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

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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 α-(1→4) linked α-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 α-(1→4) linked α-D-galacturonic acid (GalA) residues, whereas rhamnogalacturonan (RG-I) backbone consists of repeating units of α-(1→4) linked α-D-galacturonic acid and α-(1→2) linked α-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 α-(1→4) linked α-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 α-(1→2)-rhamnose and α-(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

100 and degree of acetylation and methylation by β-elimination (Rombouts & Thibault,
101 1986). It has been reported that the highest yields of pectic substances are generally
102 obtained by hot acid extractions which is also the most convenient approach for
103 industrial extraction of pectin (May, 1990; Pagan, Ibarz, Llorca & Coll, 1999).
104 Previous studies reported that the temperature, pH and time could modify the quantity
105 as well as the quality of the extracted pectins (Levigne, Ralet & Thibault, 2002;
106 Pagan, Ibarz, Llorca & Coll, 1999). Furthermore, it was shown that the variations in
107 the number of methyl-esterified groups and composition of neutral sugars of the
108 isolated fractions are primarily governed by the extraction protocol (Kjøniksen,
109 Hiorth & Nyström, 2005; Turquois, Rinaudo, Taravel & Heyraud, 1999). The
110 extracted materials typically are polydisperse heteropolymers having diverse chemical
111 structures and molecular sizes (MacDougall & Ring, 2004).

112 Okra polysaccharides are potentially a new source of natural polysaccharides,
113 which can be used as functional ingredients (thickeners, viscosity enhancers, gelling
114 agents and texture modifiers) by the food industry (Georgiadis, Ritzoulis, Sioura,
115 Kornezou, Vasiliadou & Tsioptsias, 2011). Recent studies have mainly focused on
116 characterization of okra polysaccharides obtained with sequential extractions, starting
117 with acidic hot buffers followed by chelating agents and dilute alkali buffers.
118 Nevertheless, the effect of extraction pH on the physicochemical characteristics and
119 therefore functional properties of okra isolates has not been extensively studied
120 (Georgiadis, Ritzoulis, Sioura, Kornezou, Vasiliadou & Tsioptsias, 2011;
121 Kontogiorgos, Margelou, Georgiadis & Ritzoulis, 2012; Ndjouenkeu, Akingbala &
122 Oguntimein, 1997; Sengkhamparn, Verhoef, Schols, Sajjaanantakul & Voragen,
123 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 *Abelomoschus 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 (M_p 270, 410 $\times 10^3$ 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

149 Bradford assay (Bradford, 1976). The galacturonic acid (anhydrous) content of
150 pectins was estimated colorimetrically by the *m*-hydroxydiphenyl method (Filisetti-
151 Cozzi & Carpita, 1991). The methoxyl (-OCH_3) content of pectins was determined by
152 titration (Schultz, 1965). The method is based on a titration of free carboxyl groups
153 present followed by de-esterification and titration of the carboxyl groups that have
154 been made available. A correction was made for the acetic acid liberated due to the
155 cleavage of the *O*-acetyl groups. The degree of methyl esterification (DM) was
156 calculated from the galacturonic acid and methoxyl content values determined above
157 according to the following equation (Schultz, 1965):

158 $$\text{DM (\%)} = \frac{176 \text{ x methoxyl content (\%(w/w))}}{31 \text{ x GA content (\%(w/w))}} \text{x}100 \qquad (1)$$

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

164 $$\text{DA (\%)} = \frac{176 \text{ x acetyl content (\% (w/w))}}{43 \text{ x GA content (\% (w/w))}} \text{ x } 100 \qquad (2)$$

165 where 176 and 43 are the molecular weights of anhydrous galacturonic acid (GA) and
166 acetyl, respectively. Neutral sugars were determined using methanolysis conducted
167 with 1M methanolic HCl solution at 85 °C for 24 h, as described previously (Bleton,
168 Mejanelle, Sansoulet, Goursaud & Tchapla, 1996). Sugar derivatives were analysed
169 using an Agilent 7890A GC system (Santa Clara, CA, USA) coupled to an Agilent
170 5675C quadrupole MS. The samples were eluted from a HP-5 column (30 m x 0.25
171 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):

$$HG\ (mol\%) = GalA\ (mol\%) - Rha\ (mol\%) \quad (3)$$

$$RG\text{-}I\ (mol\%) = 2Rha\ (mol\%) + Ara\ (mol\%) + Gal\ (mol\%) \quad (4)$$

2.4 FT-IR spectroscopy

Spectra were obtained between 400-4000 cm^{-1} in Attenuated Total Reflection (ATR) 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_2O (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

198 various okra pectin concentrations (1%, 2%, 4% and 5% w/v) with and without
199 sonication in order to investigate how sonication affects the primary structure of the
200 polymers. Preliminary data (not shown) demonstrated that sonication for 9 min does
201 not contribute to the structural modifications as evidenced by inspection of ^{1}H-NMR
202 spectra of sonicated and non-sonicated samples at various concentrations. Chemical
203 shifts were expressed in δ (ppm) relative to the resonance of internal standard: spectra
204 were referenced to internal or external acetone (^{13}C δ = 31.55 ppm and ^{1}H δ = 2.225
205 ppm).

206 *2.6 Molecular weight determination*
207
208 To evaluate the average molar masses (M_w, weight average molar mass; M_n,
209 number-average molar mass) samples were analyzed by size exclusion
210 chromatography (SEC). Pectins were solubilized in 50 mM $NaNO_3$ solution (3 mg
211 mL^{-1}) at room temperature under magnetic stirring overnight. Samples were injected
212 onto an analytical SEC system comprising of three columns Aquagel-OH 40, 50 and
213 60 (15µm particle size, 25cm × 4mm, Agilent, Oxford, UK) connected in series.
214 Pectins were eluted with 50 mM $NaNO_3$ (containing 0.02% NaN_3 as a preservative) at
215 a flow rate of 1 mL min^{-1} and detected with an RI detector (differential index of
216 refraction (dn/dc) equal to 0.1470 ml g^{-1}). Molecular parameters (Mw, Mn, Rg,
217 Mw/Mn) were measured with a multiangle laser light scattering (MALLS) detector
218 (mini-DAWN, Wyatt, Santa Barbara, CA, USA).

