic acid is also confirmed by a resonance at 54.18–54.21 ppm in OP2 and OP6 spectra (Figure 4a, b). The third signal attributed to methyl groups at 18.5 and 17.58 ppm corresponded to methyl groups of rhamnose. 1H and 13C-NMR spectra of both okra polysaccharides demonstrated good match with the spectrum of okra polysaccharides isolated using sequential extractions and those isolated from pumpkin, apple, flax stems and citrus plant (Bédouet, Courtois & Courtois, 2003; Cozzolino et al., 2006; Grasdalen, Bakøy & Larsen, 1988; Koštálová, Hromádková & Ebringerová, 2013; Rosenbohm, Lundt, Christensen & Young, 2003; Sengkhamparn, Verhoe, Schols, Sajjaanantakul & Voragen, 2009; Tamaki, Konishi, Fukuta & Tako, 2008).

3.4 Macromolecular characteristics of pectin

To obtain information with regards to molecular dimensions of the pectins weight average ($M_w$) and number average ($M_n$) molecular weights, radius of gyration ($R_g$), and polydispersity index ($M_w/M_n$) were determined by size exclusion chromatography (SEC) coupled to multiangle laser light scattering. The elution RI traces of OP2 and OP6 are shown in Figure 5, whereas estimates of their molecular characteristics are represented in Table 3. The elution profiles of both samples indicated broad $M_w$ distributions representing populations of polymers of high and low molecular weights as indicated by the presence of three peaks (Figure 5). Weight
average molecular weights (M<sub>w</sub>) of individual peaks were 764, 515, 508 x 10<sup>3</sup> g mol<sup>-1</sup> and 1086, 792, 608 x 10<sup>3</sup> g mol<sup>-1</sup> for samples OP2 and OP6, respectively. Moreover, it can be clearly seen that only third peak to elute was similar for both OP2 and OP6 samples. On the contrary, a shift towards a population of polymers of lower molecular sizes was observed for the first and second peak in OP2 elution profile (Figure 5). This variation in elution patterns should be attributed to the differences in the pH of the extraction that results in partial hydrolysis of OP2 something that contributes to the shift of both peaks towards lower molecular weight values. The weight-average molar mass values were much higher than those obtained for okra polysaccharides obtained by sequential extraction (10 – 100 x 10<sup>3</sup> g mol<sup>-1</sup>), sugar beet (70 – 355 x 10<sup>3</sup> g mol<sup>-1</sup>) and citrus pectins (162 x 10<sup>3</sup> g mol<sup>-1</sup>) (Leroux, Langendorff, Schick, Vaishnav & Mazoyer, 2003; Levigne, Ralet & Thibault, 2002; Sengkhamparn, Verhoef, Schols, Sajjaanantakul & Voragen, 2009) indicating that the present protocol results in especially high molecular weight pectins.