219 *2.7 Dilute solution rheology*
220
221 Okra pectin was dispersed at 0.01 – 5.0 % g dL^{-1} at pH 7.0 in Sorensen's
222 phosphate buffer or pH 3.0 citric buffers in the presence of 0.1 M NaCl with 0.02 g
223 dL^{-1} NaN_3 as a preservative. Pectins were placed in sealed glass-vials and left

224 overnight under continuous stirring to ensure complete solubilisation. Intrinsic
225 viscosity [η] of okra pectins was determined at 20 °C with an Ubbelohde capillary
226 viscometer (PSL, UK). Calculations were obtained by extrapolation of viscometric
227 data to zero concentration according to the Huggins equation: $\eta_{sp}/c = [\eta] + k_H[\eta]^2 c$
228 where $\eta_{sp}=(\eta_{solution}/\eta_{buffer}) - 1$ and k_H is the Huggins constant. Zero shear viscosity
229 measurements were carried out at 20 °C using a Bohlin Gemini 200HR Nano
230 rotational rheometer (Malvern Instruments, Malvern, UK) equipped with cone-and-
231 plate geometry (55 mm diameter, cone angle 2°) and a Peltier temperature controller.
232 All measurements were performed in a steady shear mode in the range 1–1000 s^{-1}.

233 **3. Results and discussion**
234

235 *3.1 Isolation and compositional analysis*
236
237 An isolation protocol was designed to study the influence of pH on extraction
238 yield and the molecular characteristics of pectic substances from fresh okra pods.
239 Extraction with petroleum ether (bp 40-65 °C) was performed as a first step in order
240 to obtain a lipid-free material, which was subsequently used in aqueous extractions at
241 pH 2.0 and 6.0 with 100 mM citric and phosphate buffer, respectively. The highest
242 yields of pectic substances are usually obtained at high temperatures and low pH
243 values in order to facilitate the cleavage of strong bonds between protopectin and
244 other cell wall materials (Voragen, Rolin & Marr, 2003). It has been also reported that
245 temperature has significant impact on the extraction yield of okra polysaccharides
246 (Samavati, 2013). The isolation of the present okra polysaccharides was performed at
247 80 °C in order to facilitate the solubilisation of insoluble pectic substances
248 (protopectin). Polysaccharides with different compositional characteristics can be
249 isolated depending on the pH, time and temperature of extraction. It has been reported

250 that pectic substances extracted at pH 3.0 have similar compositional characteristics to
251 water-soluble pectin but result in low yield values. Extraction at pH values below 3.0
252 leads to higher yields with pectins rich in rhamnogalacturonans (Levigne, Ralet &
253 Thibault, 2002). Therefore, the extractions of pectic substances from okra were
254 performed at two different pH values in order to obtain polysaccharides with distinct
255 molecular characteristics. Pectic substances from okra pods could not be
256 quantitatively recovered in a single extraction step and a second extraction was
257 required (Figure 1). Similar findings have been reported for the extraction of pectins
258 from other raw materials (Samavati, 2013; Sudhakar & Maini, 2000). The final stage,
259 which can significantly affect the yield and chemical characteristics of pectins, is
260 precipitation with ethanol. In the present work, precipitation was performed with
261 ethanol at a 1:2 (v/v) supernatant to ethanol ratio and resulted in higher yields of
262 pectic substances in comparison to preliminary 1:1 (v/v) ratio. It has been also
263 reported that there is a pronounced effect of ethanol volume used in precipitation step
264 on DM of isolated pectic substances (Faravash & Ashtiani, 2007). This occurs as the
265 interaction between water, the carboxylic groups of pectin and the hydroxyl groups of
266 ethanol facilitates cleavage of methyl ester linkages. Following alcohol precipitation,
267 the pectin was washed with isopropanol and extensively dialysed against distilled
268 water. Extraction with citric buffer adjusted to pH 2.0 resulted in slightly lower yield
269 compared to extraction at pH 6.0. Furthermore, these extraction protocols result in
270 pectin isolates of high purity as evidenced by low protein content (Table 1). The
271 highest yields of pectin are typically obtained by hot acid extraction in the pH range
272 1.5 to 3.0. Studies on pectin from other sources such as sugar beet pulp and banana
273 peels also showed that the pectin yield increases significantly with a decrease in the
274 pH of the extraction and the highest yields were obtained at pH around 1.5 (Happi

275 Emaga, Ronkart, Robert, Wathelet & Paquot, 2008; Levigne, Ralet & Thibault, 2002;
276 Yapo, Robert, Etienne, Wathelet & Paquot, 2007). These discrepancies with present
277 data could be attributed to the origin of the initial material and the extraction
278 conditions applied. The lower pectin yield at pH 2.0 could be attributed to partial acid
279 hydrolysis that occurs at elevated temperatures as will be discussed later.

280 The chemical composition of okra pectins is shown in Table 1. The GalA
281 content of the okra isolates varied from 46.8 to 56.9 % (Table 1). The GalA content
282 was found to be significantly higher than has been previously reported for okra hot
283 buffer soluble solids (HBSS, 35%) (Sengkhamparn, Verhoef, Schols, Sajjaanantakul
284 & Voragen, 2009) and was close to that of sugar beet pectins (29.5-52.8 %) (Levigne,
285 Ralet & Thibault, 2002). Furthermore, the highest GalA content and pectin yield were
286 obtained when okra pectins were extracted at pH 6.0. The results strongly indicate,
287 that the pectin extraction yield is related to the content of GalA reinforcing that partial
288 degradation of pectic substances can take place under extraction conditions at pH 2.0.
289 Both okra pectins were found to be low methoxyl (LM) pectins with DM of 40.0%
290 and 24.6% for OP2 and OP6, considering that DM represents methoxyl content per
291 galacturonic acid unit. The differences in DM of pectin samples could be attributed to
292 the de-esterification process caused by β-eliminative degradation of the esterified
293 homogalacturonan backbone that leads to the removal of methyl esters resulting in
294 pectin with lower degree of methylation and consequently lower molecular weight
295 (Kurita, Fujiwara & Yamazaki, 2008). Previous studies on okra extracts obtained by
296 sequential extraction also revealed the presence of low methoxyl pectins
297 (Sengkhamparn, Verhoef, Schols, Sajjaanantakul & Voragen, 2009). Okra extracts
298 also exhibited high acetyl content with marginal differences for 6.0 (OP2) and 5.2 %
299 (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).