3.5 Dilute solution viscometry

Intrinsic viscosity, a measure of the hydrodynamic volume occupied by a molecule, is a measure of the capacity of a polymer molecule to enhance the viscosity of solutions. Pectins isolated from okra pods contain substantial amounts of galacturonate residues. In aqueous solutions (pH 7.0), the expansion of individual coils by intramolecular electrostatic repulsion increases intrinsic viscosity. Therefore, to avoid complications stemming from changes in coil dimensions with polymer concentrations and to obtain intrinsic viscosity values in the absence of electrostatic interactions, all measurements were performed under the electrostatic screening provided by 0.1M NaCl (Kontogiorgos, Margelou, Georgiadis & Ritzoulis, 2012; Ndjourounekeu, Akingbala & Oguntimein, 1997). Dilute solution viscometry was also
performed at two different buffer pH values (7.0 and 3.0) in order to investigate the
changes in coil conformations with modulation of intramolecular forces. The intrinsic
viscosity values of okra pectins dispersed in phosphate buffer adjusted to pH 7.0 were
4.1 and 4.4 dL g\(^{-1}\) for OP2 and OP6, respectively (Table 3). A slight difference in \([\eta]\)
values for OP2 and OP6 could be attributed to higher degree of branching of RG-I
segments in OP2 indicating higher flexibility of RG-I regions and formation of
compact macrostructures with a shorter hydrodynamic size (Yapo, 2011). Okra pectin
\([\eta]\) values were found to be higher in comparison to those previously reported for okra
extracts obtained by sequential extractions (~ 0.9 – 2.7 dL g\(^{-1}\)) and comparable to
pectins isolated from sugar beet (~ 2.1 – 4.1 dL g\(^{-1}\)) or pumpkin (~ 3.3 – 3.4 dL g\(^{-1}\))
(Kontogiorgos, Margelou, Georgiadis & Ritzoulis, 2012; Levigne, Ralet & Thibault,
2002; Morris, Castile, Smith, Adams & Harding, 2010; Morris, Ralet, Bonnin,
Thibault & Harding, 2010; Ndjournekeu, Akingbala & Oguntimein, 1997; Ptitchkina,
Danilova, Doxastakis, Kasapis & Morris, 1994). The contribution of acetyl and
methyl groups and degree of branching of side chains can also play a significant role
to the coil dimensions of extracted pectin (Anger & Berth, 1986; Sengkhamparn,
Sagis, de Vries, Schols, Sajjaanantakul & Voragen, 2010). Lower amounts of RG-I
regions (49.0 – 59.4%) and much higher of HG segments (44.9 – 38.9%) could
account for the higher \([\eta]\) values of OP2 and OP6. It is well documented that charge
density, chain length (molecular weight) and stiffness of polymer control the
magnitude of \([\eta]\) (Morris, Cutler, Ross-Murphy & Rees, 1981). The polyelectrolyte
nature of pectin also controls the conformation of the chains. Increase of pH results in
dissociation of GalA and both samples (OP2, OP6) are negatively charged resulting in
electrostatic repulsion, extended conformations and consequently high \([\eta]\) values.
Intrinsic viscosity data obtained with citric buffer adjusted to pH 3.0 (Table 3) show
that $[\eta]$ of OP2 and OP6 were 3.3 dL g$^{-1}$ and 2.8 dL g$^{-1}$, respectively. Decrease of pH leads to protonation of GalA contributing to the decrease in net charge and strength of electrostatic repulsions resulting in more compact conformations. Changes of intramolecular forces contributed to slightly lower $[\eta]$ of OP6 indicating a decrease of the hydrodynamic volume of the macromolecular chain consequently leading to the predominance of a more flexible structure in comparison to OP2 sample where expansion of individual coils takes place.

The solution behaviour of okra pectins was investigated by measuring the zero shear specific viscosity ($\eta_{sp}$)$_0$ at different concentrations of the polysaccharide and plotting them versus the dimensionless coil overlap parameter, $c[\eta]$. Double-logarithmic plots of ($\eta_{sp}$)$_0$ vs. $c[\eta]$ were constructed to determine specific critical concentration ($c^*$) at which the transition from the dilute to concentrated regime appears and which is accompanied by significant changes in solution rheological properties (Figure 6, Table 3) (Morris, Cutler, Ross-Murphy & Rees, 1981). Critical concentration values ($c^*$, g dL$^{-1}$) for OP2 and OP6 dispersed in phosphate buffer (pH 7.0) were between 0.15 – 0.37 g dL$^{-1}$ whereas solutions prepared with citric buffer (pH 3.0) demonstrated higher values in the range 0.44 – 0.45 g dL$^{-1}$. In general, polymers that have high $[\eta]$ will also exhibit a transition from the dilute to concentrated region at lower polymer concentration due to the increased number of intermolecular interactions. For okra pectin solutions, $c^*$ values were lower than those reported for okra gum (1.5 g dL$^{-1}$), okra polysaccharides obtained by hot buffer sequential extraction (0.83 – 1.23 g dL$^{-1}$), apple pectins (1.27 – 1.39 g dL$^{-1}$) and other random coil polysaccharides (Hwang & Kokini, 1992; Kontogiorgos, Margelou, Georgiadis & Ritzoulis, 2012; Morris, Cutler, Ross-Murphy & Rees, 1981; Ndjouenkeu, Akingbala & Oguntimein, 1997; Sengkhamparn, Sagis, de Vries, Schols,
Sajjaanantakul & Voragen, 2010). The $c^*\eta$, a measurement of the total volume occupied by all coils within the polymer solution regardless of their molecular weight at the critical concentration, was also calculated. The results presented in Table 3 show the $c^*\eta$ for OP2 and OP6 in different buffer solutions. It has been reported that for most disordered linear polysaccharides double-logarithmic plots of $(\eta_{sp})_0$ vs. $c[\eta]$ superimpose closely regardless of the primary structure and molecular weight, and also fall into two linear regions with a sharp change of slopes (Morris, Cutler, Ross-Murphy & Rees, 1981; Ndjouenkeu, Akingbala & Oguntimein, 1997). However, as shown in Figure 6, the results obtained for present okra pectins do not comply well with this generalisation, particularly for dilute region ($c < c^*$) and demonstrate a significant deviation in slopes values regardless of solution pH. Moreover, slopes 1 of OP2 and OP6 were found to be significantly lower in comparison to those reported for polymers of different primary structure but with similar conformational characteristics (1.1 – 1.6) (Lapasin & Pricl, 1999). Therefore, our results indicate that the polyelectrolyte nature and differences in molecular structure of extracted pectins significantly affect conformational characteristics of polymer chains within the dilute region. However, values of slopes 2 are in a good agreement with the slopes values typical for disordered polysaccharides indicating that in dilute solutions the net charge of pectin chains plays predominant role for chain conformations (Table 3). The above findings suggest that buffer composition and extraction strategy are principal determinants of the structural characteristics of the isolated pectins and the properties of resulting solutions.
4. Conclusions