325 The ratios of constituent sugars were used in order to investigate the structure
326 of the extracted pectins at the molecular level. According to the sugar composition
327 data expressed as sugar molar ratios (Table 2) some interesting characteristics of the
328 extracted polysaccharides were observed. The molar ratio of rhamnose to galacturonic
329 acid is indicative of the presence of RG-I segments within the pectin population. The
330 RG-I backbone is typically composed of alternating units of rhamnose and
331 galacturonic acid and therefore the molar ratio of Rha/GalA is virtually 1:1 (Yapo,
332 2011). The contribution of RG-I to the pectin population was 0.18 and 0.25 for OP2
333 and OP6, respectively (Table 2). Therefore, OP2 and OP6 contained high amounts of
334 RG-I regions with higher values for OP6 (59.4%) in comparison to OP2 (49%)
335 indicating the prevalence of RG-I segments within the pectin population especially in
336 OP6 (Table 2). The HG/RG-I ratio varied from 0.9 (OP2) to 0.7 (OP6), suggesting the
337 presence of approximately equal proportions of HG and RG-I segments. These data
338 suggest structural dissimilarities of our samples compared to common pectins isolated
339 from apple or sugar beet, where RG-I segments constituted ~16.2 or ~31.9% of the
340 pectin populations (Leroux, Langendorff, Schick, Vaishnav & Mazoyer, 2003).
341 However, more than 50% of RG-I has been previously reported for hot water-
342 extracted pectins from soybean and green tea leaves and almost as the only pectic
343 component in okra polysaccharides obtained by hot buffer sequential extraction and
344 linseeds mucilages (Ele-Ekouna, Pau-Roblot, Courtois & Courtois, 2011;
345 Muralikrishna, Salimath & Tharanathan, 1987; Nakamura, Furuta, Maeda, Nagamatsu
346 & Yoshimoto, 2001; Sengkhamparn, Verhoef, Schols, Sajjaanantakul & Voragen,
347 2009). The molar ratio of (Ara+Gal)/Rha is indicative for the degree of branching of
348 RG-I segments. The molar ratio for OP2 was 2.9 and 2.5 for the OP6 suggesting
349 shorter side chains of RG-I regions in OP6 than in OP2. Generally, OP2 and OP6

350 demonstrated remarkably higher degree of branching of side chains than was
351 previously reported for okra polysaccharides obtained by sequential extractions (1.3–
352 1.4) (Sengkhamparn, Verhoef, Schols, Sajjaanantakul & Voragen, 2009). In addition,
353 the (Ara+Gal)/Rha ratio indicates the presence of galactan and arabinan side chains in
354 the RG-I segments (Table 2).

355 *3.2 FT-IR spectroscopy*
356
357 Polysaccharides extracted at pH 2.0 or pH 6.0 were analysed using FT-IR
358 spectroscopy in the wavenumber region 900 – 4000 cm^{-1} and their spectra were
359 compared to low- and high-methoxyl citrus pectin (Figure 2). The region between
360 3500 and 1800 cm^{-1} shows two major identical peaks for both samples corresponding
361 to O-H stretching absorption due to inter- and intramolecular hydrogen bonding of the
362 GalA backbone (3000 – 3500 cm^{-1}) and C-H absorption (2940 cm^{-1}), which typically
363 includes CH, CH_2 and CH_3 stretching vibrations (Chatjigakis, Pappas, Proxenia,
364 Kalantzi, Rodis & Polissiou, 1998; Gnanasambandam & Proctor, 2000). A second
365 region of the FT-IR spectra below 1800 cm^{-1} indicates the 'fingerprint' region for
366 carbohydrates and corresponds to the skeletal C-O and C-C vibration bands (ca. 900 –
367 1200 cm^{-1}) of glycosidic bonds and pyranose rings (Kamnev, Colina, Rodriguez,
368 Ptitchkina & Ignatov, 1998). The spectral regions with three bands at around 1044,
369 1072 and 1147 cm^{-1} were assigned to pyranose ring vibrations and may indicate
370 certain similarities in the monosaccharide composition of OP2 and OP6 (Figure 2).
371 Also this region of FT-IR spectra demonstrates considerable differences in neutral
372 sugars composition between commercial citrus and extracted okra pectin. While citrus
373 pectin has typical bands at around 1004, 1022, 1047, 1072 cm^{-1}, the okra pectin has
374 only at 1044, 1072 and 1147cm^{-1} with relatively higher abundance of each band. This
375 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 α-(1→2)-linked and branched a-(1→2) and α-(1→4)-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.

^{13}C-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 ^{13}C-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. ^{1}H and ^{13}C-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, Verhoef, 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

428 average molecular weights (M_w) of individual peaks were 764, 515, 508 x10^3 g mol^{-1}
429 and 1086, 792, 608 x10^3 g mol^{-1} for samples OP2 and OP6, respectively. Moreover, it
430 can be clearly seen that only third peak to elute was similar for both OP2 and OP6
431 samples. On the contrary, a shift towards a population of polymers of lower molecular
432 sizes was observed for the first and second peak in OP2 elution profile (Figure 5).
433 This variation in elution patterns should be attributed to the differences in the pH of
434 the extraction that results in partial hydrolysis of OP2 something that contributes to
435 the shift of both peaks towards lower molecular weight values. The weight-average
436 molar mass values were much higher than those obtained for okra polysaccharides
437 obtained by sequential extraction ($10 - 100 \times 10^3$ g mol^{-1}), sugar beet ($70 - 355 \times 10^3$
438 g mol^{-1}) and citrus pectins (162×10^3 g mol^{-1}) (Leroux, Langendorff, Schick,
439 Vaishnav & Mazoyer, 2003; Levigne, Ralet & Thibault, 2002; Sengkhamparn,
440 Verhoef, Schols, Sajjaanantakul & Voragen, 2009) indicating that the present protocol
441 results in especially high molecular weight pectins.

442 *3.5 Dilute solution viscometry*
443
444 Intrinsic viscosity, a measure of the hydrodynamic volume occupied by a
445 molecule, is a measure of the capacity of a polymer molecule to enhance the viscosity
446 of solutions. Pectins isolated from okra pods contain substantial amounts of
447 galacturonate residues. In aqueous solutions (pH 7.0), the expansion of individual
448 coils by intramolecular electrostatic repulsion increases intrinsic viscosity. Therefore,
449 to avoid complications stemming from changes in coil dimensions with polymer
450 concentrations and to obtain intrinsic viscosity values in the absence of electrostatic
451 interactions, all measurements were performed under the electrostatic screening
452 provided by 0.1M NaCl (Kontogiorgos, Margelou, Georgiadis & Ritzoulis, 2012;
453 Ndjouenkeu, Akingbala & Oguntimein, 1997). Dilute solution viscometry was also

454 performed at two different buffer pH values (7.0 and 3.0) in order to investigate the
455 changes in coil conformations with modulation of intramolecular forces. The intrinsic
456 viscosity values of okra pectins dispersed in phosphate buffer adjusted to pH 7.0 were
457 4.1 and 4.4 dL g^{-1} for OP2 and OP6, respectively (Table 3). A slight difference in [η]
458 values for OP2 and OP6 could be attributed to higher degree of branching of RG-I
459 segments in OP2 indicating higher flexibility of RG-I regions and formation of
460 compact macrostructures with a shorter hydrodynamic size (Yapo, 2011). Okra pectin
461 [η] values were found to be higher in comparison to those previously reported for okra
462 extracts obtained by sequential extractions (~ 0.9 – 2.7 dL g^{-1}) and comparable to
463 pectins isolated from sugar beet (~ 2.1 – 4.1 dL g^{-1}) or pumpkin (~ 3.3 – 3.4 dL g^{-1})
464 (Kontogiorgos, Margelou, Georgiadis & Ritzoulis, 2012; Levigne, Ralet & Thibault,
465 2002; Morris, Castile, Smith, Adams & Harding, 2010; Morris, Ralet, Bonnin,
466 Thibault & Harding, 2010; Ndjouenkeu, Akingbala & Oguntimein, 1997; Ptitchkina,
467 Danilova, Doxastakis, Kasapis & Morris, 1994). The contribution of acetyl and
468 methyl groups and degree of branching of side chains can also play a significant role
469 to the coil dimensions of extracted pectin (Anger & Berth, 1986; Sengkhamparn,
470 Sagis, de Vries, Schols, Sajjaanantakul & Voragen, 2010). Lower amounts of RG-I
471 regions (49.0 – 59.4%) and much higher of HG segments (44.9 – 38.9%) could
472 account for the higher [η] values of OP2 and OP6. It is well documented that charge
473 density, chain length (molecular weight) and stiffness of polymer control the
474 magnitude of [η] (Morris, Cutler, Ross-Murphy & Rees, 1981). The polyelectrolyte
475 nature of pectin also controls the conformation of the chains. Increase of pH results in
476 dissociation of GalA and both samples (OP2, OP6) are negatively charged resulting in
477 electrostatic repulsion, extended conformations and consequently high [η] values.
478 Intrinsic viscosity data obtained with citric buffer adjusted to pH 3.0 (Table 3) show

479 that [η] of OP2 and OP6 were 3.3 dL g^{-1} and 2.8 dL g^{-1}, respectively. Decrease of pH
480 leads to protonation of GalA contributing to the decrease in net charge and strength of
481 electrostatic repulsions resulting in more compact conformations. Changes of
482 intramolecular forces contributed to slightly lower [η] of OP6 indicating a decrease of
483 the hydrodynamic volume of the macromolecular chain consequently leading to the
484 predominance of a more flexible structure in comparison to OP2 sample where
485 expansion of individual coils takes place.

486 The solution behaviour of okra pectins was investigated by measuring the zero
487 shear specific viscosity $(\eta_{sp})_o$ at different concentrations of the polysaccharide and
488 plotting them *versus* the dimensionless coil overlap parameter, $c[\eta]$. Double-
489 logarithmic plots of $(\eta_{sp})_o$ *vs.* $c[\eta]$ were constructed to determine specific critical
490 concentration (c^*) at which the transition from the dilute to concentrated regime
491 appears and which is accompanied by significant changes in solution rheological
492 properties (Figure 6, Table 3) (Morris, Cutler, Ross-Murphy & Rees, 1981). Critical
493 concentration values (c^*, g dL^{-1}) for OP2 and OP6 dispersed in phosphate buffer (pH
494 7.0) were between 0.15 – 0.37 g dL^{-1} whereas solutions prepared with citric buffer
495 (pH 3.0) demonstrated higher values in the range 0.44 – 0.45 g dL^{-1}. In general,
496 polymers that have high [η] will also exhibit a transition from the dilute to
497 concentrated region at lower polymer concentration due to the increased number of
498 intermolecular interactions. For okra pectin solutions, c^* values were lower than those
499 reported for okra gum (1.5 g dL^{-1}), okra polysaccharides obtained by hot buffer
500 sequential extraction (0.83 – 1.23 g dL^{-1}), apple pectins (1.27 – 1.39 g dL^{-1}) and other
501 random coil polysaccharides (Hwang & Kokini, 1992; Kontogiorgos, Margelou,
502 Georgiadis & Ritzoulis, 2012; Morris, Cutler, Ross-Murphy & Rees, 1981;
503 Ndjouenkeu, Akingbala & Oguntimein, 1997; Sengkhamparn, Sagis, de Vries, Schols,