In the present work, the molecular features of okra pectins as affected by extraction conditions were studied. Extraction conditions influenced the fine structure of pectins resulting in isolates with distinct molecular characteristics. The present isolation protocols resulted in high molecular weight pectins with low degree of methylation (DM) and high degree of acetylation (DA). Galacturonic acid (GalA) amount varied by altering the pH of the extraction with higher pH values (pH6.0) resulting in greater GalA content. Both isolates contained high amounts of branched RG-I segments as indicated by the ratio of rhamnose to galacturonic acid and the high content of galactose to rhamnose. Dilute solution viscometry revealed changes in the coil dimensions for both of the isolated biopolymers with changes in pH as evidenced by intrinsic viscosity measurements. The high molecular weight and degree of acetylation as well as the influence of pH on the conformation of the chains introduces a new source of pectins with potentially high emulsifying and emulsion-stabilizing capacity.

Acknowledgements: The authors gratefully acknowledge the financial support provided by the GEN Foundation.
6. References


### Table 1. Chemical composition of okra pectins extracted at pH 2.0 or 6.0.

<table>
<thead>
<tr>
<th></th>
<th>OP2</th>
<th>OP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (g pectin/100 g okra pods)</td>
<td>13.3 ± 0.3</td>
<td>15.7 ± 0.2</td>
</tr>
<tr>
<td>Total sugars(^a)</td>
<td>70.0 ± 3.7</td>
<td>81.8 ± 6.4</td>
</tr>
<tr>
<td>D-GalA(^a)</td>
<td>46.8 ± 2.1 (55.0(^b))</td>
<td>56.9 ± 6.9 (51.6(^b))</td>
</tr>
<tr>
<td>Methoxyl (-OCH(_3))(^a)</td>
<td>3.3 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Degree of methylation (DM%)</td>
<td>40.0 ± 1.6</td>
<td>24.6 ± 1.0</td>
</tr>
<tr>
<td>Acetyl (-COCH(_3))(^a)</td>
<td>6.0 ± 0.6</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>Degree of acetylation (DA%)</td>
<td>52.2 ± 5.5</td>
<td>37.6 ± 3.0</td>
</tr>
<tr>
<td>D-Gal(^a)</td>
<td>17.0 ± 3.3 (21.7(^b))</td>
<td>26.1 ± 1.5 (25.7(^b))</td>
</tr>
<tr>
<td>L-Rha(^a)</td>
<td>7.1 ± 2.0 (10.1(^b))</td>
<td>12.1 ± 0.9 (13.2(^b))</td>
</tr>
<tr>
<td>L-Ara(^a)</td>
<td>4.5 ± 3.1 (7.1(^b))</td>
<td>6.0 ± 3.3 (7.3(^b))</td>
</tr>
<tr>
<td>D-Glc(^a)</td>
<td>2.4 ± 0.5 (3.1(^b))</td>
<td>2.2 ± 0.1 (2.2(^b))</td>
</tr>
<tr>
<td>D-Xyl(^b)</td>
<td>2.0 ± 0.7 (3.0(^b))</td>
<td>n/a</td>
</tr>
<tr>
<td>Protein(^a)</td>
<td>4.3 ± 0.0</td>
<td>6.3 ± 0.1</td>
</tr>
</tbody>
</table>

\(^a\)All values are expressed as % on wet basis of pectin powder.  
\(^b\)Values in brackets are mol%.