504 Sajjaanantakul & Voragen, 2010). The $c^*[\eta]$, a measurement of the total volume
505 occupied by all coils within the polymer solution regardless of their molecular weight
506 at the critical concentration, was also calculated. The results presented in Table 3
507 show the $c^*[\eta]$ for OP2 and OP6 in different buffer solutions. It has been reported that
508 for most disordered linear polysaccharides double-logarithmic plots of $(\eta_{sp})_o$ *vs.* $c[\eta]$
509 superimpose closely regardless of the primary structure and molecular weight, and
510 also fall into two linear regions with a sharp change of slopes (Morris, Cutler, Ross-
511 Murphy & Rees, 1981; Ndjouenkeu, Akingbala & Oguntimein, 1997). However, as
512 shown in Figure 6, the results obtained for present okra pectins do not comply well
513 with this generalisation, particularly for dilute region ($c < c^*$) and demonstrate a
514 significant deviation in slopes values regardless of solution pH. Moreover, slopes 1 of
515 OP2 and OP6 were found to be significantly lower in comparison to those reported for
516 polymers of different primary structure but with similar conformational characteristics
517 (1.1 – 1.6) (Lapasin & Pricl, 1999). Therefore, our results indicate that the
518 polyelectrolyte nature and differences in molecular structure of extracted pectins
519 significantly affect conformational characteristics of polymer chains within the dilute
520 region. However, values of slopes 2 are in a good agreement with the slopes values
521 typical for disordered polysaccharides indicating that in dilute solutions the net charge
522 of pectin chains plays predominant role for chain conformations (Table 3). The above
523 findings suggest that buffer composition and extraction strategy are principal
524 determinants of the structural characteristics of the isolated pectins and the properties
525 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.

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Tables

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

	OP2	OP6
Yield (g pectin/100 g okra pods)	13.3 ± 0.3	15.7 ± 0.2
Total sugars[a]	70.0 ± 3.7	81.8 ± 6.4
D-GalA[a]	46.8 ± 2.1 (55.0)[b]	56.9 ± 6.9 (51.6)[b]
Methoxyl (-OCH_3)[a]	3.3 ± 0.1	2.5 ± 0.1
Degree of methylation (DM%)	40.0 ± 1.6	24.6 ± 1.0
Acetyl (-$COCH_3$)[a]	6.0 ± 0.6	5.2 ± 0.4
Degree of acetylation (DA%)	52.2 ± 5.5	37.6 ± 3.0
D-Gal[a]	17.0 ± 3.3 (21.7)[b]	26.1 ± 1.5 (25.7)[b]
L-Rha[a]	7.1 ± 2.0 (10.1)[b]	12.1 ± 0.9 (13.2)[b]
L-Ara[a]	4.5 ± 3.1 (7.1)[b]	6.0 ± 3.3 (7.3)[b]
D-Glc[a]	2.4 ± 0.5 (3.1)[b]	2.2 ± 0.1 (2.2)[b]
D-Xyl[a]	2.0 ± 0.7 (3.0)[b]	n/a
Protein[a]	4.3 ± 0.0	6.3 ± 0.1

[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.

Sample	GalA/(Rha+Ara+Gal+Xyl)	Rha/GalA	(Ara+Gal)/Rha	HG	RG–I	HG/RG
OP2	1.3	0.18	2.9	44.9	49	0.9
OP6	1.1	0.25	2.5	38.9	59.4	0.7

773 **Table 3**. Molecular characteristics of OP2 and OP6. Slopes, intrinsic viscosity ([η]),
774 critical concentration (c*) and coil overlap parameter (c*[η]) of OP2 or OP6 at two
775 different buffer pH values.
776

Parameter	OP2		OP6	
Mw x 10^3 (g/mol)	641		767	
Mn x 10^3 (g/mol)	628		715	
Rg (nm)	108		121	
Mw/Mn	1.02		1.07	
	pH 7	pH 3	pH 7	pH 3
Slope 1	0.71	0.44	0.31	0.20
Slope 2	1.97	2.13	1.75	2.04
[η] (dL g^{-1})	4.1	3.3	4.4	2.8
c* (g dL^{-1})	0.37	0.45	0.15	0.44
c*[η]	1.51	1.49	0.66	1.24

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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_2O at 70 ^{o}C. Acetone reference at 2.22 ppm.

Fig. 4. ^{13}C-NMR spectra of OP2 (a) and OP6 (b) samples in D_2O at 70 ^{o}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 $(\eta_{sp})_o$ *vs.* reduced concentration $c[\eta]$ of OP2 and OP6 at pH 3 and pH 7.

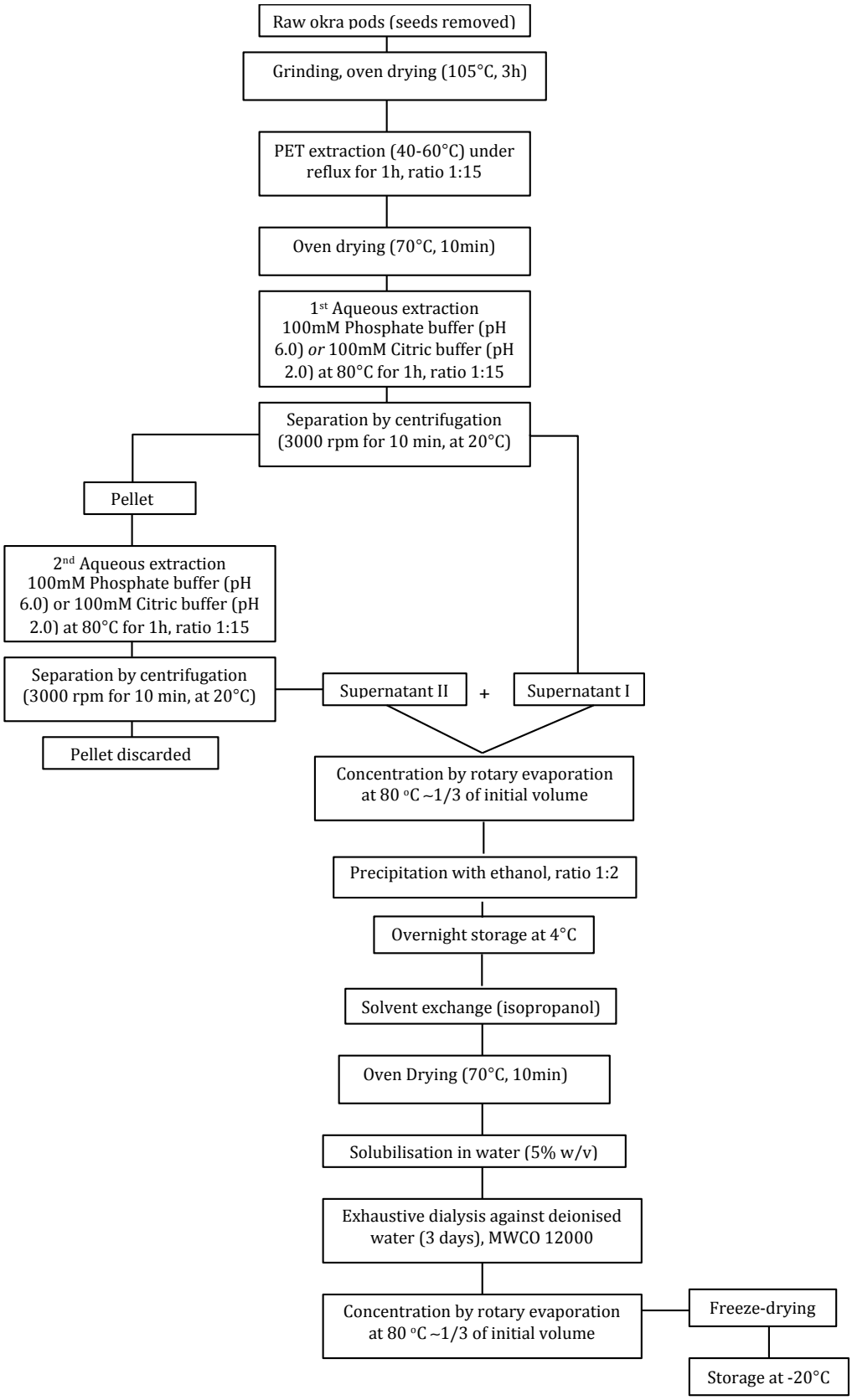
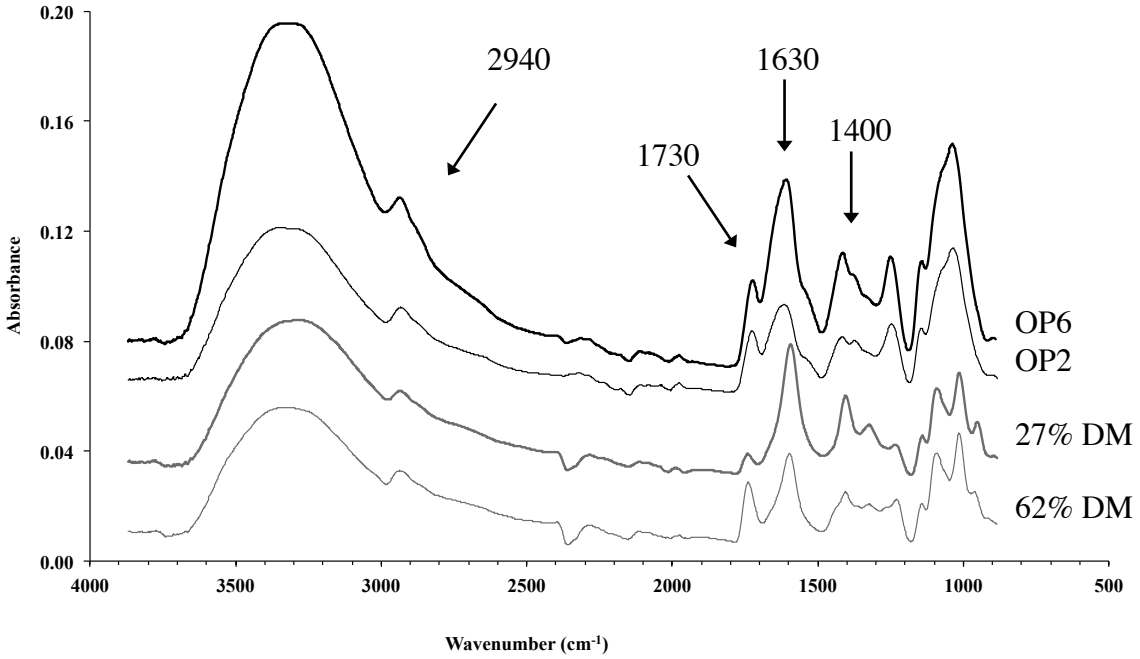

Raw okra pods (seeds removed)
Grinding, oven drying (105°C, 3h)
PET extraction (40-60°C) under reflux for 1h, ratio 1:15
Oven drying (70°C, 10min)
1st Aqueous extraction 100mM Phosphate buffer (pH 6.0) or 100mM Citric buffer (pH 2.0) at 80°C for 1h, ratio 1:15
Separation by centrifugation (3000 rpm for 10 min, at 20°C)
Pellet
2nd Aqueous extraction 100mM Phosphate buffer (pH 6.0) or 100mM Citric buffer (pH 2.0) at 80°C for 1h, ratio 1:15
Separation by centrifugation (3000 rpm for 10 min, at 20°C)
Pellet discarded
Supernatant II
+
Supernatant I
Concentration by rotary evaporation at 80 °C ~1/3 of initial volume
Precipitation with ethanol, ratio 1:2
Overnight storage at 4°C
Solvent exchange (isopropanol)
Oven Drying (70°C, 10min)
Solubilisation in water (5% w/v)
Exhaustive dialysis against deionised water (3 days), MWCO 12000
Concentration by rotary evaporation at 80 °C ~1/3 of initial volume
Freeze-drying
Storage at -20°C

Figure 1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3000
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2000
1500
1000
500
Wavenumber (cm-1)
2940
1730
1630
1400
OP6
OP2
27% DM
62% DM