### Table 2. Sugar molar (%) ratios for OP2 and OP6.

<table>
<thead>
<tr>
<th>Sample</th>
<th>GalA/(Rha+Ara+Gal+Xyl)</th>
<th>Rha/GalA</th>
<th>(Ara+Gal)/Rha</th>
<th>HG</th>
<th>RG-I</th>
<th>HG/RG</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP2</td>
<td>1.3</td>
<td>0.18</td>
<td>2.9</td>
<td>44.9</td>
<td>49</td>
<td>0.9</td>
</tr>
<tr>
<td>OP6</td>
<td>1.1</td>
<td>0.25</td>
<td>2.5</td>
<td>38.9</td>
<td>59.4</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Table 3. Molecular characteristics of OP2 and OP6. Slopes, intrinsic viscosity ([η]), critical concentration (c*) and coil overlap parameter (c*[η]) of OP2 or OP6 at two different buffer pH values.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>OP2</th>
<th>OP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mw x 10^3 (g/mol)</td>
<td>641</td>
<td>767</td>
</tr>
<tr>
<td>Mn x 10^3 (g/mol)</td>
<td>628</td>
<td>715</td>
</tr>
<tr>
<td>Rg (nm)</td>
<td>108</td>
<td>121</td>
</tr>
<tr>
<td>Mw/Mn</td>
<td>1.02</td>
<td>1.07</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>pH 7</th>
<th>pH 3</th>
<th>pH 7</th>
<th>pH 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope 1</td>
<td>0.71</td>
<td>0.44</td>
<td>0.31</td>
<td>0.20</td>
</tr>
<tr>
<td>Slope 2</td>
<td>1.97</td>
<td>2.13</td>
<td>1.75</td>
<td>2.04</td>
</tr>
<tr>
<td>[η] (dL g⁻¹)</td>
<td>4.1</td>
<td>3.3</td>
<td>4.4</td>
<td>2.8</td>
</tr>
<tr>
<td>c* (g dL⁻¹)</td>
<td>0.37</td>
<td>0.45</td>
<td>0.15</td>
<td>0.44</td>
</tr>
<tr>
<td>c*[η]</td>
<td>1.51</td>
<td>1.49</td>
<td>0.66</td>
<td>1.24</td>
</tr>
</tbody>
</table>
Figure captions

**Fig. 1.** Isolation protocol for pectins isolated from okra pods.

**Fig. 2.** Fourier transform–infrared spectra (FT-IR) of commercial pectin standards with different DM and OP2, OP6.

**Fig. 3.** $^1$H-NMR spectra of OP2 (a) and OP6 (b) samples in D$_2$O at 70 °C. Acetone reference at 2.22 ppm.

**Fig. 4.** $^{13}$C-NMR spectra of OP2 (a) and OP6 (b) samples in D$_2$O at 70 °C. Acetone reference at 31.25 ppm.

**Fig. 5.** Refractive index (RI) and MALLS traces (LS) of size exclusion chromatograms of OP2 and OP6.

**Fig. 6.** Double logarithmic plots of zero shear specific viscosity $(\eta_p)_o$ vs. reduced concentration $c[\eta]$ of OP2 and OP6 at pH 3 and pH 7.
Figure 1
Figure 2
Figure 3

a) 
- GalA-OCH₃ 3.84 ppm
- O-Acetyl 2.10 ppm
- Rha-CH₃ 1.27, 1.36 ppm
- Acetone 2.22 ppm

b) 
- GalA-OCH₃ 3.84 ppm
- O-Acetyl 2.10 ppm
- Rha-CH₃ 1.27, 1.36 ppm
- Acetone 2.22 ppm
Figure 4
Figure 5
Figure 6