Figure 2

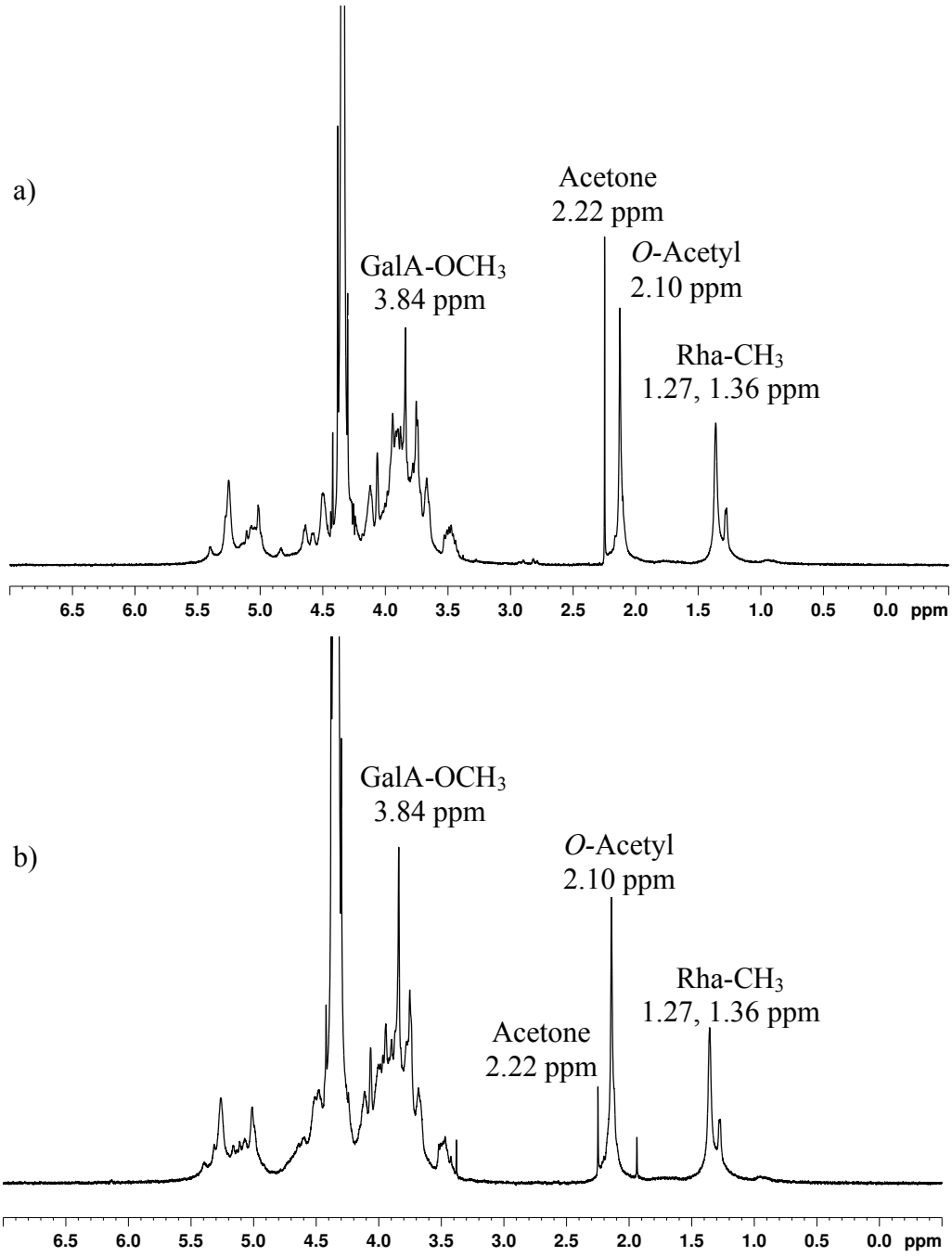
a)
Acetone
2.22 ppm
GalA-OCH3
3.84 ppm
O-Acetyl
2.10 ppm
Rha-CH3
1.27, 1.36 ppm
6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 ppm
b)
GalA-OCH3
3.84 ppm
O-Acetyl
2.10 ppm
Rha-CH3
1.27, 1.36 ppm
Acetone
2.22 ppm
6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 ppm

Figure 3

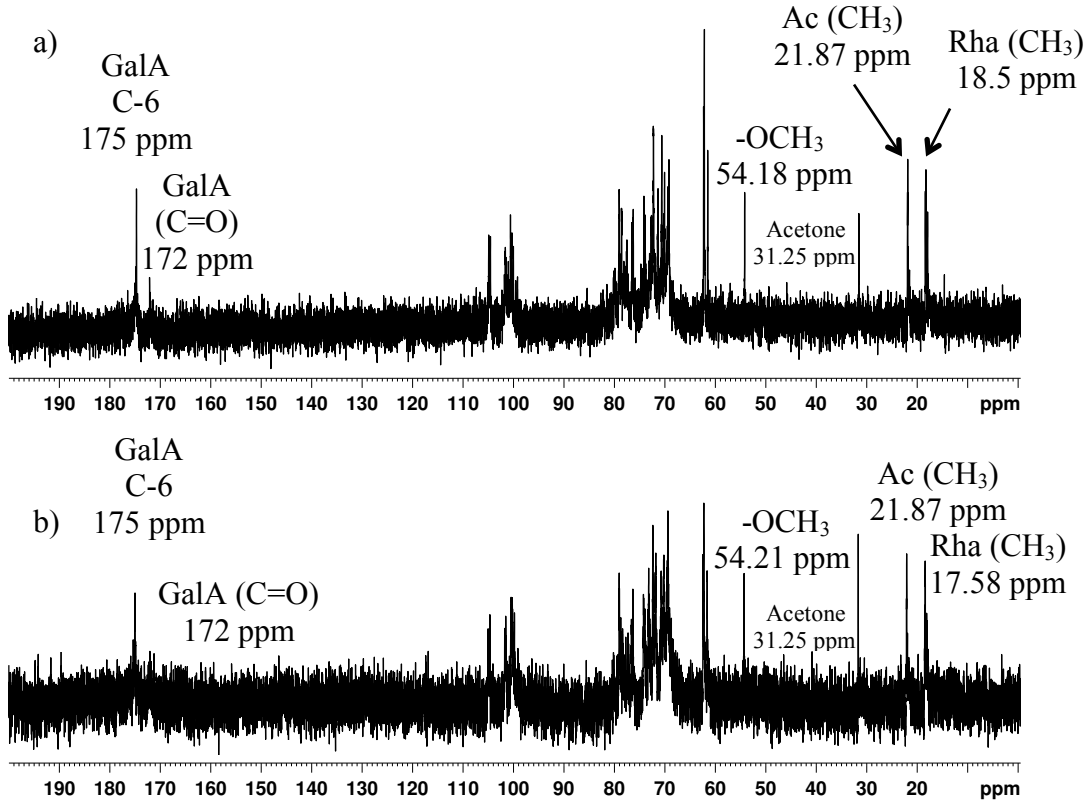
a)
GalA
C-6
175 ppm
GalA
(C=O)
172 ppm
Ac (CH3)
21.87 ppm
Rha (CH3)
18.5 ppm
-OCH3
54.18 ppm
Acetone
31.25 ppm
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm
b)
GalA
C-6
175 ppm
GalA (C=O)
172 ppm
-OCH3
54.21 ppm
Ac (CH3)
21.87 ppm
Rha (CH3)
17.58 ppm
Acetone
31.25 ppm
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm

Figure 4

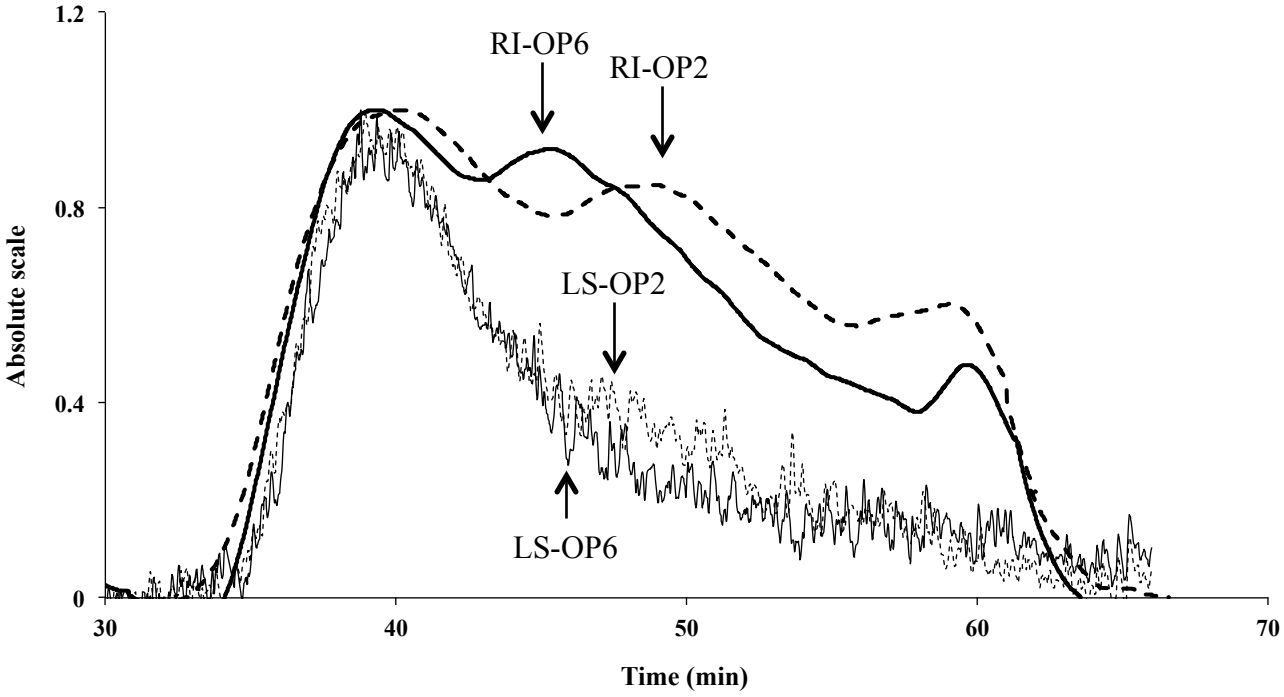
1.2
0.8
0.4
0
Absolute scale
RI-OP6
RI-OP2
LS-OP2
LS-OP6
30
40
50
60
70
Time (min)

Figure 5

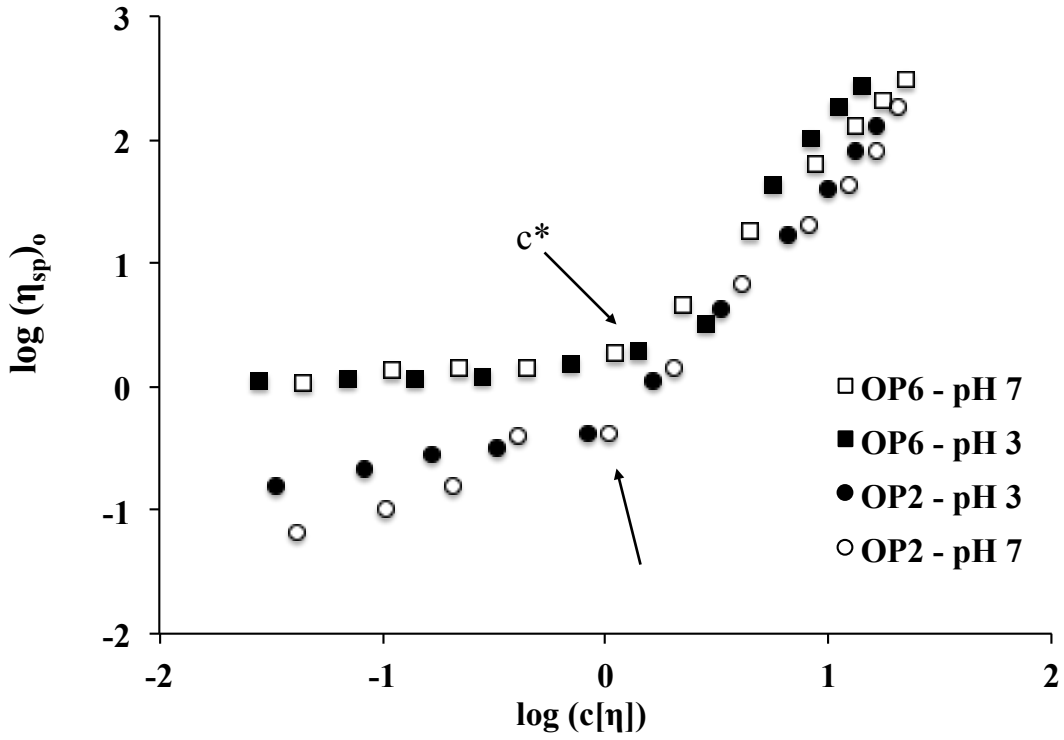
3
2
1
0
-1
-2
-2
-1
0
1
2
log (η_{sp})$_o$
log (c[η])
c*
OP6 - pH 7
OP6 - pH 3
OP2 - pH 3
OP2 - pH 7

Figure 6